



Variations in gut microbiome and metabolites of dogs with acute diarrhea in poodles and Labrador retrievers

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Abstract

For different breeds of dogs with acute diarrhea, the gut microbiota and metabolome profiles are unclear. This prospective observational study analyzed the gut microbiomes of poodles with acute diarrhea and Labrador retrievers with acute diarrhea based on 16S amplicon sequencing, with respective healthy dogs as controls. Fecal non-target metabolomics and metagenomics were performed on poodles with acute diarrhea. This study found that the diversity and structure of the microbial community differed significantly between the two breeds in cohorts of healthy dogs. Two breeds of dogs with acute diarrhea demonstrated different changes in microbial communities and metabolic functions. The metabolism of starch and sucrose was significantly decreased in dogs with acute diarrhea, which may be attributed to the reduced activity of dextran dextrinase. Non-targeted metabolomics identified 21 abnormal metabolic pathways exhibited by dogs with acute diarrhea, including starch, amino acid, bile acid metabolism, etc., and were closely related to specific intestinal flora. This study provided new insights into breed specificity and the development of dietary treatment strategy in canine gastrointestinal disease.

Keywords QIIME2 · Domestic canine · Acute diarrhea · Microbiome · Metabolome · Metagenomics

Introduction

Acute diarrhea in dogs is a common gastrointestinal disorder, which episodes were induced by complex factors, such as dietary, infectious, and systemic (Pilla and Suchodolski 2021). Intestinal microorganisms are important subjects for the study of acute gastrointestinal disorders since they play an important role in defending against pathogens, stimulating the immune system, supporting host digestive metabolism, and providing intestinal energy nutrition (Pilla and Suchodolski 2021; Suchodolski 2011). The metabolome is the collection of all small molecule metabolites in a biological system, providing key information for clinical diagnosis and physiological assessment (Singh 2020; Suchodolski 2022).

Recent advances have been made in characterizing the microbiome and its metabolites in dogs with acute diarrhea using sequencing technologies and metabolomics (Pilla and Suchodolski 2019). For example, studies have shown

that dogs with acute diarrhea developed severe dysbiosis, with a reduction in bacteria producing SCFA such as *Blautia* and *Faecalibacterium*, and an increase in *Clostridium* (Suchodolski et al. 2012). Not only that, fecal, serum, and urine metabolites also changed significantly with the development of acute diarrhea (Guard et al. 2015). These suggest that the occurrence of acute diarrhea can have a severe impact on the host's gut microbiota and overall metabolic profile, although the clinical manifestations appear to be mild or even without complications. These microbiomic and metabolomic features, which are manifested in acute diarrhea for dogs, are also present in chronic enteropathy (CE) (e.g., idiopathic inflammatory bowel disease, IBD) in dogs and humans (Minamoto et al. 2019). Notably, the long-term effects of acute diarrhea may contribute to the development of CE. Regarding breed differences, the breeds of dog included within the case-control group were mixed, such as mixed breed, border terrier and samoyed (Pilla and Suchodolski 2021; Suchodolski et al. 2012). Although acute or chronic gastrointestinal disease can affect all canine breeds, certain breeds exhibit specific phenotypes. For example, studies have reported that yorkshire dogs with chronic enteropathy exhibited breed-specific patterns regarding flora dysbiosis and fecal metabolomics (Galler et al.

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2022). To date, studies on comparing the fecal microbiome and metabolomic characteristics of dogs with acute diarrhea under different breeds have not been performed. In addition, there is only limited information upon the metagenomics of dogs with acute diarrhea.

This work was aimed at investigating the gut microbiome of acutely diarrheal poodles and acutely diarrheal Labrador retrievers, in comparison to healthy that of their respective breeds. Furthermore, the impact of acute diarrhea on fecal metagenomics and metabolomics of poodles were also investigated. It is hypothesized that the diversity of microbiomes differed between the two breeds of dogs under acute diarrhea.

Materials and methods

Dog recruitment and fecal collection

This is a case–control study from two breeds of dogs suffering from acute diarrhea. We recruited poodles and Labrador retrievers in various regions of China and divided them into four groups: healthy poodles (PH), poodles with acute diarrhea (PD), healthy Labradors (LH), and Labradors with acute diarrhea (LD). All dogs came from private households and were fed a variety of commercial and homemade foods. During fecal sample collection, pet owners were given a questionnaire to assess whether the dog’s feces met the inclusion criteria. The Bristol score was used to identify the type of stool, which is pellet-like (1 score), sausage-like but with raised clumps on the surface (2 scores), sausage-like but with cracks on the surface (3 scores), snake-like with a smooth surface (4 scores), marshmallow-like (5 scores), porridge-like (6 scores), and liquid (7 scores) (Zhou et al. 2019). The identification of obese dogs was based on the Nestle Purina body condition evaluation criteria (Ramos et al. 2022). The inclusion criteria of healthy dogs were: (1) no gastrointestinal diseases at least 4 weeks before sampling and 3 weeks after sampling; (2) Bristol score of collected feces was 1–4. The inclusion criteria for dogs with acute diarrhea were as follows: (1) all dogs were relieved or cured within 3 weeks from the date of onset of diarrhea symptoms; (1) feces were collected during the diarrhea period, and the Bristol score was 5–7. Dogs using antibiotics, probiotics, prebiotics, or other medications within 1 month prior to sample collection were excluded. After the dogs defecated freely, the fecal samples were collected by the pet owners and sent to the laboratory for storage at –80 °C. The study was conducted with written informed consent from all pet owners. This study did not require specific ethical review and approval as only freely excreted fecal samples were collected from dogs.

16S rRNA gene sequencing and data analysis

Sample treatment and 16S rRNA gene sequencing

The collected fecal samples were ground into powder with liquid nitrogen and extracted genomic DNA by the cetyl-trimethylammonium bromide method (Yang et al. 2021). Subsequently, the obtained DNA was amplified as a template by polymerase chain reaction (PCR) in Phusion High-Fidelity PCR Master Mix with GC Buffer using the bacterial 16S V4 region (515F and 806R) as primers (Johnson et al. 2019). Sequencing libraries were constructed employing the IIDNA Library Prep Kit (E7645, New England Biolabs), and then evaluated on Qubit fluorometer (Thermo Scientific). Lastly, library sequencing was undertaken on the Illumina NovaSeq6000 platform to obtain 250 bp paired-end reads.

Data processing and species annotation

Paired-end reads were split, merged and spliced using FLASH software (V1.2.11) for obtaining raw tags (Magoč et al. 2011). Raw tags were filtered using fastp software (V0.20.0), and chimeras were removed using Vsearch software (V2.15.0) (Haas et al. 2011). Next, to acquire the initial amplicon sequence variation (ASV), we denoised these tags and removed ASVs with abundance less than 5, using DADA2 module in QIIME2 software (V202006) (Li et al. 2020). Species annotation was carried out with the Silva database of QIIME2 software (<https://www.arb-silva.de/>).

