Journal of Oceanology and Limnology
Vol. 40 No. 5, P. 2068-2078, 2022
https://doi.org/10.1007/s00343-021-1278-4

16S rRNA gene sequencing analysis on changes in the intestinal flora of *Procambarus clarkii* with "Black May" disease*

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Received Sep. 3, 2021; accepted in principle Oct. 8, 2021; accepted for publication Nov. 26, 2021

Abstract The morbidity and mortality peak of farmed *Procambarus clarkii* occurs around May every year, a phenomenon known as "Black May" disease (BMD). Increasing evidence shows that the intestinal flora is closely related to host health. We analyzed and compared the microbiota of healthy and BMD-affected *P. clarkii* intestines. The results show that there was no significant difference in bacterial α-diversity (richness P=0.59; evenness P=0.43; and diversity P=0.052) between the diseased group and the control group. Four dominant phyla in the intestines of crayfish in the control group, namely Tenericutes (30.86%), Bacteroidetes (29.99%), Firmicutes (22.23%), and Proteobacteria (15.23%), were identified. However, a striking shift in the microbial composition were found in the intestines of *P. clarkii* with BMD. Bacteroidetes was a dominant phylum in healthy *P. clarkii*, whereas the prevalence was low in diseased *P. clarkii* (1.87%). By contrast, the prevalence of Proteobacteria was significantly higher (P<0.05) in *P. clarkii* with BMD than in *P. clarkii* without BMD. *Candidatus Bacilloplasma*, *Bacteroides*, *Vibrio*, and *Aeromonas* showed significant differences (P<0.05) at the genus level. Tax4Fun function prediction indicated that the relative abundance of genes involved in energy metabolism in the intestinal flora of *P. clarkii* with BMD was significantly reduced (P<0.05). Therefore, BMD can change the composition of the intestinal microbiota of *P. clarkii*. This study contributes to the understanding of the relationship between intestinal flora and host especially in aquatic animals.

Keyword: *Procambarus clarkii*; "Black May" disease (BMD); intestinal flora; high-throughput sequencing

1 INTRODUCTION

Convincing evidence suggests that the intestinal microbiota plays an important role in maintaining host health, including nutrient absorption, pathogen defense, and antibiotic resistance (Ott et al., 2004; Sekirov et al., 2010; Dai et al., 2017). In addition, the intestinal microbial flora, which produces signaling molecules, can influence host metabolism, such as short-chain fatty acid (SCFA) production and vitamin synthesis (Watanabe et al., 2006). Therefore, the balance of the intestinal ecosystem is very important for host health, and a change in bacterial diversity may lead to host disease (Guarner, 2007; Othman et al., 2008). The outbreak of diseases in aquaculture is usually accompanied by disorder of the intestinal flora. Therefore, characterizing the differences in intestinal flora between healthy and diseased hosts can be a first step in improving disease prevention and treatment (Berry et al., 2012; Berry and Reinisch, 2013). Studies have explored the differences in intestinal microflora between healthy and diseased aquatic animals. In *Penaeus vannamei* with acute hepatopancreatic necrosis disease (AHPND), *Vibrio*

* Supported by the Natural Science Fund Project of Jiangsu Province (No. BK20181138) and the Central Public-Interest Scientific Institution Basal Research Fund, Freshwater Fisheries Research Center, CAFS (No. 2019JBFZ09)
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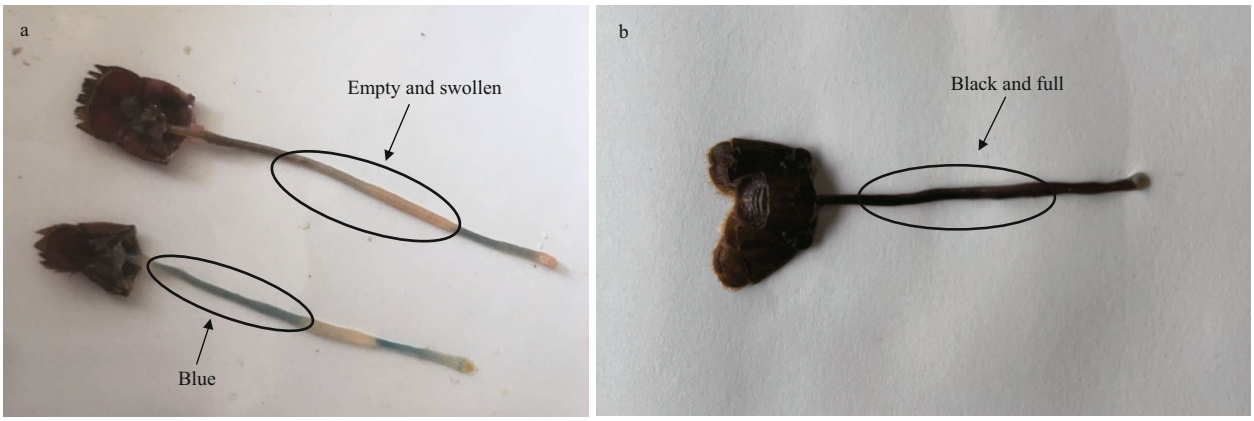

Fig.1 The intestines of diseased (a) and healthy (b) *P. clarkii*

The intestines of healthy *P. clarkii* was black and full, and the intestines of diseased *P. clarkii* was blue, empty, and swollen.

was enriched in the intestine (Dong et al., 2021). There were significant differences in intestinal microflora between healthy and diseased (suffering from furunculosis) *Coreius guichenoti* (Li et al., 2016). Some studies have shown that changes in the intestinal bacterial community are closely related to the severity of *P. vannamei*, and the indicator group can be used for health assessment (Xiong et al., 2015). However, the analysis of crustacean intestinal microbiota is still in its infancy, and it is not clear how diseases affect the intestinal microbiota.

