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Species diversity of fecal microbial flora in *Canis lupus familiaris* infected with canine parvovirus

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ABSTRACT

Parvovirosis is a highly contagious disease in dogs, often causing acute hemorrhagic enteritis and altering the intestinal microflora. In this study, real-time PCR was used to detect the viral copy numbers in dogs diagnosed with the disease. Hematological and hemobiochemical parameters were also determined. The species and abundances of the fecal microbial flora in both sick and healthy dogs were determined and compared via metagenomic sequencing. The viral copy numbers in the sick dogs were infected with little difference in the positive samples. The blood coagulation time was significantly shorter and the number of white blood cells was significantly greater in the sick dogs. The serum calcium content was slightly increased and the phosphorus content was reduced in the sick dogs. The LDH and CK activities were significantly elevated in the sick dogs. Metagenomic sequencing and analysis revealed relatively more *Escherichia*, *Lachnoclostridium*, *gnavus* group (*Ruminococcus*), and uncultured_bacterium_f_lachnospiraceae in the infected dogs, whereas the abundance of *Collinsella* was relatively reduced. *Alloprevotella* and *Sutterella* were absent among the fecal microorganisms of the infected dogs. The relative abundances of *Romboutsia*, *Erysipelatoclostridium*, *Anaerotruncus*, and *Blautia* were significantly increased in the infected dogs. Functional analysis of the metagenomes of the samples indicated a significant enrichment of the 'replication, recombination and repair', 'nucleotide transport and metabolism', 'transcription', and 'defense metabolism' functions in the fecal microbial flora of the infected dogs. In summary, this study provides a scientific theoretical basis for preventing and controlling diarrhea caused by the canine parvovirus.

1. Introduction

Canine parvovirus (CPV) is a single-stranded DNA virus belonging to the genus *Protoparvovirus* in the family *Parvoviridae*. The virus replicates autonomously, without a capsule, and encodes 5,323 amino acids. It has the smallest molecular weight of all DNA viruses (Reed et al., 1988; Chinchkar et al., 2006). Its genome consists of two open reading frames, each with a promoter region, which encode nonstructural proteins (NS1, NS2) and structural proteins (VP1, VP2). VP2 is the main component of the viral capsid and determines the host range and antigenicity of the virus (Doki et al., 2006; Filipov et al., 2011).

Canine parvovirus is lethal and infects dogs as well as cats and weasels (Chang et al., 1992; Mochizuki et al., 2008; Harbison et al., 2009). CPV-2c antigenic variants were found in Vietnamese leopard cats in 2004. Routine immunization is an effective protective measure, but studies have found that immunized dogs can be reinfected with parvovirus (Decaro et al., 2008; Filipov et al., 2011). A genetic dynamics analysis of the CPV genome showed that the evolutionary rate of its VP2 gene can reach 2×10^{-4} replacements/site/year, which is near that of some RNA viruses and may be related to the rapid evolution of CPV. Therefore, many dogs remain infected with the disease (Jeoung et al., 2008).

The animal intestinal gut contains many microorganisms that have dynamically balanced and complex relationships, both symbiotic and competitive, with their hosts. Microorganisms and their metabolites help the animal body decompose and absorb nutrients, thus affecting host health (Kanauchi et al., 2005; Decaro et al., 2007; Friswell et al., 2010; Suchodolski et al., 2010). Parvovirus mainly infects puppies, and infected puppies may show typical signs of hemorrhagic enteritis within 5–7 days, such as fever, vomiting, loss of appetite, changes in fecal consistency from soft to watery, and bloody stools. These symptoms are attributable to the intestinal infection of the host by the canine parvovirus, which can damage the microbial balance in the host's gut, leading to secondary enteritis (Joao Vieira et al., 2008).

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In this study, the pathogenic bacterial species and quantities in the fecal tracts of infected dogs were studied by analyzing their feces and then compared with those of normal dogs. The community dynamics of intestinal microbes are an important factor in ensuring the physiological homeostasis of the host. Here, we used high-throughput 16S-rRNA gene sequencing technology to comprehensively analyze and compare the composition and structure of the fecal microbial communities in healthy and infected dogs. This study provides a scientific theoretical basis for preventing and controlling diarrhea caused by the canine parvovirus and is of great significance to improving the health of dogs.

2. Materials and methods

2.1. Experimental materials

Stool samples were collected from 15 infected dogs with similar symptoms (fever, depression, anorexia, vomiting and severe diarrhea) and 15 healthy dogs of the same breed and similar ages at a dog-breeding base in Sichuan Province, China.