Diversity analysis and community function prediction

Six indicators—Observed otus, Chao1, Shannon, Simpson, Dominance, and Pielou e—were estimated in QIIME2 to examine the alpha diversity of the samples (Liu et al. 2021). Using the psych package and pheatmap package of R program (Version 2.15.3), we conducted Spearman correlation analysis to examine the association between clinical indicators (body weight and age) and alpha diversity index. In QIIME2, beta diversity was evaluated in relation to bray–curtis dissimilarity to examine differences across groups. Using the ade4 package and ggplot2 package in R software (V2.15.3), principal coordinates analysis (PCoA) was carried out to depict differences between groups in complicated multidimensional data. To evaluate the significance of differences across groups for all diversity markers, the Wilco rank sum test in QIIME2 program was employed. We used multivariate analysis by linear models (MaAsLin, <https://huttenhower.sph.harvard.edu/galaxy/>) with the associated metadata as covariates to account for the effects of sex, age, and body weight on the abundance of microbial

communities within subgroups. MetaStat analysis was utilized to identify taxa that were substantially different between groups at the phylum and genus taxonomic levels with R software (V3.5.3). Linear discriminant analysis (LDA) with effect size (LEfSe) was used to identify biomarkers across various groups, with the LDA score cutoff set at 3. Furthermore, to investigate the functions of microbial communities in different groupings, functional annotation analysis was performed using the Tax4Fun R package (<http://tax4fun.gobics.de/>). The functional differences among different groups were tested by *T* test method.

Metagenomic sequencing and analysis

Metagenomic sequencing of eight fecal samples (from four healthy poodles and four poodles with acute diarrhea) was performed. The genomic DNA was isolated using the Magnetic Stool DNA Kit (TIANGEN, China) and randomly interrupted into fragments of around 350 bp in length. The entire library was constructed by end repaired, A-tailed, ligated with Illumina adapter, purification and PCR amplification. Finally, sequencing was performed on the Illumina HiSeq PE150 platform.

The raw data of sequencing were preprocessed to remove low quality reads using Readfq software (V8). Reads from the host (domestic canine) were filtered out using Bowtie2 software (V2.2.4) (Karlsson et al. 2012). Subsequently, the clean data obtained above was assembled using MEGAHIT software (v1.0.4-beta) to obtain scaftigs without N (Feng et al. 2015). The scaftigs (≥ 500 bp) of each sample were performed ORF prediction using MetaGeneMark (V2.10, <http://topazatech.edu/GeneMark/>), and the information of length less than 100 nt in the prediction results was filtered out (Qin et al. 2010). ORF prediction results were deduplicated to obtain non-redundant initial gene catalogue (Unigenes) using CD-HIT software (V4.5.8, <http://www.bioinformatics.org/cd-hit/>). The clean data were aligned to the original gene catalog, and Bowtie2 software (Bowtie2.2.4) was used to determine the number of reads on the alignment of the gene in each sample. We compared unigenes with bacterial sequences taken from NR database of NCBI (V20180102, <https://www.ncbi.nlm.nih.gov/>), using DIAMOND software (V0.9.9.110). Based on the lowest common ancestor (LCA) algorithm in the MEGAN software, the species annotation information of the sequence and the abundance information at each taxonomic level were calculated. In order to obtain functional data for individual samples, we compared unigenes with functional databases, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (V2018-01-01) (Kanehisa et al. 2000), the database of evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) (V4.5) (Huerta-Cepas et al. 2019),

and the carbohydrate-active enzymes database (CAZy) (V201801) (Treiber et al. 2020).

Fecal non-target metabolomic analysis

We performed untargeted metabolomics assays with liquid chromatography-mass spectrometry (LC–MS) techniques on 16 fecal samples (from 8 healthy poodles and 8 poodles with acute diarrhea). Sample preparation and assay, raw data preprocessing and metabolite annotation were performed by Novogene Co. Ltd (Tianjin, China). Details were provided in the Online Resource 1.

To identify biological differences in metabolite sets between subgroups, partial least squares discriminant analysis (PLS-DA) was conducted using MetaX software (Wen et al. 2017). The value of variable importance in the projection (VIP), which was determined using PLS-DA, represented the metabolite's contributions to the group. The fold change (FC) value of each metabolite was obtained by calculating the ratio of the mean values of all repeated quantitative values of the metabolite in the two groups. *P* value was the significance level of the difference between metabolites between groups using *T* test. VIP > 1.0 , FC > 1.2 , or FC 0.833 were evaluated as differentiating metabolites across groups with *P* value < 0.05 . To explore the role of differential metabolites in biochemical metabolic pathways, we performed KEGG enrichment analysis. We annotated metabolic pathways for all identified metabolites based on the KEGG pathway database. The enriched pathways in differential metabolites were tested using the hypergeometric test method, and *P* value ≤ 0.05 was considered to be significantly enriched. KEGG-enriched pathways are presented with plotted bubble plots from the ggplot2 package in R software. In order to further explore the extent of association between microbial communities and metabolites, we selected 21 genus-level differences in flora and 22 significantly different metabolites between PH and PD groups, and performed Pearson correlation analysis. Pearson's correlation coefficient (rho) and *P* values were computed and the correlation heat map was drawn in the metabolism and 16 s correlation analysis module in the NovoMagic platform (<https://magic.novogene.com/customer/main#/ms-menu-metabolize16s-association-analysis/>). $|r_{ho}| \geq 0.4$ and $P \leq 0.05$ were considered significant correlations.

Statistical analysis

The age, body weight, fecal score, defecation frequency and diarrhea duration of dogs in each group were expressed as mean \pm standard deviation. Statistical significance analysis among multiple groups was performed based on SPSS software (R26.0.0.0). The Kruskal–Wallis test was used for

continuous variables, and Fisher's exact test was used for Boolean variables.

Results

Clinical and statistical characteristics of animals

In the current case-control investigation, a total of 48 fecal samples from 48 canines in seven different locations of China were obtained (Table 1). Four groups—PH ($n=12$), PD ($n=12$), LH ($n=12$), and LD ($n=12$)—were formed from them. Between the four groups, there were generally no discernible differences in terms of age, gender, or frequency of defecation ($P>0.05$). Significant differences were found in obese subjects, fecal scores and geographical origin ($P<0.05$). There were significant differences in body weight ($P<0.05$) between the four groups due to

differences in the two breeds of small dogs (Poodle) and large dogs (Labrador retriever). Duration of persistent diarrhea was 13.5 ± 3.5 days for PD and 12.6 ± 4.0 days for LD, respectively.