Procambarus clarkii, a species of crayfish, is classified into arthropods, crustaceans, Decapoda, and the family Labridae. *P. clarkii* is native to northern Mexico and the southern USA, and it has spread to Europe, Africa, Asia, and other parts of the Americas (Gherardi, 2006). The species was introduced to China in 1929 (Yue et al., 2008). In recent years, it has become an important species in the aquaculture industry due to its high protein, rich nutrition, and delicious flavor. However, the peak of the disease season typically occurs in May, during the farming of crayfish, and the phenomenon occurred in May is commonly known as "Black May" disease (BMD) (Huang et al., 2020). Diseased crayfish usually exhibit reduced food intake. Their carapace is easily peeled off and movement is slow. Dissection of diseased individuals reveals that the intestine is empty, blue, and swollen (Fig.1a). BMD has caused tremendous economic losses to the crayfish farming industry. Once a pond becomes affected, nearly 90% mortality would be typically reported. At present, there are few studies on BMD in *P. clarkii* in China, so little is known about how BMD affects *P. clarkii*. Considering that the intestines of *P. clarkii* with BMD are empty and swollen, it is necessary to characterize the changes in intestinal microbiota.

To explore the influence of BMD on the intestinal flora of *P. clarkii*, we compared the intestinal flora of healthy and BMD-diseased *P. clarkii*, and found associated changes in the intestinal flora. The results provide a theoretical reference for the study of the intestinal bacteria of *P. clarkii* and will help in the prevention and treatment of this disease.

2 MATERIAL AND METHOD

2.1 Sampling

The diseased crayfish used in the experiment were collected from a *P. clarkii* farm with BMD in Xuyi County, Jiangsu Province, China. The organisms exhibited reduced food intake and lethargy. Dissection of diseased individuals reveals that the intestine is empty, blue, and swollen. Our samples (control and diseased) were collected from different ponds. The intestines of 10 healthy (control) and 10 diseased *P. clarkii* were collected, frozen in liquid nitrogen, and stored at -80 ℃. And our laboratory through PCR amplification and observation with transmission electron microscopy to detect the pathogens in the sampled crayfish, including white spot syndrome virus (WSSV) and *Procambarus clarkii* dicistro-like virus (PcDV, a novel Dicistro-like virus discovered in *Procambarus clarkii* with "Black May"disease). In the end, both pathogens were positive (Huang et al., 2020).

2.2 Extraction of genomic DNA and PCR amplification of 16S rDNA

The total genomic DNA of *P. clarkii* intestine was extracted by the cetyltrimethylammonium bromide (CTAB) method, and the concentration of isolated DNA was measured with a NanoDrop 2000

spectrophotometer (GE Healthcare, USA). The samples were diluted to 1 ng/μL with sterile water and used to amplify the 16S rDNA of intestinal microorganisms.

The diluted genomic DNA was used as the template for PCR amplification. PCR amplification of the 16S rRNA gene was performed using PCR primers (515*F*-806*R*) specific for the V3–V4 regions. The primer sequences were as follows: 5′-GTGCCAGCMGCCGCGGTAA-3′/5′-GGACTACHVGGGTWTCTAAT-3′. PCR products were detected by electrophoresis with 2% agarose gels. Then, PCR products were purified with GeneJET Gel Extraction Kit (Thermo Scientific). Before sequencing, a spectrophotometer was used to determine the DNA concentration of each PCR product, and the DNA was mixed in an appropriate ratio according to the sequencing requirements.

2.3 High-throughput sequencing

Twenty samples were sequenced in a commercial company (Novogene, Beijing), of which 10 samples were from the control group and 10 were from the diseased group. Sequencing libraries were generated using an Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index adapters were added. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina Nova Seq platform and 250-bp paired-end reads were generated.

2.4 Sequence analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) to obtain the Raw tags (the spliced tags sequence) (Magoč and Salzberg, 2011). According to the tag quality control process of Qiime (V1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) (Caporaso et al., 2010), the following operations were carried out: tags interception: truncate raw tags from the first low-quality base site with continuous low-quality value (the default quality threshold is ≤19) and the base number reaches the set length (the default length is 3); tags length filtering: filter out tags with continuous high-quality base length less than 75% of tags length. Effective tags were obtained through read splicing, quality control, and removal of chimeric sequences (https://github.com/torognes/vsearch/) (Rognes et al., 2016).

2.5 Statistical method

To study the species composition of each sample, the effective tags of all samples were clustered into operational taxonomic units (OTUs) with 97% identity (Uparse v7.0.1001, http://www.drive5.com/uparse/) (Haas et al., 2011). The OTU sequences were taxonomically annotated by Mothur and SILVA132 (http://www.arb-silva.de/). According to the OTU clustering results, a Venn diagram was drawn to show common and unique OTUs between different groups (http://bioinformatics.psb.ugent.be/webtools/Venn/). According to the species annotation results, the relative abundances of species at different taxonomic levels were plotted.