2.2. Real-time PCR and conditions

DNA was extracted from an aliquot of each dog's blood using a TIANamp Virus DNA/RNA Kit (Tiangen, Beijing, China) (Zhu et al., 2013). The upstream and downstream primers used were 5′-CATTGG GCTTACCACCATTT-3′ and 5′-AAATGGCCCTTGTGTAGACG-3′, respectively. The amplified fragment length was 209 bp. Real-time PCR was performed in a 25-μl volume, containing 10 μl of SYBR Green PCR Master Mix (Takara, Dalian, China), 1 μl each of the upstream and downstream primers (final concentration, 0.1 μM each), 2 μL of template (10 ng of DNA), and diethyl pyrocarbonate (DEPC)-treated water. In the no-template (control) group, the template was substituted with 2 μl of ultrapure water. The reaction conditions were denaturation at 95 °C for 10 min and 40 cycles for 15 s at 95 °C and 1 min at 60 °C.The conditions for the dissolution curve analysis were 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) was used.

2.3. Hemobiochemical analysis

Blood (10 mL) was collected in a centrifuge tube and left at room temperature for 1–2 h, then stored at 4 °C for 3–4 h. After blood coagulation and contraction, the serum was extracted with a capillary tube and centrifuged for 15 min at 3200 × g. The lactate dehydrogenase (LDH) and creatine kinase (CK) activities were measured as per the corresponding kit instructions (Jiancheng, Nanjing, China). The serum calcium content was determined via EDTA titration, and the serum phosphorus content was determined using the reduced molybdenum blue method.

2.4. Metagenomic analysis of fecal microbial flora

Samples were collected from the rectums of the sick and healthy dogs. The cotton swabs dipped in feces were placed in 10 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) diluted 10 times. The medium was then placed in a 1.5-ml freezing tube and cryopreserved at −80 °C. The 15 infected dogs were randomly allocated to one of three groups (three biological replicates; n = 5 per group), and the samples collected within each group were pooled in equal proportions. The control dogs were similarly analyzed. The Illumina HiSeq sequencing platform was used to construct a small-fragment library to be sequenced with the dual-end sequencing method. Total bacterial DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, United States) per the manufacturer's protocol. The quality and concentration of the extracted DNA were measured using a NanoDrop spectrophotometer (ND-2000c, NanoDrop Technologies, Wilmington, DE, United States). DNA amplification, homogenization and library construction were performed per the manufacturer's protocol. Sequencing libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) and quantified with a Qubit 2.0 Fluorometer ((Life Technologies, CA, USA). Finally, paired-end sequencing was conducted using an Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, CA, United States) at Biomarker Bioinformatics Technology, Co., Ltd. (Beijing, China). The species analysis was performed by filtering the original sequences, assembling the split-ends, and retrieving the optimized sequences (tags), which were then clustered to identify the operational taxonomic units (OTUs), and the species were classified according to the OTU sequence compositions (Caporaso et al., 2010; Edgar, 2010). From the OTU analysis results, the samples were classified at all taxonomic levels, and the community structure maps, species heat maps, phylogenetic trees, and taxonomic trees for each sample were obtained at the phylum, class, order, family, genus, and species levels. The species diversity within a single sample was determined via alpha diversity analysis, and the ACE, Chao1, Shannon, and Simpson indices for each sample were calculated at a 97% similarity level (Grice et al., 2009). The differences in species diversity (community composition and structure) between the samples were compared via beta diversity analysis. Distance matrices were used to obtain the unweighted pair group method with arithmetic mean (UPGMA) trees and sample clustering heat maps (Lozupone and Knight, 2005). Statistically significant biomarkers among the groups were identified by analyzing the significance of the between-group differences (Segata et al., 2011). The estimated bacterial functions and their abundances were calculated via predictive analysis of the bacterial 16S rRNA genes (Parks et al., 2014).

2.5. Statistical analysis

Bacterial abundance variance analysis and t-tests were used to compare significant differences between groups of data. $P < 0.05$ was considered statistically significant. All physiological index data were subjected to one-way analysis of variance (ANOVA) and tested for significant differences between treatments using SPSS software (version 20.0; SPSS Inc., Chicago, IL, United States). The treatment effects were evaluated using Duncan's test ($P < 0.05$).

3. Results

3.1. Real-time PCR

Real-time PCR can accurately detect viral species. In this study, no fluorescent signals were detected in the samples from healthy control dogs after 40 cycles of PCR. However, the cycle threshold (CT) values of all samples from the sick dogs were approximately 27–28. The results confirmed that 15 dogs were infected with parvovirus, with little difference in the viral numbers in the positive samples (Fig. 1).