Community diversity of the gut across breeds and disease states

To assess differences in richness, diversity, and evenness of samples, we performed alpha diversity analysis (Fig. 1a). Overall, it was difference in alpha diversity between PH and LH. The Chao1 index and Observed otus index were considerably higher in LH ($P<0.05$) than in PH, demonstrating that low-abundance species were more prevalent in Labrador retrievers than in poodles. But the Dominance, Shannon, Simpson and Pielou e indices were not significantly different between PH and LH. Additionally, no significant differences were observed in the Observed otus, Chao1, Dominance,

Table 1 Demographic and clinical details of samples

| Groups | PH | PD | LH | LD | P |
|--|-------------|--------------|---------------|--------------|--------|
| Sample size | 12 | 12 | 12 | 12 | NA |
| Age (years) | | | | | |
| Overall | 4.4 ± 3.6 | 4.3 ± 2.8 | 5.4 ± 4.9 | 4.1 ± 3.2 | 0.827 |
| Child | 0.6 ± 0.3 | 0.8 ± 0.2 | 0.5 ± 0.3 | 0.6 ± 0.2 | 0.468 |
| Adult | 4.1 ± 1.4 | 5.0 ± 1.4 | 4.9 ± 1.7 | 3.9 ± 1.8 | 0.686 |
| Elderly | 8.5 ± 1.7 | 7.0 ± 0.0 | 10.8 ± 3.8 | 7.8 ± 0.3 | 0.116 |
| Gender | | | | | |
| Male | 8 | 6 | 5 | 5 | 0.417 |
| Female | 4 | 6 | 7 | 7 | |
| Weight (kg) | 4.2 ± 0.9 | 4.0 ± 1.8 | 28.9 ± 10.9 | 33.1 ± 9.5 | <0.001 |
| Obese subjects | 0 | 4 | 0 | 4 | 0.001 |
| Stool score | 2 ± 1 | 6 ± 1 | 2 ± 1 | 6 ± 1 | <0.001 |
| Frequency of defecation (times/day) | 1.8 ± 0.5 | 2.2 ± 0.7 | 2.1 ± 0.5 | 2.3 ± 0.5 | 0.085 |
| Duration of diarrhea (days) | 0 | 13.5 ± 3.5 | 0 | 12.6 ± 4.0 | NA |
| Dosing history (antibiotics, probiotics, prebiotics and other drugs) | None | None | None | None | NA |
| Other illnesses | None | None | None | None | NA |
| Vaccination | 12 | 12 | 12 | 12 | NA |
| Dewormed | 12 | 12 | 12 | 12 | NA |
| Region | | | | | |
| Northeast China | 0 | 2 | 0 | 0 | <0.001 |
| North China | 3 | 4 | 3 | 5 | |
| Northwest China | 1 | 0 | 0 | 0 | |
| Central China | 1 | 0 | 2 | 0 | |
| Eastern China | 6 | 4 | 6 | 4 | |
| Southern China | 0 | 1 | 1 | 0 | |
| Southwest China | 1 | 1 | 0 | 3 | |

Format of age, weight, stool scores, frequency of defecation and duration of diarrhea: mean \pm standard deviation. Kruskal-Wallis test for the continuous factors. Fisher's exact test was performed on the categorical factors. P value ≤ 0.05 means significant difference

PH Health group of poodles, PD Acute diarrhea group of poodles, LH Health group of Labrador retriever, LD Acute diarrhea group of Labrador retriever, NA Not applicable

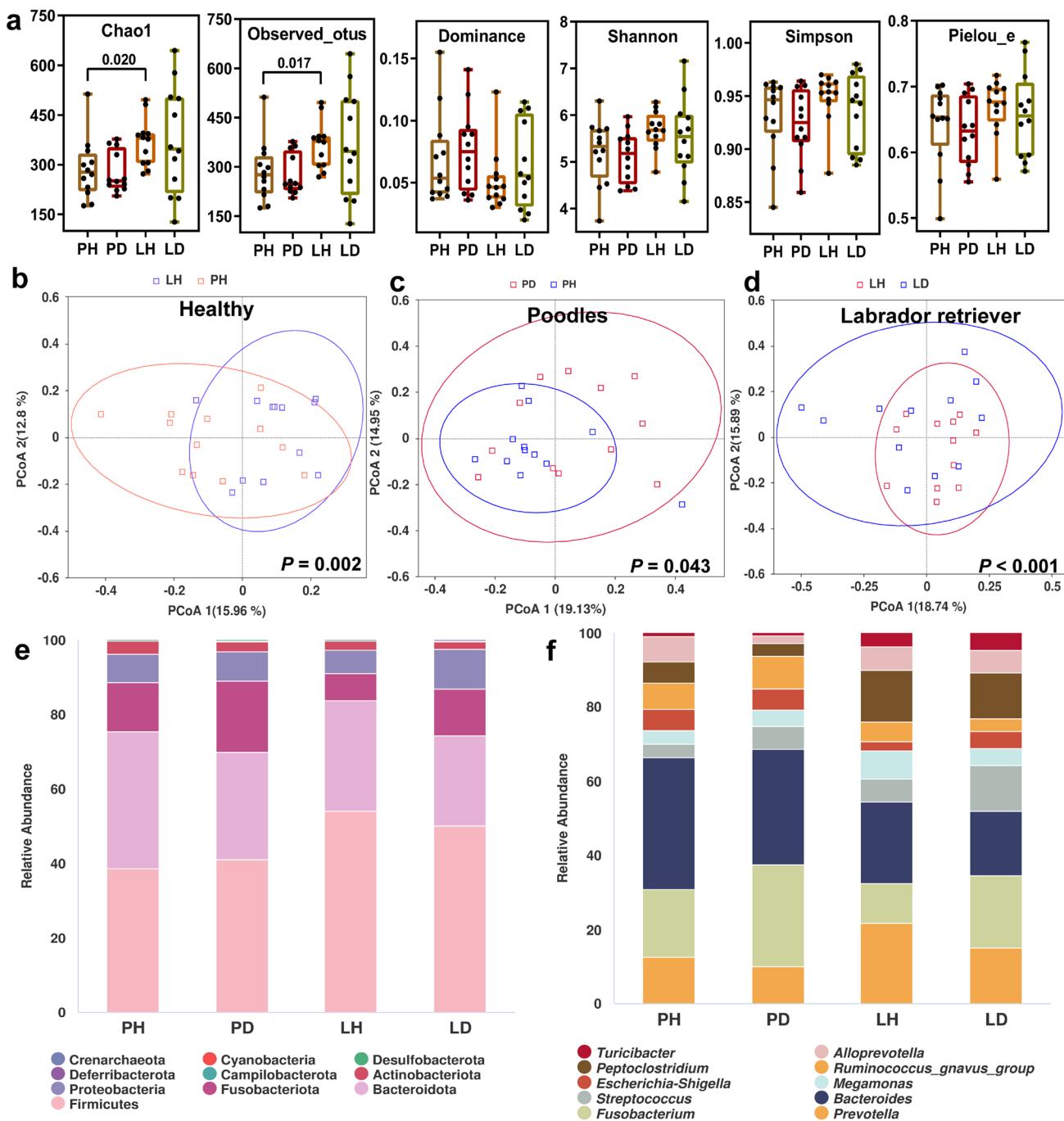


Fig. 1 Community diversity analysis and species relative abundance histograms between different groups. **a** Alpha diversity index between healthy and acute diarrhea groups from poodles and Labrador retrievers. $P < 0.05$ for significant differences and no labeling i. e. no significant differences (Wilco rank sum test). **b-d** Principal coordinate analysis (PCoA) according to bray-curtis distance algorithm. Wilco

rank sum test was employed to assess the significance of differences in beta diversity index between different groups, $P < 0.05$ indicates significant differences. **e-f** Histogram of the top 10 taxa ranked in relative abundance at the phylum (**e**) and genus (**f**) taxonomic levels for different subgroups

Shannon, Simpson, and Pielou e indices between disease-control groups in poodles (PH and PD) or in Labrador retriever (LH and LD). This suggested that the species richness, diversity, and evenness of the intestinal tract of

dogs with acute diarrhea did not differ from those of healthy dogs in both breeds. Spearman correlation analysis (Fig. S1 in Online Resource 1) found a significant positive correlation between body weight and Observed otus ($\rho = 0.0797$,

$P=0.008$) and Chao1 indices ($\rho=0.084$, $P=0.007$), which suggested that the higher the body weight, the higher the abundance of low-abundance species. Additionally, we discovered a negative correlation between age and Shannon ($\rho=-0.132$, $P>0.05$) and Simpson indices ($\rho=-0.154$, $P>0.05$), which may indicate that the diversity of gut microbiota tends to decrease with age, although no significant differences were seen (Fig. S1 in Online Resource 1).