The evaluation of microbial diversity in a natural environment involves two aspects: species richness and evenness information. Alpha diversity was used to analyze the richness and diversity of the within-community microbial community, as shown by rank abundance. Alpha diversity indices (Good's coverage, the Chao1 estimator, the ACE, the Simpson index, and the Shannon index) were calculated in QIIME (Version 1.9.1) to analyze the abundance and diversity of microbial communities in the two groups. R (version 2.15.3) was used to analyze the difference of alpha diversity index between groups. Beta diversity was used to analyze the microbial community composition of different samples. QIIME was used to calculate Unweighted UniFrac distance and construct unweighted pair group method with arithmetic (UPGMA) sample clustering tree. Principal coordinate analysis (PCoA) was conducted by the WGCNA packages, STAT packages, and GGPLOT2 packages of R (Version 2.15.3), based on Unweighted UniFrac distance. Moreover, linear discriminant analysis (LDA) effect size (LEfSe) was used to identify biomarkers with significant differences between the groups (the default filter value of the LDA score is 4).

The 16S rRNA gene sequences of prokaryotes in the kyoto encyclopedia of genes and genomes (KEGG) database were extracted and compared to those in the SILVA SSU Ref NR database (BLAST bitcore >1 500) by the BLASTN algorithm to establish the correlation matrix. The KEGG database prokaryotic whole genome function information annotated by UProC and PAUDA was mapped to the SILVA database to realize the functional annotation of the SILVA database.

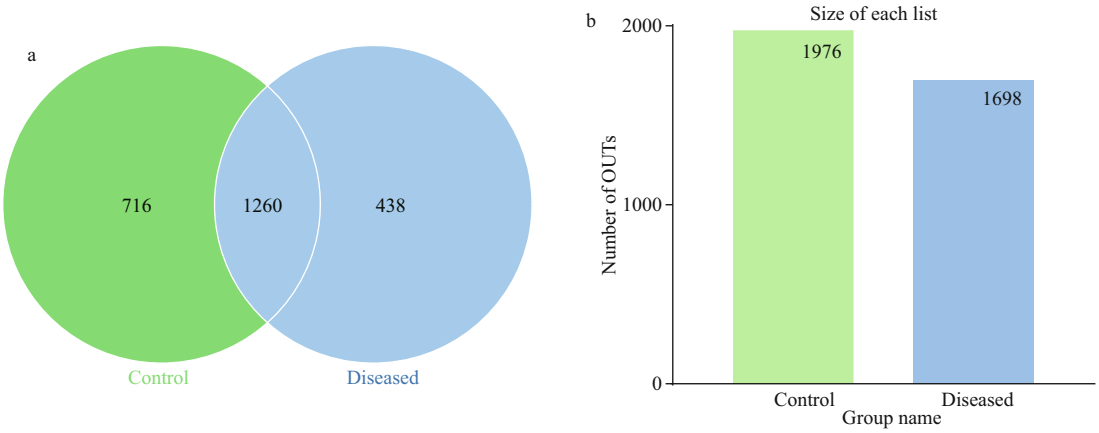

Fig.2 The Venn diagrams (a) and the number of OTUs in control and diseased group (b)

The shared and unique OTUs are represented through Venn diagrams; 1 260 OTUs are shared among samples.

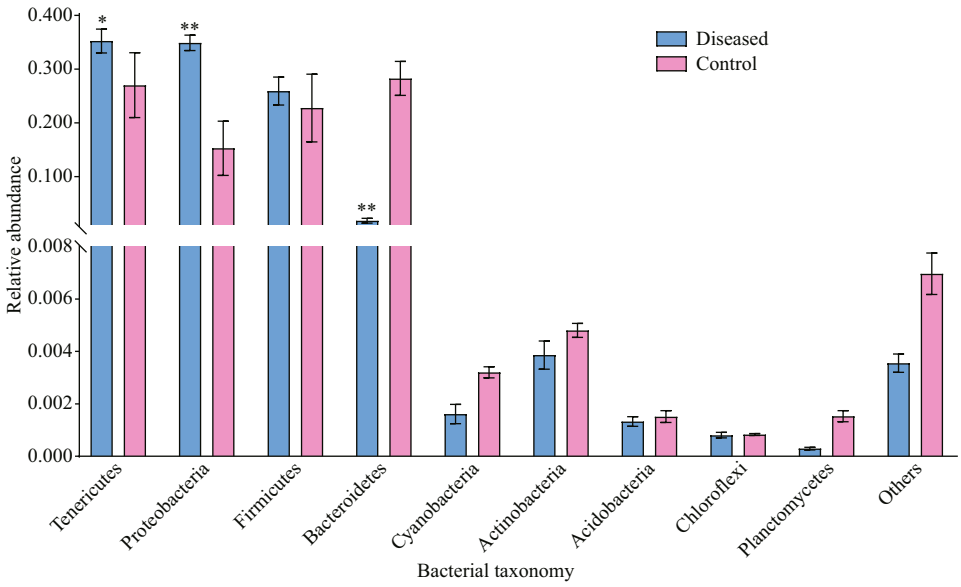

Fig.3 Abundance of intestinal microflora of *P. clarkii* at the phylum level in the control and diseased groups

*: $P \leq 0.05$; **: $P \leq 0.01$.