3.2. Effects of CPV infection on blood physiology

The blood coagulation time was significantly shorter, and the number of white blood cells was significantly greater in the sick dogs than in the healthy control dogs ($P < 0.01$). The serum calcium content was also slightly increased, and the phosphorus content was reduced in the sick dogs. The LDH and CK activities were significantly elevated in the sick dogs. These phenotypes were consistent with previously reported effects of canine parvovirus infections. However, the physiological parameters of the infected dogs did not significantly differ (Tables 1 and 2).

3.3. Quality evaluation of metagenomic sequencing data

The quality of the high-throughput sequencing data was statistically

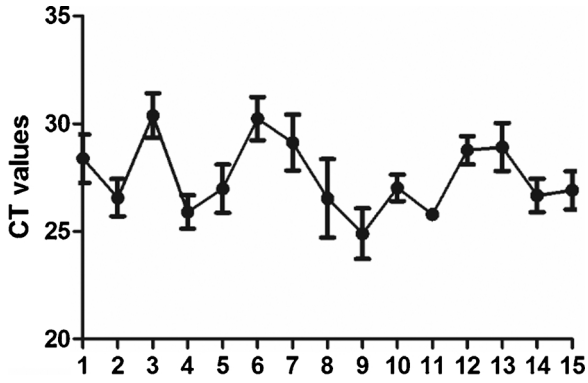

Fig. 1. Relative viral contents were detected via real-time PCR in 15 infected dogs.

Table 1
Changes in blood parameters of dogs infected with parvovirus.

	Leukocytes $\times 10^9.L^{-1}$	Erythrocytes $\times 10^9.L^{-1}$	Thrombocytes $\times 10^{12}.L^{-1}$	blood coagulation time /s
Ref	2.82a ± 0.24	14.78a ± 0.64	1107a ± 39,14	298.08b ± 11.58
Parvo	7.97b ± 0.31	14.73a ± 0.48	1141a ± 27.56	221.87a ± 13.38

evaluated by analyzing the sequence number, sequence length, GC content, Q20 and Q30 mass values, and effectiveness. The Q20 of the sample sequences was approximately 97%, and the Q30 was approximately 94% (Table 3). Therefore, the sequence quality met the requirements of the subsequent analysis.

3.4. Classification and analysis of fecal microbial species

An OTU is an artificial designation used to classify a specific unit (e.g., strains, species, genera, groupings) in phylogenetic research or population genetics for ease of analysis. All sequences were divided into OTUs according to their similarity levels. If the similarity between sequences exceeded 97%, the sequence was defined as an OTU, and each OTU corresponded to a representative sequence. Using the UCLUST program in the QIIME software package (Edgar, 2010; Parks et al., 2014), the tags were clustered at the 97% similarity level, and the OTUs were identified and taxonomically annotated as per the SILVA (bacteria) and UNITE (fungi) taxonomic databases. The total number of OTUs obtained in this experiment was 90, and the number of fecal microbial OTUs was higher in the healthy dogs than in the sick dogs (Fig. 2a).

Fig. 2b shows the relative species abundances in the two groups, with different colors representing different species. The infected dogs had relatively more *Escherichia*, *Lachnoclostridium*, (*Ruminococcus*) *gnavus* group, and uncultured_bacterium_f_lachnospiraceae but fewer *Collinsella*. *Alloprevotella* and *Sutterella* were absent among the fecal microorganisms in the infected dogs.

3.5. Species abundance and diversity analysis

The alpha diversity reflects the single-sample species abundance, richness, and species diversity. Various measures of alpha diversity include the Chao1, ACE, Shannon, and Simpson indices. The Chao1 and ACE indices measure species abundance, and the Shannon and Simpson indices measure species diversity, which is affected by species abundance and community evenness in the sampled communities. When the species abundances of two communities are the same but the species uniformity in one community is greater, the species diversity of that community is greater, and it will have a larger Shannon index and a smaller Simpson index (Grice et al., 2009). The OTU coverage was also calculated. Higher values indicate a higher probability that a species in the sample will be measured and a lower probability that it will not be measured. In this study, the Chao1 and ACE indices were both lower in the fecal microbial samples of the infected dogs and higher in those of the healthy dogs, indicating that the fecal microbial samples of the healthy dogs had more species. The Shannon and Simpson indices showed no significant differences between the two samples, and the coverage value indicated that the sequencing results represented the true situation of the microorganisms in the samples (Table 4).