In order to observe whether there are differences in community diversity and structure in different breeds and different disease states, we performed PCoA and beta diversity analysis in different cohorts.

For breed, microbial community diversity differed significantly between poodles and Labrador retrievers in the cohort of healthy dogs ($P=0.002$) (Fig. 1b), but no significant differences were observed in the cohorts of all dogs ($P=0.071$) (Fig. S2a in Online Resource 1) or dogs with acute diarrhea ($P=0.542$) (Fig. S2b in Online Resource 1). This difference remained significant ($P=0.002$) between PH and LH after adjusting for the effects of body weight, age, and gender using MaAsLin. From these data, it can be seen that the gut microbial community structure and diversity of different breeds of dogs are significantly different.

In addition, community diversity was markedly different in dogs with acute diarrhea compared to healthy dogs, whether in cohorts of all dogs (Fig. S2c in Online Resource 1, $P<0.001$), poodles (Fig. 1c, $P=0.043$), or Labrador retrievers (Fig. 1d, $P<0.001$). After adjustment for weight, age and gender, this difference was still significant in the cohort of all dogs ($P<0.001$) and Labrador retrievers ($P<0.001$). This suggested that acute diarrhea has led to alterations in gut community structure and diversity in dogs.

Differential microbial communities of the gut in different species and disease states

From the relative abundance histogram of the microbial community, we can see that at the phylum level, the intestinal flora of dogs are mainly Firmicutes (38.6–53.9%), Bacteroidota (23.7–36.6%), Fusobacteriota (7.4–19.0%), Proteobacteria (6.3–10.6%) and Actinobacteriota (1.8–3.5%) (Fig. 1e). At the genus level, the dominant intestinal bacterial communities of dogs were mainly *Prevotella* (6.5–12.4%), *Fusobacterium* (6.1–18.1%), *Bacteroides* (10.0–23.3%), *Streptococcus* (2.4–7.0%), *Megamonas* (2.6–7.3%), *Escherichia-Shigella* (1.3–3.7%) and *Ruminococcus gnavus group* (2.0–5.7%) (Fig. 1f).

To further explore which microbial communities were altered, Metastats analysis was performed on different subgroups and LEfSe was performed to find signature communities (Fig. 2).

We discovered that in different breeds, LH had greater levels of the phylum Firmicutes ($P=0.002$), Deferribacterota

($P=0.011$), and Acidobacteriota ($P=0.029$), as well as the genus *Peptoclostridium* ($P=0.025$), *Holdemanella* ($P=0.009$), and *Turicibacter* ($P=0.002$) (Supplementary Table 1). The genus *Bacteroides*, *Mogibacterium*, and *Anaerofilum*, however, exhibited higher PH values when compared to LH ($P=0.003$, $P=0.001$, and $P=0.001$, respectively) (Online Resource 2). LEfSe (Fig. 3a) found that the genus *Bacteroides* ($LDA=4.7$, $P=0.013$) under the family Bacteroidaceae and the species *Fusobacterium mortiferu* ($LDA=4.6$, $P=0.037$) under the phylum Fusobacteriota were more abundant in PH. The LH was dominated by the phylum Firmicutes ($LDA=4.9$, $P=0.002$) (Fig. 3b), including the genus *Holdemanella* ($LDA=3.9$, $P=0.002$) and *Turicibacter* ($LDA=3.8$, $P=0.004$) under the family Erysipelotrichaceae, the genus *Peptoclostridium* ($LDA=4.3$, $P=0.020$) and *Romboutsia* ($LDA=3.5$, $P=0.049$) under the family Peptostreptococcaceae, and the species *Blautia caecimuris* ($LDA=3.7$, $P=0.020$) under the family Lachnospiraceae. In addition, the species *Bacillus bogoriensis* ($LDA=3.6$, $P=0.006$) under the phylum Fusobacteriota were more abundant in LH than in PH.

According to metastats analysis (Online Resource 2), PD saw a significant increase ($P<0.05$) in the phyla Campylobacterota, Spirochaetota, and Deinococcota, as well as the genera *Actinomyces* and *Pseudonocardia*, while PH saw a significant decrease in the genus *Stenotrophomonas*, *Allisonella*, and *Muribaculum*. In PD compared to PH, there was a substantial decrease in the family Prevotellaceae ($LDA=4.5$, $P=0.037$) under the phylum Bacteroidota and the genus *Faecalibacterium* ($LDA=3.9$, $P=0.049$) under the phylum Firmicutes, according to LEfSe (Fig. 3c).

On the other hand, the phylum Patescibacteria, Planctomycetota and Armatimonadota, and the genus *Corynebacterium*, *Providencia*, *Brevibacterium*, *Kurthia* and *Acidovorax* were considerably higher in LD, whereas the genus *Ruminococcus gnavus group*, *Roseburia*, *Prevotellaceae NK3B31 group*, *Rubrobacter* and *Mesorhizobium* were significantly reduced in comparison to LH ($P<0.05$) (Online Resource 2). The results of LEfSe showed (Fig. 3e and f) that the differentially enriched microbial communities in LD were the family Brevibacillaceae ($LDA=3.1$, $P=0.032$) and the genus *Brevibacillus* ($LDA=3.1$, $P=0.032$) under the order Brevibacillales, the family Clostridiaceae ($LDA=4.0$, $P=0.015$) and the species *Clostridium colicanis* ($LDA=3.1$, $P=0.024$) under the order Clostridiales, the genus *Muribaculaceae* ($LDA=3.7$, $P=0.032$) under the family Muribaculaceae, and the genus *Nesterenkonia* ($LDA=3.1$, $P=0.032$) under the phylum Actinobacteriota. However, the genus *Ruminococcus gnavus group* ($LDA=3.7$, $P=0.032$) and species *Blautia caecimuris* ($LDA=4.0$, $P=0.028$) under the phylum Firmicutes, the genus *Prevotellaceae NK3B31 group* ($LDA=3.7$, $P=0.037$) and species *Bacteroides*