3 RESULT

3.1 The high-throughput sequencing data analysis

In total, through the splicing of reads, 85 772 tags per sample were measured, and 82 002 valid data were obtained through quality control, with the amount of effective data reaching 77 703 tags after removing chimaera sequences. With 97% identity, the sequences were grouped into OTUs, with 2 432 OTUs. In the Venn diagram, the two circles represent the diseased group and the control group, and the numbers represent the number of OTUs. Among them, 1 260 OTUs were shared between the two groups, 438 OTUs were unique to the diseased group, and 716 OTUs were unique to the control group (Fig.2).

3.2 The intestinal flora composition

In this study, 47 phyla were recognized in the intestinal flora of control and diseased groups. As shown in Fig.3, we identified four dominant phyla in the intestines of crayfish in the control group, namely Tenericutes (30.86%), Bacteroidetes (29.99%), Firmicutes (22.23%), and Proteobacteria (15.23%). We also identified three dominant phyla in the diseased group, namely, Tenericutes (35.71%), Proteobacteria (34.59%), and Firmicutes (26.65%). Although Tenericutes had the highest abundance in both groups, and there was no significant difference ($P>0.05$), a striking shift in the microbial composition was found in the intestines of *P. clarkii* with BMD. Bacteroidetes was a dominant phylum in healthy *P. clarkii*, whereas its prevalence was low in diseased

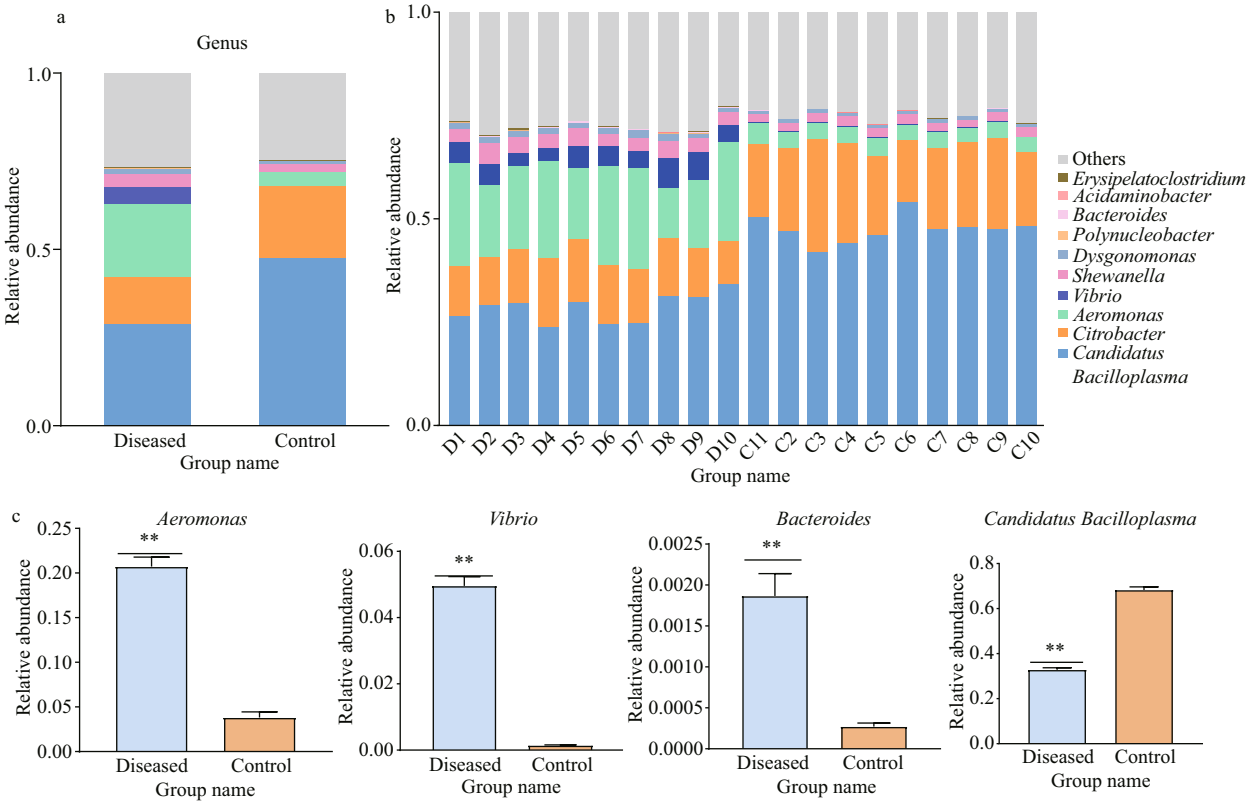

Fig.4 The relative abundances of the intestinal bacterial community among the diseased and control samples at the genus level

The top 10 most abundant genera are shown. Other minor taxa and unclassified bacteria are listed under "Other". Means for each group (a); means for each sample (b); bacteria with significant differences (c). D1–D10: diseased group; C11–C1: control group. **: $P \leq 0.01$.

P. clarkii (1.87%). By contrast, the prevalence of Proteobacteria was significantly higher ($P<0.05$) in *P. clarkii* with BMD than in *P. clarkii* without BMD. The percentage of bacteria belonging to the phylum Proteobacteria in diseased crayfish was higher than that in healthy crayfish, which was mainly due to the contributions of Alphaproteobacteria ($P<0.05$). In contrast, the percentage of Bacteroides in diseased crayfish was significantly lower than that in healthy crayfish ($P<0.05$).