An UPGMA clustering tree was combined with an abundance histogram (Lozupone and Knight, 2005). Fig. 3 (left) shows the clustering analysis results of six samples (three infected dogs and three uninfected dogs). The three fecal microbial samples from the infected dogs clustered together, as did the three fecal microbial samples from the uninfected dogs, and these two clusters were distinctly separated. These results indicate that the species composition of the fecal microorganisms in the infected dogs differed from that in the healthy dogs. Fig. 3 (right) shows the 10 most abundant OTUs in the samples from the six groups (three groups of infected dogs, each n = 5; three groups of uninfected dogs, each n = 5). These results were basically consistent with the abundance and diversity analyses, with a few differences between samples in the same groups.

3.6. Significance analysis of between-group differences

A significance analysis of the intergroup differences was used to identify biomarkers that differed significantly between the two groups. The qualified biomarkers were identified with the established biomarker screening standard, a linear discriminant analysis (LDA) score > 4. In this study, linear discriminant analysis effect size (LEfSe) was used to identify the species that differed significantly between the two samples (Segata et al., 2011). Fig. 4a and b show these results. *Clostridium* had the greatest effect in the fecal microorganisms in the infected dogs, followed by uncultured_bacterium_f_lachnospiraceae. The effect of *Alloprevotella* was greatest in the healthy dogs, which is consistent with the proposition that *Alloprevotella* is only found among the fecal microorganisms of healthy dogs.

3.7. ANOVA of the relative species abundances

ANOVA was used to compare the bacterial species abundances in the two samples to determine whether the mean values of the species abundances in the two samples (fecal microorganisms from infected and uninfected dogs) were equal and to determine the significance of the mean difference. Fig. 5 shows the 20 most abundant species with the lowest P values. Table S1 shows all taxa that differed significantly at the genus level. These results showed large numbers of *Collinsella* and *Sutterella* among the fecal microorganisms of the healthy dogs. The relative abundances of these genera were significantly higher in the healthy dogs than in the infected dogs. In the fecal microorganisms of the infected dogs, the relative abundances of *Romboutsia*,

Table 2
Changes in blood biochemical markers of dogs infected with parvovirus.

	Serum calcium $\times 10^{-2}$mg. ml^{-1}	Serum phosphorus $\times 10^{-2}$mg. ml^{-1}	Lactate dehydrogenase $U.L^{-1}$	Creatine kinase $U.L^{-1}$
Ref	9.91a ± 0.37	3.66b ± 0.78	271.8a ± 6.69	18.31a ± 0.99
Parvo	10.04a ± 0.55	2.29a ± 0.28	417.1b ± 10.89	31.09b ± 3.27

Table 3
Statistics for the sample sequencing results.

Sample ID	PE Reads	Raw Tags	Clean Tags	Effective Tags	AvgLen(bp)	GC(%)	Q20(%)	Q30(%)	Effective(%)
parvo 1	174,039	163,142	149,372	141,393	411	51.16	97.05	94.27	81.24
parvo 2	99,734	91,997	83,701	79,537	412	51.1	96.94	94.08	79.75
parvo 3	141,993	131,949	120,425	113,339	411	50.49	97.01	94.17	79.82
ref1	104,268	96,336	87,295	75,690	413	51.46	96.96	94.05	72.59
ref2	102,038	93,491	84,480	73,510	413	51.38	96.86	93.9	72.04
ref3	118,750	108,630	97,783	85,425	414	51.34	96.85	93.88	71.94