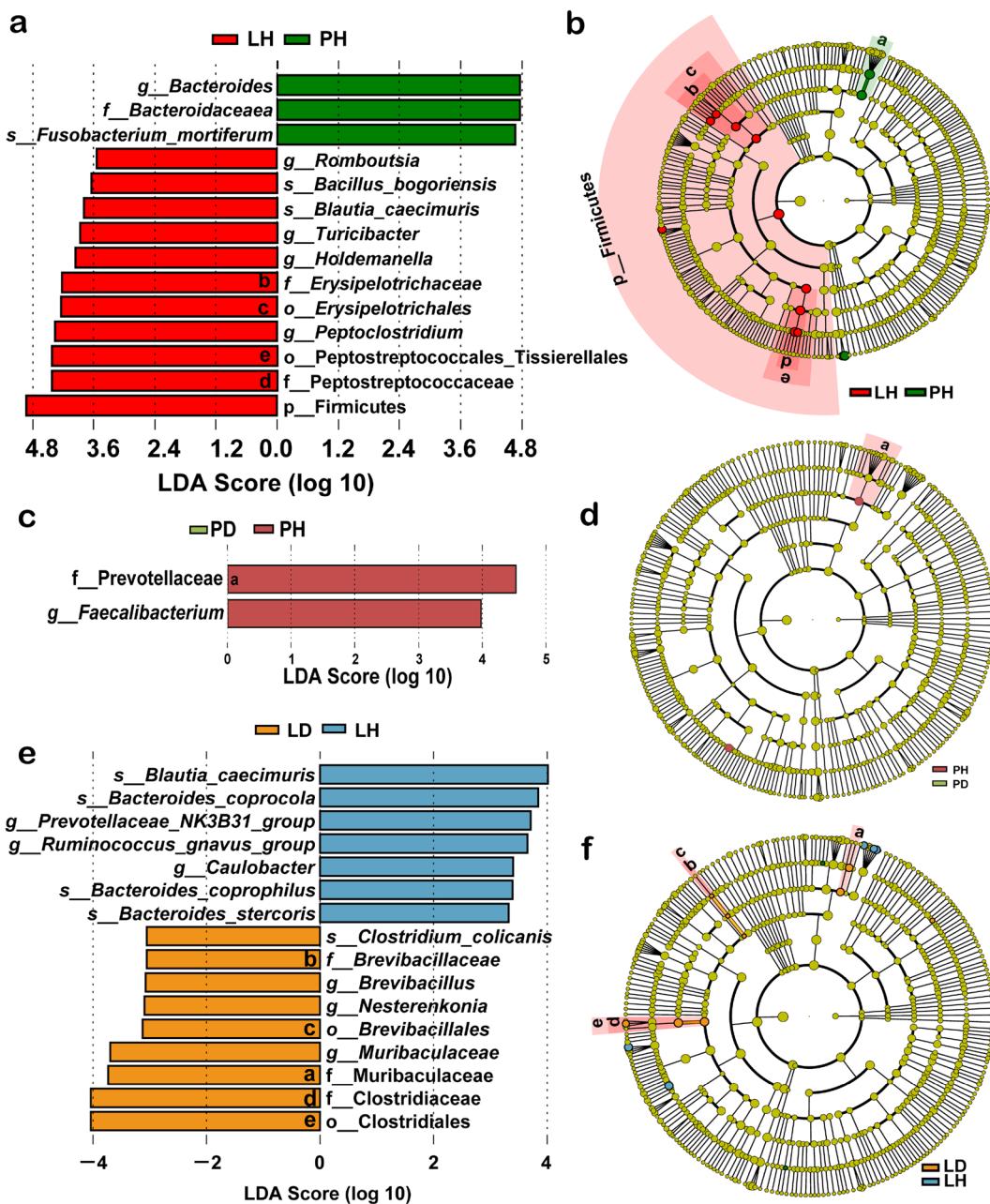


Fig. 2 LDA effect size analysis (LEfSe) between different comparison groups (LDA threshold > 3). **a, b** The distribution histogram of LDA values and evolutionary branching between PH and LH, the letters labeled in **b** correspond to **a**. **c, d** The distribution histogram of LDA values and evolutionary branching between PH and PD, the letters labeled in **d** correspond to **c**. **e, f** The distribution histogram of LDA values and evolutionary branching between LH and LD,

with the letters labeled in **f** corresponding to **e**. The circles radiating from the inside out in the evolutionary branching diagram represent the taxonomic level from phylum to species. Nodes represent taxon at that taxonomic level, and their size is proportional to the relative abundance size. All yellow nodes are species that do not differ significantly between comparison groups (color figure online)

stercoris (LDA = 3.3, $P = 0.017$), *Bacteroides coprophilus* (LDA = 3.4, $P = 0.037$) and *Bacteroides coprocola* (LDA = 3.8, $P = 0.015$), and the genus *Caulobacter* (LDA = 3.4, $P = 0.032$) under the phylum Proteobacteria were significantly lower in LD, compared to LH.

Functional prediction of the gut microbiome

To explore the gut microbiome function in different breeds and acute diarrhea states, we performed Tax4Fun analysis. From the first-level hierarchy of functional annotation

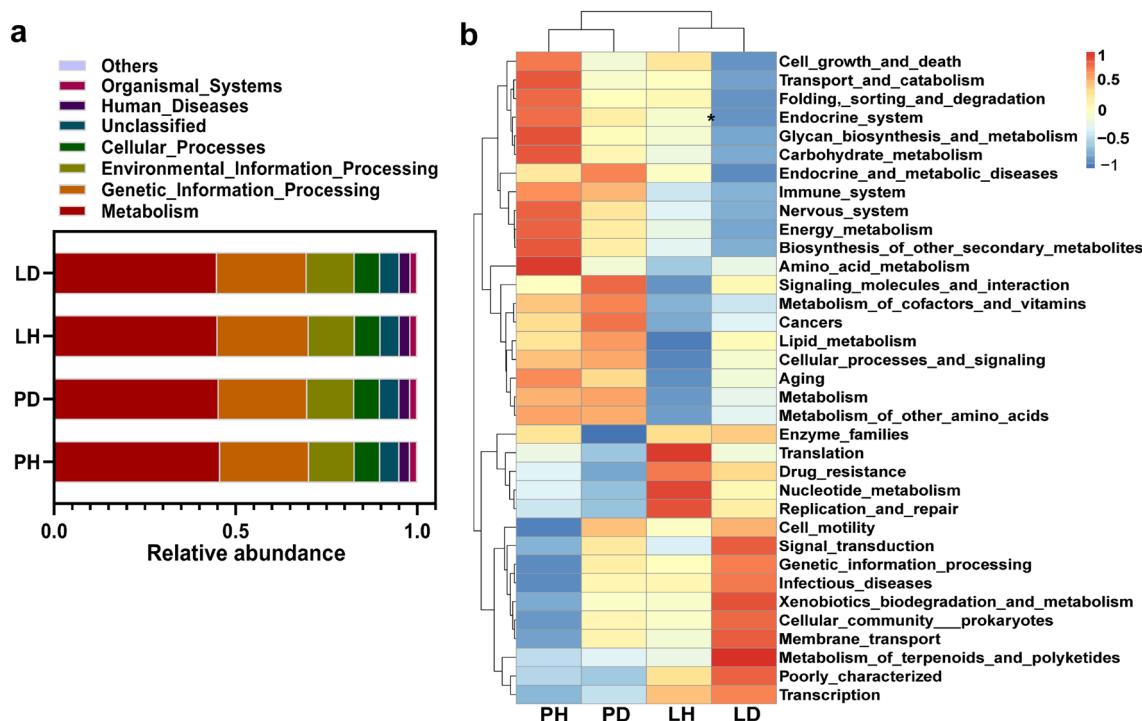


Fig. 3 Community functional annotation and clustering heat map of gut microorganisms under each subgroup based on Tax4Fun analysis. **a** Histogram of the relative functional abundance of different subgroups ranked in the top 10 at the first annotation level. **b** Cluster heat

map of the top 35 functional abundances at the second annotation level. Horizontal is the group, vertical is the functional annotation information, and left is the functional clustering tree

(Fig. 3a), it can be seen that the functions of the intestinal flora in dogs are mainly metabolism (44.9–45.7%), genetic information processing (24.3–25.0%) and environmental information processing (12.5–13.2%). Functional clustering heatmaps of the second hierarchy (Fig. 3b) revealed significant alterations in gut microbial community function across breeds and acute diarrhea states.