As shown in Fig.4a, the composition of the intestinal flora of *P. clarkii* showed more relative differences at the genus level. In the annotation results, a total of 1 150 (47.29%) OTUs annotations were at the genus level. Among them, the dominant intestinal bacteria of *P. clarkii* in the control group were *Candidatus Bacilloplasma* (47.56%) *and Citrobacter* (20.30%). The dominant intestinal bacteria in the diseased group were *Candidatus Bacilloplasma* (28.72%), *Citrobacter* (13.30%), and *Aeromonas* (20.76%). Although *Candidatus Bacilloplasma* was the dominant intestinal bacterium in both the control group and the diseased group, its abundance was significantly lower ($P<0.01$) in the diseasedd group. In addition, other genera with significant enrichment in the diseased group were *Aeromonas* ($P<0.01$) and *Vibrio* ($P<0.01$) (Fig.4c). The individual relative abundances for samples in the control and diseased group were shown in Fig.4b, and the detailed data were shown in Supplementary Tables S1 & S2.

3.3 Diversity analysis

The results of alpha diversity analysis show that the Simpson, ACE, Chao1, and Shannon indices in the diseased group were slightly lower than those in the control group (Supplementary Table S3), but there was no significant difference in alpha diversity indices between the groups (richness $P=0.59$; evenness $P=0.43$; and diversity $P=0.052$) (Fig.5a).

PCoA of all 20 samples showed that the clustering of sample points in the control group and the diseased group were relatively close, indicating that the difference in intestinal flora among individuals within each group was small. In contrast, the clusters of sample points of the control group and the diseased group were far apart, indicating a certain difference between intestinal flora groups (Fig.5b). PCoA

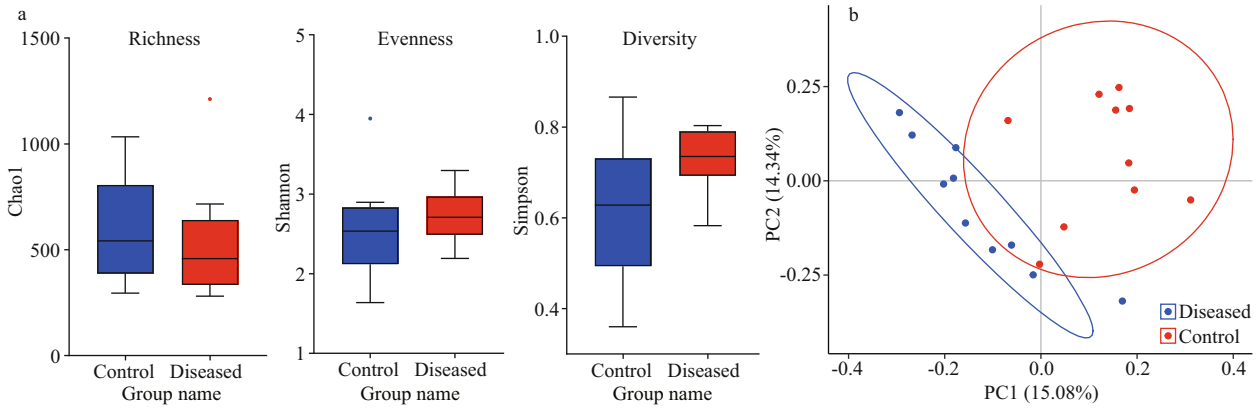

Fig.5 Measures of α-biodiversity, including richness (Chao1), evenness (Shannon), and diversity (Simpson) (a), and principal component analysis (PCA) (b)

In (b), the first two PCs are used as the *x* and *y* coordinates. Each sample is represented as a point. Samples from different sources are colored differently: diseased (red); control (blue).

indicated that PC1, which reflected the bacterial community, could separate the healthy group from the diseased group, and the total contribution of other principal components was 15.08%.

In addition, a multi response permutation procedure (MRPP, based on Bray-Curtis distance) analysis was performed to examine the differences in intestinal microbiota between the two groups, as shown in Table 1. The difference between groups was expressed as the expected-delta, which was greater than the difference within groups (significance=0.031).

To further analyze the intestinal flora of *P. clarkii* related to BMD, LEfSe was used to compare the relative abundance of intestinal flora between the control group and the diseased group (Fig.6a). There were 5 significantly different flora constituents between the control group and the diseased group, belonging to three phyla: Cyanobacteria, Firmicutes, and Proteobacteria. Among them, we found that the bacteria of the diseased group showed a significant increase in the abundances of Enterobacteriaceae and Vibrionaceae of Proteobacteria. Their relationship is shown in Fig.6b.