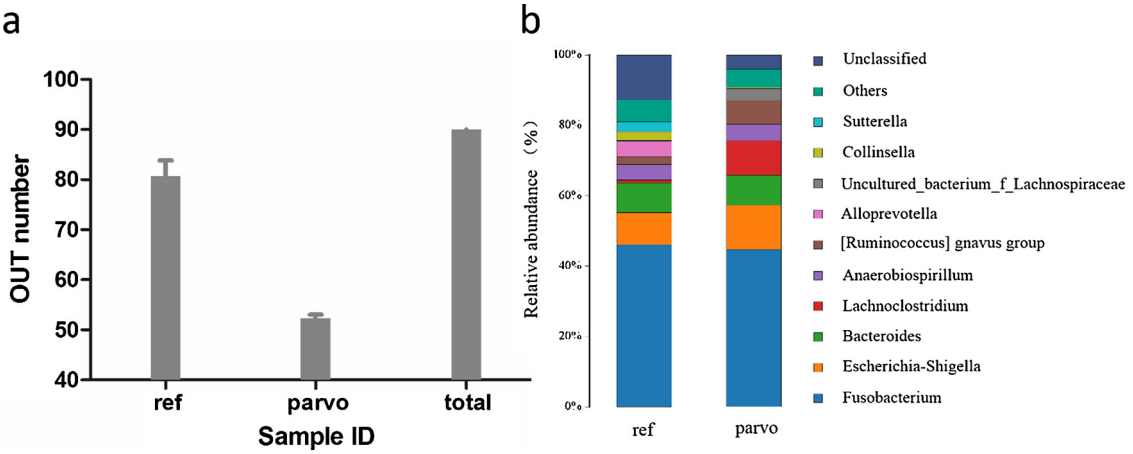

Fig. 2. Numbers and distributions of OTUs in infected and uninfected dogs. The sick dog sample group was named 'parvo', and the healthy (control) dog group was named 'ref'. a) Numbers of OTUs in infected and uninfected dogs. b) OTU distributions in infected and uninfected dogs.

Table 4
Alpha diversity indices.

Sample ID	OTU	ACE	Chao1	Simpson	Shannon	Coverage
ref1	78	80.85	81	0.1783	2.4659	1
ref2	77	77.825	77.33	0.1656	2.5223	1
ref3	87	87.321	87	0.1488	2.6396	1
parvo1	53	54.921	53.25	0.152	2.3487	1
parvo2	53	55.986	59	0.1592	2.3611	0.9999
parvo3	51	51.737	52	0.1729	2.3137	1

Erysipelatoclostridium, *Anaerotruncus*, and *Blautia* were significantly higher than those in the healthy dogs.

3.8. Functional trends of fecal bacteria in infected and healthy dogs predicted using metagenomic analysis

PICRUSt software was used to compare the species composition information obtained from the 16S rRNA sequencing data and determine the functional bacterial compositions of the samples to analyze the functional differences between them (Parks et al., 2014). Fig. 6 shows the results. Microorganisms associated with the categories 'replication, recombination and repair', 'nucleotide transport and metabolism', 'transcription', and 'defense metabolism pathway' were significantly enriched in the microbial flora of the infected dog feces, whereas those associated with 'amino acid transport and metabolism', 'carbohydrate transport and metabolism', and 'energy generation and conversion' were significantly enriched in the fecal microbial flora of the healthy dogs. These data suggest that the functional gene composition of the gut microbiome was significantly altered in the infected dogs.

4. Discussion

Kelly in Australia (1978) and Thomson and Gagnon in Canada (1978) isolated canine parvovirus from the feces of dogs suffering

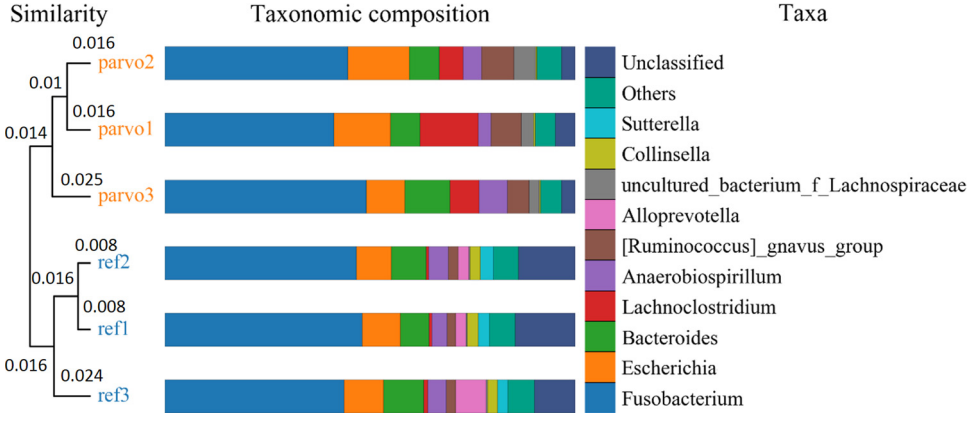

Fig. 3. Similarity of species compositions and abundances in all samples.