To further investigate the differences in community function between different subgroups, we performed *T* test for the function of the 3rd hierarchy. We observed that carbohydrate metabolism, transport and catabolism, metabolism of other amino acids, cellular processes and signaling, and biosynthesis of other secondary metabolites were more abundant in poodles (PH), while functions such as replication and repair, nucleotide metabolism, drug resistance, genetic information processing and viral protein family were higher in Labrador retrievers (LH) (Fig. 4a).

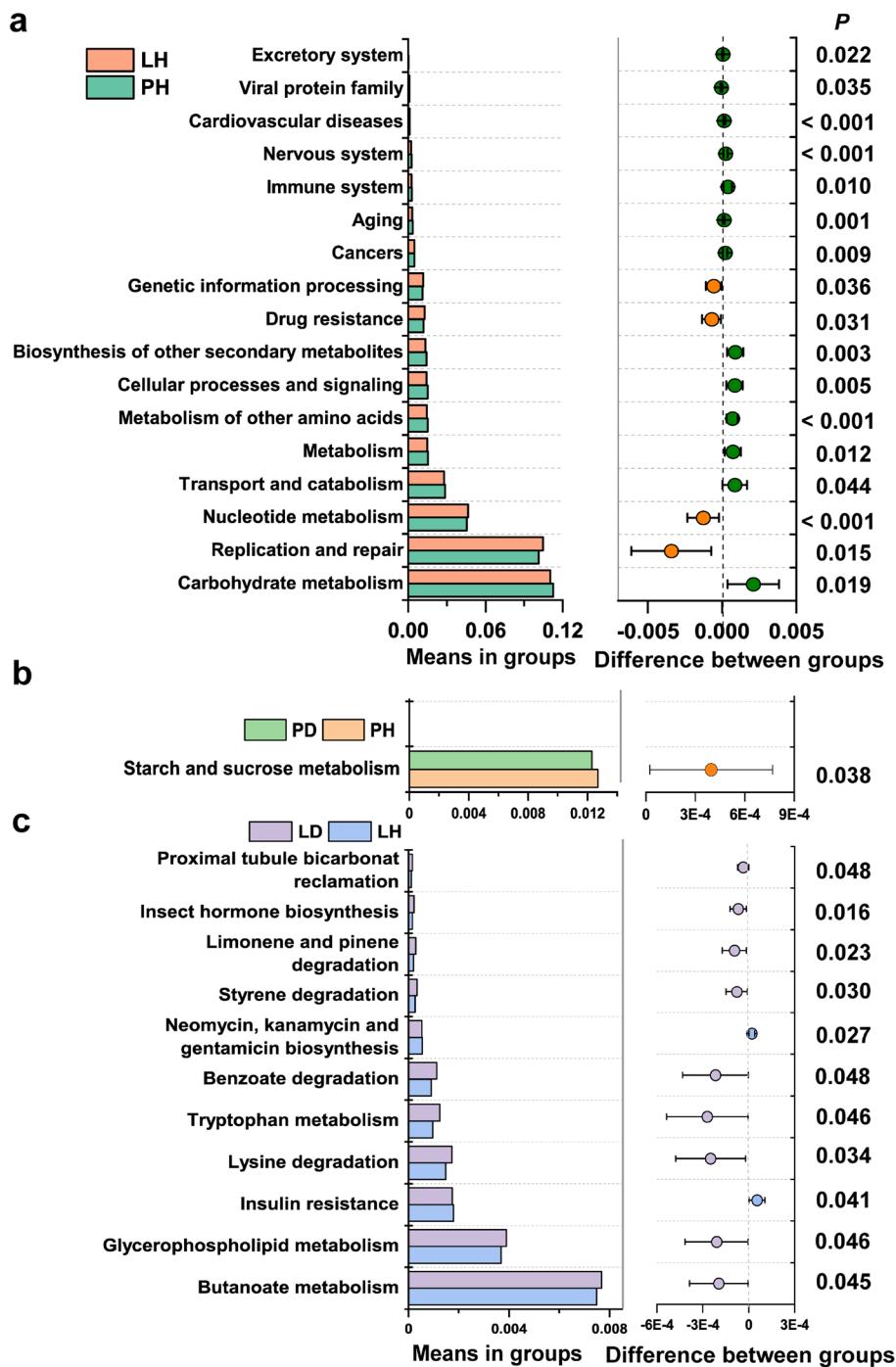
Furthermore, it is noteworthy that starch and sucrose metabolism function was significantly reduced in poodles with acute diarrhea (PD) in contrast to PH ($P = 0.038$). We further explored the differential carbohydrase and gene ortholog clusters between PH and PD using Metastat analysis based on the functional annotation of metagenomic sequencing in the CAZy and eggNOG database. We noticed that the levels of dextran dextrinase ($P = 0.040$),

alpha-1,6-L-fucosyltransferase ($P = 0.041$) and glucose-6-phosphate 1-dehydrogenase ($P < 0.001$) were noticeably lower in PD with respect to PH, while the levels of arabinan alpha-L-arabinosyltransferase ($P = 0.041$) and heparan beta-glucuronyltransferase ($P = 0.041$) were elevated in PD (Online Resource 3). Moreover, analysis (Fig. 4c) confirmed that in Labradors with acute diarrhea (LD), the functional abundance of insulin resistance ($P = 0.041$) and neomycin, kanamycin and gentamicin biosynthesis ($P = 0.027$) were significantly reduced, while butanoate metabolism ($P = 0.045$), glycerophospholipid metabolism ($P = 0.046$), lysine degradation ($P = 0.034$), and tryptophan metabolism ($P = 0.046$) was significantly enhanced, compared with LH.

Untargeted metabolomics of stool

To understand the possible metabolic changes caused by acute diarrhea, we next performed a non-target metabolomic assay on eight selected feces of poodles with acute diarrhea, using feces from healthy poodles as controls. 16 canine fecal samples yielded a total of 1661 identified compounds. The screening criteria for differential metabolites led to the identification of 115 significantly different metabolites, of which 7 were elevated in BD and 108 were downregulated in BD (Online Resource 4). We