3.4 16S rRNA functional prediction analysis

To analyze the functional changes in intestinal microbiota in *P. clarkii* with BMD, Tax4Fun was used to predict the metagenomic potential between the two groups. The results show that the high abundance of bacterial metagenomes in the intestine of healthy and BMD-diseased *P. clarkii* was mainly related to the metabolic pathway group, genetic information processing group and environmental information processing group at KEGG level 1. There

Table 1 MRPP and Adonis analysis of the control group and diseased group (based on Bray-Curtis distance)

MRPP			Adonis	
Observed-delta	Expected-delta	*P* value	*R* value	*P* value
0.515 4	0.543 3	0.031	0.857 55	0.033

The smaller the observed-delta value, the smaller the difference within the group; the larger the expected-delta value, the greater the difference between the groups. The *R*-value was greater than 0, indicating significant differences between groups. *P* value less than 0.05 indicates a significant difference.

was no significant difference between the KEGG level 1 microbial function of the diseased group and the control group ($P>0.05$). For the metabolism group, the largest abundance was found in amino acid metabolism, carbohydrate metabolism, and energy metabolism in level 2. For the genetic information processing group, the highest abundance was found in replication and repair and translation. In the environmental information processing group, the greatest abundance was observed in the membrane transport pathway. The specific data analysis of the above results is shown in Supplementary Fig.S1. Specifically, the microbial functions related to replication and repair in diseased group were significantly more abundant ($P<0.05$) than those in the control group. More detailed changes were observed at KEGG Level 2. As shown in Fig.7, the microbial functions related to Glycan biosynthesis and metabolism and Enzyme families in the diseased group were significantly more enriched ($P<0.05$) than those in the control group, while those assigned to the Amino acid metabolism, Energy metabolism, and Metabolism of cofactors and vitamins were significantly more enriched ($P<0.05$) in the control group than in the diseased group.

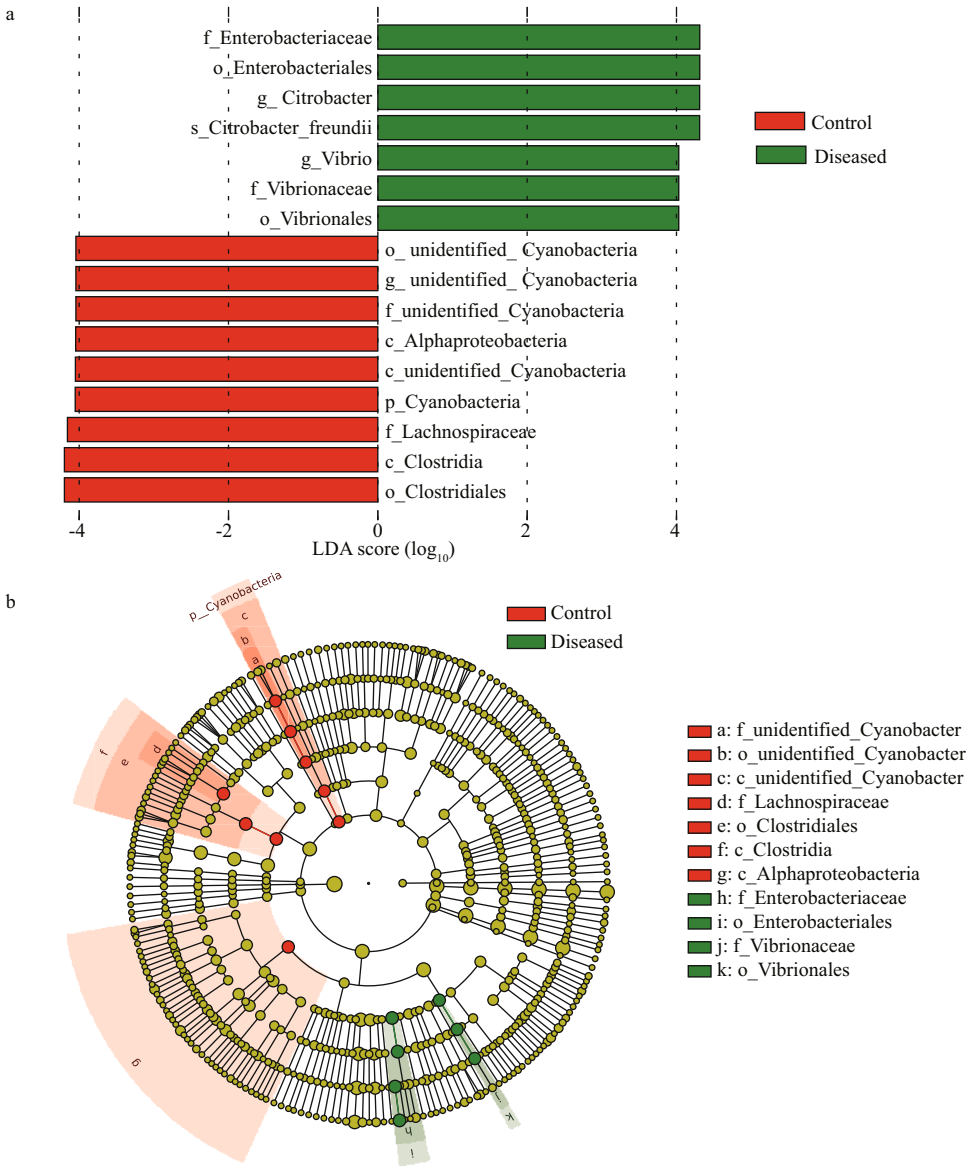

Fig.6 Linear discriminant analysis (LDA) (a) and Cladogram analysis (b)

The histogram of LDA value distribution shows species with LDA score greater than the set value (the default setting is 4), and the length of the histogram represents the impact of different species. In this branching diagram of evolution, the concentric circles from the inside to the outside represents the taxonomic rank from phylum to genus (or species). Each node represents a classification at that level, and the diameter of the node is proportional to the relative abundance.