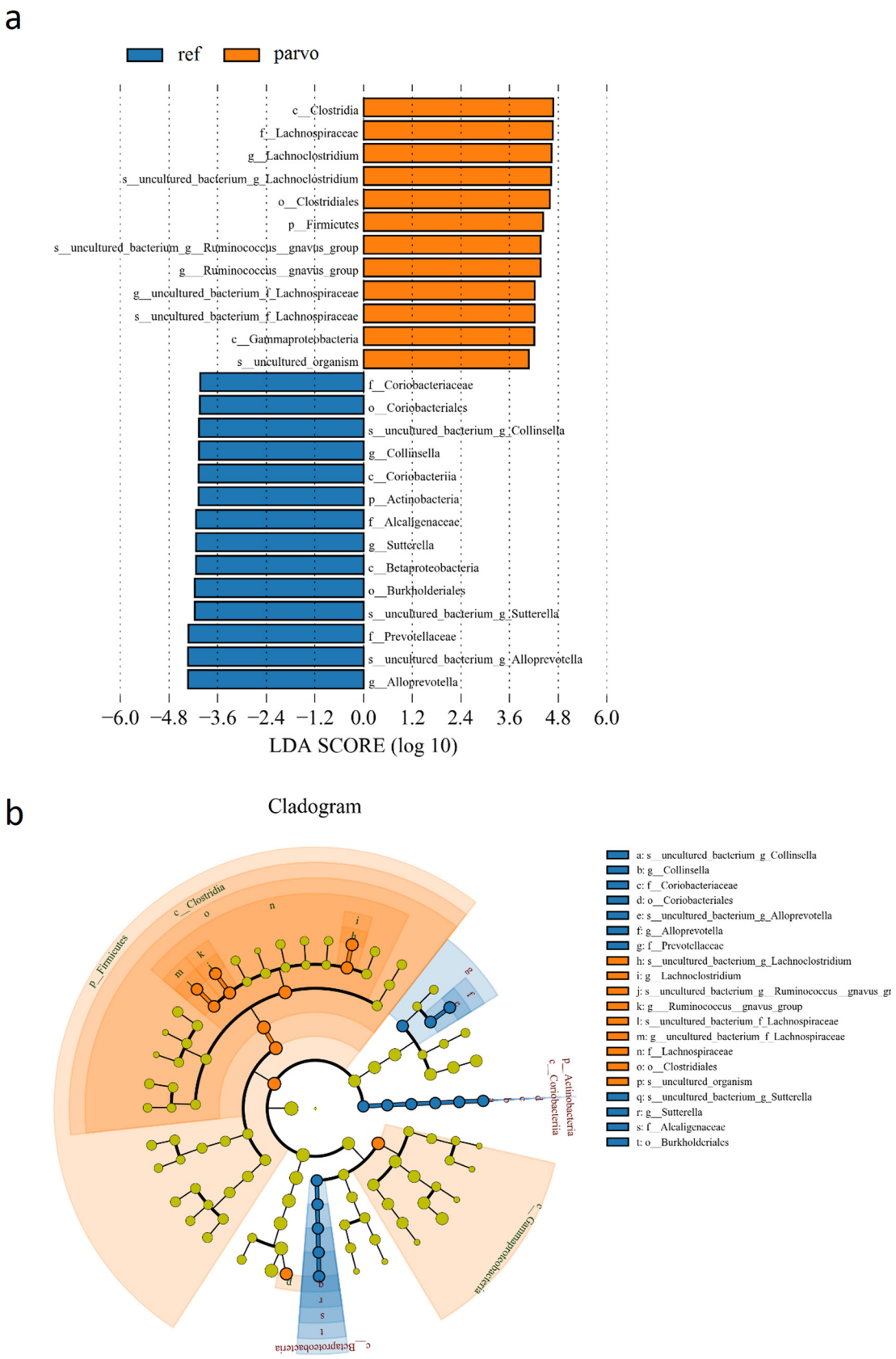

Fig. 4. Taxa whose abundances differed between the parvo-infected dogs and the healthy controls were identified using LEfSe. a) LDA value distribution histogram. b) LEfSe analysis of evolutionary branches.