Fig. 4 Differential community functions between different comparison groups based on Tax4Fun analysis. Histograms of the relative abundances of community functions significantly different at the third annotation level between **a** PH and LH, **b** PH and PD, and **c** LH and LD. Each bar on the left represents the average abundance of the difference function between different groups; the right is the confidence level of the difference between groups, the circle represents the mean difference, and its two endpoints represent the lower limit and upper limit of the 95% confidence interval. $P < 0.05$ was considered to be significantly different (T test)



observed that 23-norcholesterol, glycocholesterol and taurocholic acid were significantly elevated in PD ($P < 0.05$), while maltotriose, N6-acetyl-L-lysine, L-saccharopine, N-methyltryptamine, 1-methylnicotinamide, orotic acid, 4-acetamidoxylic acid, lysine butyrate and hydroquinone were significantly lower in PD ($P < 0.05$), compared with PH. According to PLS-DA (Fig. 5a), the fecal metabolite profiles were found to be significantly different between PH and PD. To further explore metabolic

pathways underlying differential metabolites, we performed KEGG enrichment analysis. Collectively, these differential metabolites were involved in 21 metabolic pathways (Fig. 5b and Online Resource 5). The lysine degradation pathway was substantially aberrant ($P = 0.027$) among the 12 pathways involved in amino acid metabolism. The aberrant metabolism of the digestive system was tightly correlated with three metabolic pathways: bile

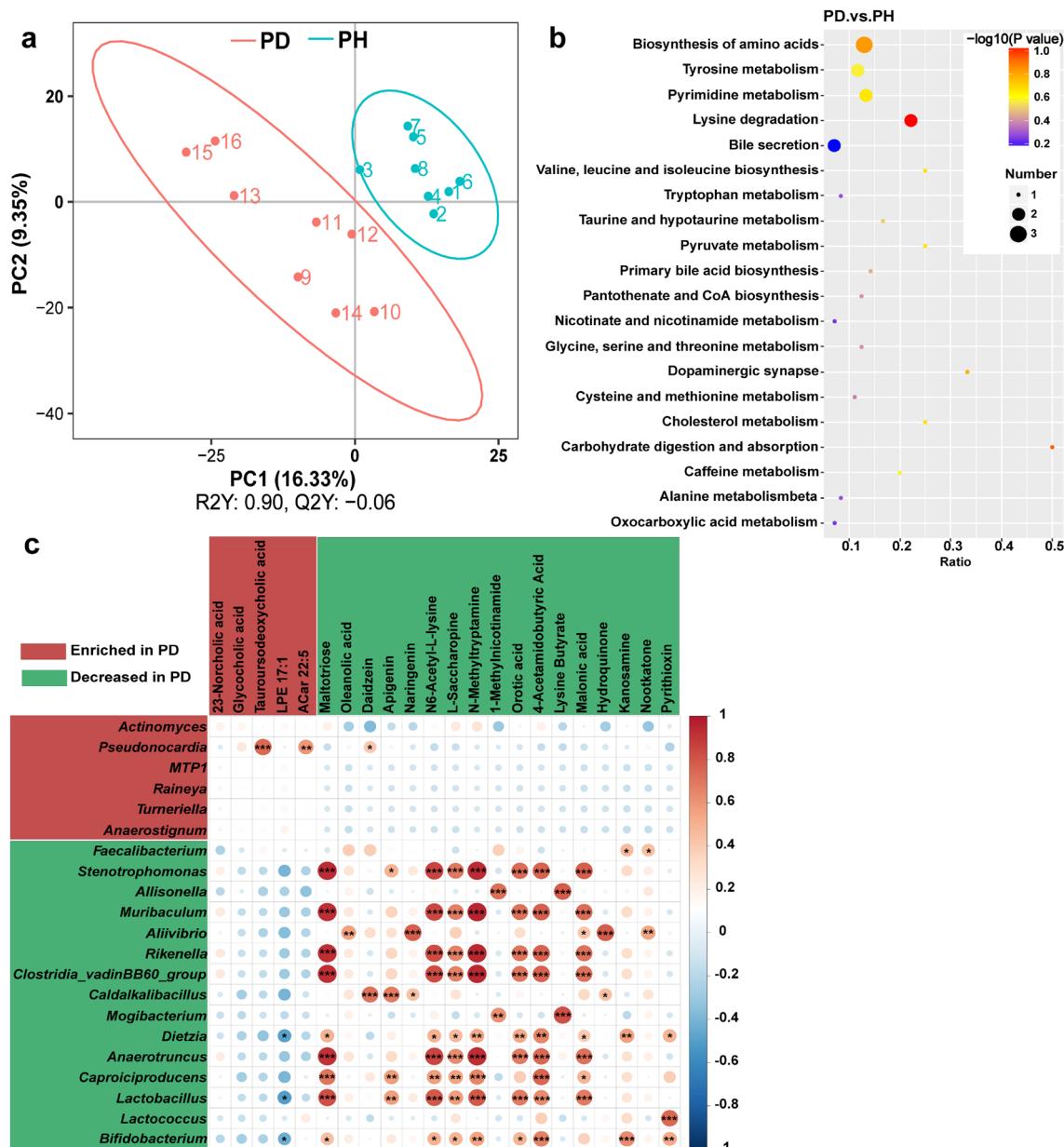


Fig. 5 Fecal untargeted metabolomics analysis of healthy group (PH) and acute diarrhea group (PD) from poodles. **a** Partial least squares discriminant analysis (PLS-DA) of fecal non-target metabolites between groups. $R^2 > 0$, indicating that the grouping model is more stable and reliable. **b** Bubble plot of KEGG pathways enriched for differential metabolites between PH and PD groups. The horizontal axis is the number ratio of differential metabolites to total metabolites in that metabolic pathway. The size of the dots represents the

number of differential metabolites in that pathway. The color of the dots denotes the P value (hypergeometric test). **c** Pearson correlation analysis of differential genera and differential metabolites between PH and PD groups. The color of the points indicates the Pearson correlation coefficient (r_{hol}), where blue indicates a negative correlation and red indicates a positive correlation. The size of the point indicates the level of significant difference (T test). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (color figure online)

secretion, cholesterol metabolism, and primary bile acid biosynthesis. In addition, abnormalities in the metabolism of pyruvate and the digestion and absorption of carbohydrates were shown.

Association analysis of fecal microbiome and metabolome

We performed Pearson correlation analysis in an attempt to investigate whether the gut microbiome of dogs with acute

diarrhea is associated with differential metabolites. The association analysis (Fig. 5c) disclosed that genera reduced in PD were positively correlated with metabolites significantly downregulated in PD and negatively correlated with metabolites significantly upregulated in PD, whereas vice versa. Specifically, the reduced genus *Stenotrophomonas*, *Muribaculum*, *Rikenella*, *Clostridia vadinBB60 group*, *Caldalkalibacillus*, *Dietzia*, *Anaerotruncus*, *Caproiciproducens*, *Lactobacillus* and *Bifidobacterium* in PD exhibited a significant positive correlation with maltotriose, N6-acetyl-L-lysine, L-saccharopine, N-methyltryptamine, orotic acid, 4-acetamidoxylic acid and malonic acid ($\rho > 0.4$, $P < 0.05$). The genus *Faecalibacterium* reduced in PD presented a significant positive correlation with kanosamine ($\rho = 0.45$, $P < 0.05$) and nootkatone ($\rho = 0.44$, $P < 0.05$). The genus *Allisonella* reduced in PD exhibited significant positive correlations with 1-methylnicotinamide ($\rho = 0.72$, $P < 0.05$) and lysine butyrate ($\rho = 0.78$, $P < 0.05$). The genus *Aliivibrio* decreased in PD showed significant positive correlation with oleanolic acid ($\rho = 0.56$, $P < 0.05$), naringenin ($\rho = 0.78$, $P < 0.05$) and hydroquinone ($\rho = 0.79$, $P < 0.05$). The genus *Caldalkalibacillus*, which decreased in PD, showed significant positive correlation with daidzein ($\rho = 0.71$, $P < 0.05$) and apigenin ($\rho = 0.68$, $P < 0.05$). The genus *Pseudonocardia*, which increased in PD, showed a significant positive correlation with taurooursodeoxycholic acid ($\rho = 0.74$, $P < 0.05$) and ACar 22:5 ($\rho = 0.60$, $P < 0.05$).