4 DISCUSSION

Procambarus clarkii is widely cultivated in China because of its strong environmental adaptability and great commercial value. As the scale of production has increased, outbreaks of disease in *P. clarkii* have occurred frequently, especially around May, when the incidence rate and mortality rate of *P. clarkii* reach their peak, phenomenon known as “Black May” disease (BMD). At present, there are many controversies about the cause of BMD. It is generally considered that BMD in *P. clarkii* is caused by large fluctuations in temperature and water temperature in May. WSSV and some Vibrio species reproduce in large numbers and cause proliferative diseases in *P. clarkii* (Chen, 2019). BMD is also believed to be caused by the accumulation of ammonia nitrogen and nitrite due to the rapid rise of water temperature and low dissolved oxygen in the water (Shen et al., 2020). According to the symptoms of the empty, swollen intestines of *P. clarkii* with BMD, the characteristics and structure of intestinal bacteria in crayfish with

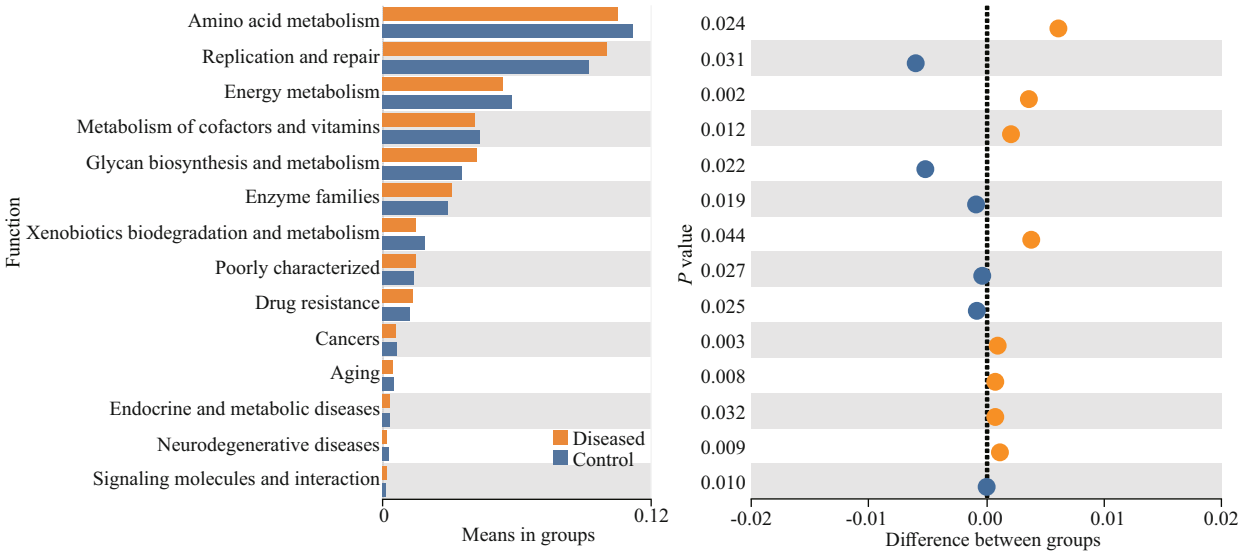

Fig.7 Extended error bar chart to determine the significant difference (95% confidence interval) between the average proportion of functional genes in the control (blue) and diseased (orange) samples

The corrected *P* value is displayed on the right side.

BMD and healthy crayfish were determined by 16S rRNA gene sequencing.

Crustaceans, such as *P. clarkii*, have a large number of microorganisms inhabiting their intestines. These microorganisms are the products of long-term coevolution with the host (Serrano-Villar et al., 2016). We identified four dominant phyla, namely, Proteobacteria, Bacteroidetes, Firmicutes, and Tenericutes, in the intestines of healthy crayfish, which confirms a previous study on the dominance of these taxa in the intestinal bacterial community of *P. clarkii* (Liu et al., 2020a; Zhang et al., 2020, 2021). In the present study, the alpha diversity of the bacteria in the intestinal tract of *P. clarkii* did not differ significantly ($P>0.05$) between control and diseased groups. However, there was a significant difference in the prevalence of the main bacterial phyla in the intestine of the groups with and without BMD, which changed the composition of the intestinal flora. We found that compared with healthy crayfish, there was a significant enrichment of Proteobacteria ($P<0.05$) in the intestines of diseased crayfish (Fig.3). In contrast, *Bacteroides* was prevalent in the intestines of healthy crayfish, while its prevalence was low in diseased crayfish. As the most abundant bacteria in fishponds, Proteobacteria participates in various biogeochemical processes in aquatic ecosystems (such as carbon, nitrogen, and sulfur cycles) (Klase et al., 2019; Li et al., 2021). Studies have shown that Proteobacteria may be essential for certain physiological and biochemical functions of crustacean intestines, such as *Portunus trituberculatus* (Zeng et al., 2016; Li et al., 2018). And an increased abundance of Proteobacteria was a potential risk of disease in *L. vannamei* (Fan and Li, 2019). *Bacteroides* may be related to intestinal fat storage, and the nutrient absorption of *Bacteroides* is negatively correlated with the presence of fat deposits (Xu et al., 2007; Guo et al., 2008). The balance of the intestinal microbial population is very important to the health of the host, and a loss or increase in intestinal microbial diversity can lead to host diseases (Semova et al., 2012; Li et al., 2021). The enrichment of Proteobacteria abundance and the decrease in *Bacteroides* indicate that BMD may affect the fat metabolism and nutrient absorption function of the intestinal microbiota of *P. clarkii*. In addition, it was reported that Alphaproteobacteria may cause local infection of tissue in *Litopenaeus vannamei*(Huang et al., 2016). Alphaproteobacteria are enriched in the intestines of diseased crayfish ($P<0.05$). However, further studies are needed to determine whether Alphaproteobacteria is related to damage to the intestinal tissues of *P. clarkii* with BMD.