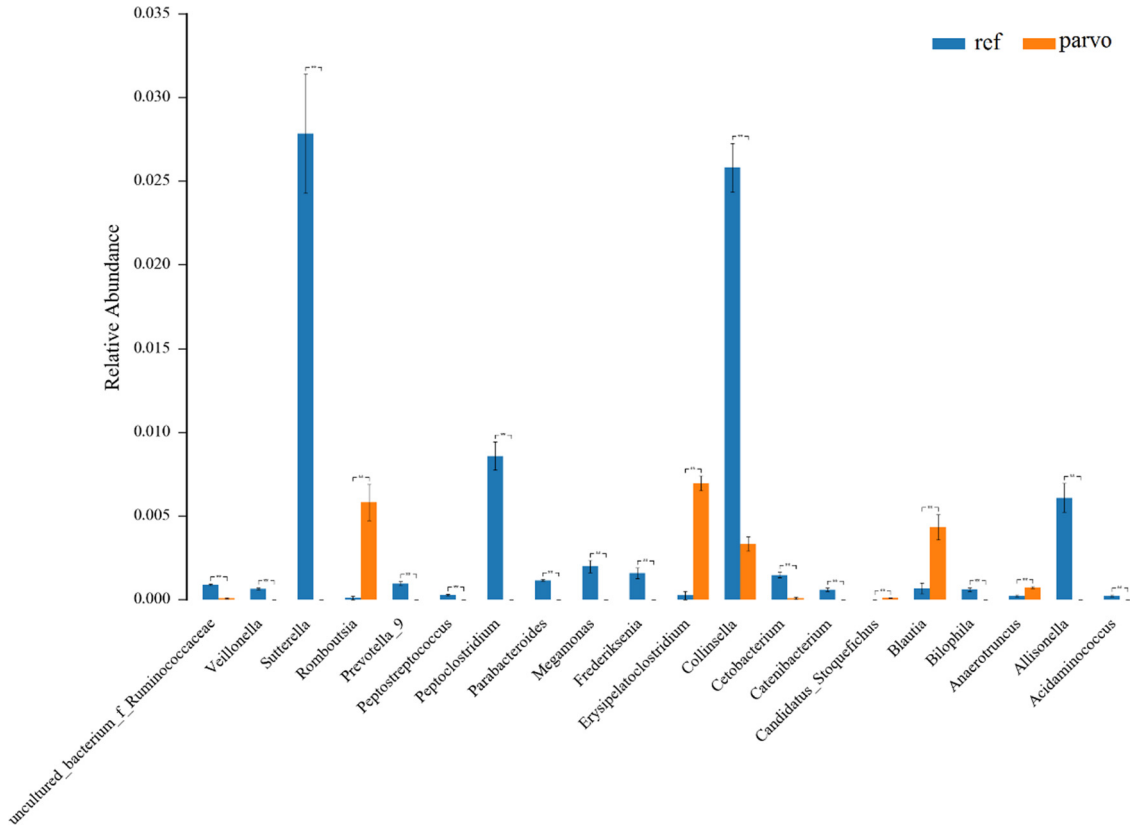

Fig. 5. Chart of significant intergroup species differences determined with ANOVA.

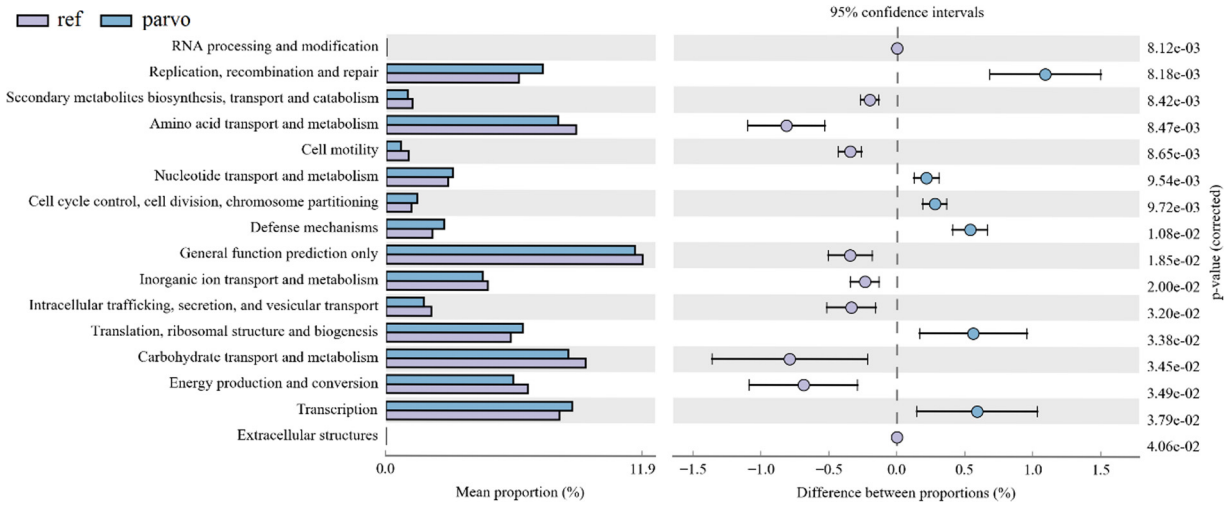

Fig. 6. Mean proportions and their differences in predicted functional metagenomes of the gut microbiota performed with Clusters of Orthologous Groups of proteins (COG).