Discussion

We systematically investigated the gut microbiota variations in dogs of various breeds and acute diarrhea episodes in this study. As previously reported (Honneffer et al. 2017), the phyla Firmicutes, Bacteroidota, Fusobacteriota, and Proteobacteria dominated the intestines of both poodles and Labrador retrievers. The diversity and structure of the microbial communities, however, were noticeably different between the two dog breeds. Compared to poodles, Labrador retrievers have richer communities and more representatives of the phylum Firmicutes. Additionally, Labrador retrievers have a greater number of replication, repair, and nucleotide metabolism processes than do poodles. The level of carbohydrate metabolism and transport and catabolism of poodle is more vigorous. It is evident that the gut microbiota has undergone adaptive changes during the long-term evolution of different breeds.

Dogs with acute diarrhea exhibit several typical features of increased pathogenic and decreased beneficial bacteria. First, *Actinomyces* and *Pseudonocardia* were significantly elevated in PD. *Providencia*, *Clostridium colicanis* were significantly increased in LD, which is consistent with previous

reports on dogs with acute diarrhea (Pilla and Suchodolski 2019). *Actinomyces* has been reported to be significantly increased in human patients with BID (e. g. ulcerative colitis and Crohn's disease) (Li et al. 2018). The current study showed that although *Actinomyces* was not associated with the pathogenesis of inflammatory bowel disease, it caused alterations in immune factors, which can exacerbate the intestinal inflammatory response (Tajima et al. 2015). Numerous studies have demonstrated that *Providencia* can cause diarrhea in humans and dogs, with invasive infection being the most established pathogenesis. For example, *Providencia alcalifaciens* invaded the intestinal mucosa mainly through the polymerization of cytoskeletal components to achieve endocytosis, and additionally by disrupting tight junctions (Shah et al. 2019). On the other hand, we found that the genus *Faecalibacterium*, a recognized butyrate-producing bacterium (Pryde et al. 2002), was significantly decreased in PD. To date, *Faecalibacterium* is considered a bioindicator of human intestinal health and its reduction is associated with the development of inflammatory bowel disease and colorectal cancer. It has been shown that *Faecalibacterium* was involved in the regulation of the host intestinal inflammatory response through the production of anti-inflammatory metabolites (butyrate and salicylate, etc.) using glucose and prebiotics (e. g. arabinogalactan, etc.) (Guard et al. 2015; Suchodolski et al. 2012). These anti-inflammatory metabolites inhibited interleukin-8 production by blocking NF κ B activation, thereby promoting host intestinal homeostasis (Ferreira-Halder et al. 2017). In addition, we noticed that several known probiotics such as *Lactobacillus*, *Lactococcus* and *Bifidobacterium* were decreased in dogs with acute diarrhea. These potential beneficial bacteria have been proven to ferment and produce acid, reduce intestinal pH, inhibit the reproduction of pathogenic bacteria, and play an important role in promoting digestion and absorption, immune regulation, etc. (Guo et al. 2019).

As is known, the microbiota functions through symbiosis with the host, providing nutrients and participating in the metabolism of exogenous substances. Conversely, metabolites (molecules and compounds) that affect the flora can also alter the nature of the microbiota, which can have beneficial or detrimental effects on host health. The altered flora of dogs with acute diarrhea was closely associated with significantly altered metabolites. First, studies showed that kanosamine inhibited the growth of phytopathogenic oomycetes and certain fungi (Vetter et al. 2021). Our study found that kanosamine was significantly reduced in dogs with acute diarrhea and was positively correlated with reduced *Faecalibacterium*. This may provide a basis for further studies on whether *Faecalibacterium* regulates host intestinal flora and metabolism through the production of antimicrobial active substances such as kanosamine. Secondly, maltotriose is an oligosaccharide that can act as a prebiotic to

promote the growth of a variety of probiotic bacteria. We found that maltotriose was significantly reduced in dogs with acute diarrhea and showed a significant positive correlation with reduced genera of *Muribaculum*, *Lactobacillus* and *Bifidobacterium*. Maltotriose is a metabolite produced by exogenous macromolecules (such as starch and dextrin) in the digestive hydrolysis of the gastrointestinal tract, which requires the involvement of important sugar hydrolases such as dextran dextrinase. Through metagenomic analysis, we found that the activities of enzymes related to carbohydrate metabolism, such as dextran dextrinase, alpha-1,6-L-fucosyltransferase and glucose-6-phosphate 1-dehydrogenase, were significantly reduced in dogs with acute diarrhea. We hypothesized that these bacteria may have an impact on host health through their involvement in starch and sucrose metabolism.

Normally, bound and unbound bile acids are reabsorbed along the entire intestine into the portal vein by passive diffusion and active transit in the distal ileum, and are rapidly absorbed by hepatocytes, recombined and re-secreted into bile. This process is called enterohepatic circulation (Begley et al. 2005). We detected a significant increase in bound primary bile acids including glycocholic acid and taurooursodeoxycholic acid in the feces of dogs with acute diarrhea, which resulted in abnormalities in various biliary metabolic pathways such as bile secretion, cholesterol metabolism and primary bile acid biosynthesis. Notably, taurooursodeoxycholic acid was closely associated with an increase in *Pseudonocardia*. This suggested that the hepatic-intestinal circulation of bile is altered in dogs with acute diarrhea and that intestinal flora played a role in this (Suchodolski 2022). For example, beneficial gut bacteria convert primary bile acids into secondary bile acids by secreting bile salt hydrolyzing enzymes, thereby inhibiting the overgrowth of potential pathogens such as *Clostridium difficile* and promoting intestinal homeostasis (Weingarden et al. 2014).

In conclusion, this study showed significant breed-specific differences in intestinal flora diversity and structure between poodles and Labrador retrievers. The gut microbiome and fecal metabolic characteristics were altered in dogs with acute diarrhea. Acute diarrheal disease in dogs was associated with abnormalities in the metabolism of starch, sucrose, bile acids, and amino acids as well as an overgrowth of opportunistic pathogenic microorganisms. These findings offer a theoretical framework for the investigation of certain microbiome gut types and the formulation of nutritional and pharmacological approaches in companion animals with gastrointestinal disorders.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00203-023-03439-6>.

Author contributions HSB and ZZW designed and supervised the study. TL, SJW, and LYS collected samples, recorded information,

and generated data. HSB performed data analysis. ZZW conducted a data review. HSB wrote the manuscript with contributions from ZZW.

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Data availability The datasets generated in this study are all submitted to publicly available repositories. The 16 s and metagenomic high-throughput sequencing data generated in this study have been submitted to NCBI's Sequence Read Archive repository with accession ID PRJNA917802. The metabolome data generated in this study have been submitted to MetaboLights database with the unique identifier MTBLS6823.

Declarations

Conflict of interest The authors declare no competing interests.

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