At the genus level, the difference in the abundance of the intestinal flora of *P. clarkii* between the control group and the diseased group was mainly observed in *Candidatus Bacilloplasma* ($P<0.01$), *Bacteroides* ($P<0.01$), *Aeromonas* ($P<0.01$) and *Vibrio* ($P<0.01$) (Fig.4c). *Candidatus Bacilloplasma* is considered to be a novel lineage of Mollicutes associated with the

hindgut wall of the terrestrial isopod *Porcellio scaber* (Crustacea: Isopoda) and was first discovered in *Porcellio scaber* (Kostanjšek et al., 2007). The enrichment of *Candidatus Bacilloplasma* was also previously found in the intestine of *P. vannamei* (Hou et al., 2018), suggesting that *Candidatus Bacilloplasma* is prevalent in the intestines of crustaceans. Members of the genus *Bacteroides* are necessary to break down complex molecules in the intestines and help the body's immune system fight against potentially harmful pathogens (Gregory et al., 2015). In addition, *Bacteroides* is important for maintaining intestinal homeostasis, especially through the production of short-chain fatty acids (SCFA) (Zhong et al., 2015). Moreover, studies have shown that *Bacteroides* is rich in enzymes related to carbohydrate transport and protein metabolism, which are essential processes of digestion (Karlsson et al., 2011; Zhou and Zhi, 2016). The over-enrichment of *Bacteroides* may be related to the regulation of the homeostasis of the intestinal flora by *P. clarkii*. The Tax4Fun function prediction further supports the view that the abundance of genes involved in metabolism (Energy metabolism and Metabolism of cofactors and vitamans) in the intestinal flora of *P. clarkii* with BMD is significantly lower ($P<0.05$) than that of control *P. clarkii* (Fig.7), which indicates that energy metabolism is disrupted by BMD. This also further explains the symptoms of the jejunum and reduced food intake of *P. clarkii* with BMD.

In the intestine of *P. clarkii* in BMD, we detected significant upregulation of some opportunistic pathogens: *Vibrio* (0.152%–4.939%, $P<0.01$) and *Aeromonas* (from 3.965% to 20.758%, $P<0.01$) (Fig.4c). Generally, many members of the *Vibrio* genus are considered the main pathogens causing disease and death in aquatic animals. For example, *Vibrio parahaemolyticus* can cause AHPND and "gill rot disease" in shrimp and crayfish (Alloul et al., 2021). Studies on *P. clarkii* show that pathogenic bacteria such as *Vibrio* may be an opportunistic factor for disease (Shui et al., 2020). When the host's immunity decreases or the number of bacteria exceeds the normal value, host disease can occur (Hou et al., 2018). *Aeromonas hydrophila*, a more common conditional pathogen, exists widely in aquatic environments. Wang et al. (2004) noted that pathogenic *Aeromonas hydrophila* can cause many diseases, such as tremor and oedema, in *Eriocheir sinensis*, and it is also one of the main bacterial pathogens in shrimp and crab farming operation. Although conditional pathogenic bacteria are significantly enriched in the intestine of crayfish with BMD, further studies are needed to determine whether the two are related to the pathogenesis of BMD in crayfish.

In the present research, the functional prediction of the intestinal flora indicated that the metagenomic potential of the intestinal flora of *P. clarkii* has a higher abundance in the Carbohydrates metabolism and Amino and acids, which may be attributed to the carbohydrates and proteins from the host being the main energy-related nutrients for bacterial colonization (El Asmar et al., 2000; Shi and Walker, 2004; Hou et al., 2018). In addition, some important pathways related to Cellular processes, Environmental information processing, and Human disease functions also changed, indicating that BMD may affect the biochemical processes of *P. clarkii* intestinal bacteria at the cellular and molecular levels.

5 CONCLUSION

Overall, our study demonstrated that BMD causes changes in the intestinal flora of *P. clarkii*. Specifically, we found that the prevalence of bacteria belonging to the phylum Proteobacteria in the intestine of *P. clarkii* increased, while the prevalence of bacteria belonging to the phylum Bacteroidetes decreased, reflecting the abnormal metabolism and nutrient absorption of crayfish with BMD, which provides new insights for further research on the pathogenesis of BMD.

6 DATA AVAILABILITY STATEMENT

All raw data were submitted to the NCBI Sequence Read Archive (SRA) under accession number PRJNA692534. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Electronic supplementary material

Supplementary material (Supplementary Tables S1–S3 and Supplementary Fig.S1) is available in the online version of this article at https://doi.org/10.1007/s00343-021-1278-4.