enteritis. Since this virus was discovered, it has been detected worldwide and is one of the most severe infectious diseases affecting dogs (Nelson et al., 1979; Reed et al., 1988). After the virus infects a healthy dog through the dog's digestive tract, the virus predominantly attacks the cardiomyocytes and intestinal epithelial cells. The former are affected in puppies, and infected intestinal epithelial cells cause dogs to display enteritis symptoms, which can be fatal when severe (HaliGur et al., 2009). In this study, 15 infected dogs were tested using real-time PCR and physiological and biochemical methods. The viral copy numbers in these 15 infected dogs were nearly the same, and the physiological and biochemical indices were similar. The microbial diversity in the fecal specimens from the infected dogs was slightly less than that in the feces of the healthy dogs, but the Shannon and Simpson indices did not differ significantly between the two groups. An abundance histogram showed the differences in species compositions and abundances between the two groups, which were consistent with those of previous reports (Zheng et al., 2018). LEfSe significance analysis of the differences between the two groups was basically consistent with the abundance histogram. ANOVA of the relative species abundances showed that the relative abundances of several bacterial genera, including *Romboutsia*, E*rysipelatoclostridium*, *Anaerotruncus*, and *Blautia*, were significantly increased in the fecal bacteria of the diseased dogs.

Identifying microorganismal changes in sick dogs is difficult because available samples are limited, and the ages, breeds, and degrees

of infection of the infected dogs vary greatly. These microbial changes have been predominantly studied by artificially inoculating dogs with viral strains. However, in this study, we obtained similarly matched sick and healthy dogs from a dog-breeding base and confirmed the diagnosis with real-time PCR and physiological tests. Variations in the microorganisms in the fecal samples from the sick and healthy dogs were determined via several methods. Although the results sometimes deviated in repeated samples, a clustering analysis and ANOVA showed that the data were authentic and reliable.

Campylobacter (Table S1), *Bacteroides* (Fig. 2b), and *Clostridium* (Table S1) were also elevated in the fecal microorganisms of the infected dogs. Previous studies have also linked these bacteria to inflammatory bowel disease in dogs (Bell et al., 2008; Jia et al., 2010), but in these cases, bacteria such as *Bacteroides* were less abundant (Jia et al., 2010; Suchodolski et al., 2010; Hooda et al., 2012). This suggests that the fecal microbial flora composition and species may vary with the disease involved.

Interestingly, *Fusobacterium* and *Streptococcus* were slightly reduced in the fecal tracts of the parvovirus-infected dogs, and *Peptoclostridium* and *Peptostreptococcus* were undetected, which is inconsistent with some previous studies (Zheng et al., 2018). We speculate that this may be attributable to differences in the breeds, ages, and long-term living environments of the dogs studied (Parrish, 1995; Zheng et al., 2018), or these species may not be characteristic of the fecal microbes of dogs infected with parvovirus.

Relatively more *Lachnoclostridium*, *Ruminococcus gnavus*, and unculturable bacterium_f_lachnospiraceae were found among the fecal microorganisms of the infected dogs (Fig. 2b). *Alloprevotella* was only found among fecal microorganisms of healthy dogs. The fecal tracts of the infected dogs contained no new microorganisms, and all bacteria in the infected dogs were also present in the healthy dogs. The intestinal environment of the infected dogs is unsuitable for the survival of genera such as *Alloprevotella*, which decomposes sugars in the intestinal tract (Qu et al., 2017). The abundances of other species, such as *Collinsella* and *Sutterella*, were relatively reduced or undetected in the infected dogs (Fig. 2b). *Collinsella* usually occurs in healthy individuals (Bag et al., 2017), whereas *Sutterella* is related to gastrointestinal diseases (Suchodolski et al., 2012). Therefore, these two bacterial genera are significantly inhibited by environmental influences, whereas other genera are more adapted to the intestinal environment of sick dogs. At present, the functions of these genera in the intestinal tracts of dogs remain unclear and require further study.

In summary, the bacterial taxa, *Romboutsia*, *Erysipelatoclostridium*, *Anaerotruncus*, *Blautia*, *Lachnoclostridium*, *Ruminococcus gnavus*, and uncultured bacterium_f_lachnospiraceae, were greatly increased in the parvovirus-infected dogs. We speculate that the intestinal microbiota of the infected dogs is involved in molecular events that interfere with DNA replication in the bacterial population. Because no samples were uniquely infected with an enteritis-associated bacterial taxon, the study could not determine whether the enteritis caused by the canine parvovirus had unique characteristics, and the corresponding roles of the canine intestinal microorganisms remain unclear. In our next study, we will conduct in-depth research in these directions.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. supplementary data

Supplementary material related to this article can be found in the online version

Supplementary Table S1. Chart of significant intergroup species differences detected with ANOVA.

Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108390.

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