Tail fell syndrome impacts intestinal microbiota in porcupinefish (*Diodon hystrix*)

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Introduction: Frequent disease outbreaks seriously affect porcupinefish (*Diodon hystrix*), which is a new aquaculture breed in China. Recently, a novel disease, termed tail fell syndrome (TFS) was increasingly observed in fish stocks, resulting in stalk ulceration, tail loss, ascites and white feces in the intestine. Intestinal microbiota homeostasis is crucial to host health because it influences host and environmental factors, and responds to various internal and external stimuli. However, changes in intestinal microbiota induced by TFS are yet to be elucidated.

Methods: In the present study, we analyzed and compared the intestinal microbiota of normal *D. hystrix* with TFS fish.

Results: Though microbiota richness and diversity were not affected by TFS, deviations in diversity indices increased and taxa distribution evenness decreased, suggesting TFS lowered microbiota community stability in *D. hystrix*. Furthermore, at the genus level, *Brevibacterium*, *Mesorhizobium*, *Ochrobactrum*, *Ralstonia*, *Anaerococcus*, and *Alistipes* abundances were significantly increased in TFS *D. hystrix*, plus, we observed significant decreases in *Halomonas*, *Prevotellaceae_NK3B31_group,* and *Psychrobacter*. Functional comparison predictions between normal and TFS *D. hystrix* revealed significantly altered pathways were mainly associated with metabolism (biotin metabolism, steroid hormone biosynthesis, flavonoid biosynthesis, biosynthesis of type II polyketide products, and steroid biosynthesis). Overall, the results revealed that TFS impacted intestinal microbiota composition and function in *D. hystrix*, which should expand our knowledge on diseases associated with porcupine pufferfish aquaculture.

KEYWORDS

aquaculture, high-throughput sequencing, *Diodon hystrix*, intestinal microbiota, tail fell syndrome

1 Introduction

Diodon hystrix (porcupinefish) is a commercially important fish species mainly distributed in the Indo-West Pacific region (Soto et al., 2013). The fish is a new aquaculture species in China due to its considerable nutritional and medicinal characteristics. With the rapid development of *D. hystrix* aquaculture in recent years, several diseases have appeared, including the novel tail fell syndrome (TFS), particularly in *D. hystrix* aquaculture. The disease was first discovered in normal aquaculture environments, with TFS and normal fish emerging from the same environment. The most dominant disease feature is tail stalk festering with entire tail loss, accompanied by ascites and white feces in the intestines, and a high death rate. These observations suggest TFS may be pathogenic in nature.

Under intensive aquaculture conditions, many diseases are caused by pathogens, for example, white spot disease *in Penaeus monodon* caused by *Vibrio alginolyticus* infections (Selvin and Lipton, 2003; Zhou et al., 2020; Zhao et al., 2021). Several diseases are caused by a variety of pathogens, including viruses and bacteria (Zhu et al., 2000; Huang et al., 2012). Furthermore, growing evidence suggests that many aquaculture animal diseases induce changes in intestinal bacterial communities (Pérez et al., 2010; Zhou et al., 2020; Zhao et al., 2021). As a complex ecosystem, intestinal microbiota promotes host health by participating in immune responses, nutrient absorption, disease resistance, and the establishment of a balanced community structure (Lin et al., 2014). In *Litopenaeus vannamei*, infection with white spot syndrome virus altered intestinal microbiota composition and function (Wang et al., 2019). In zebrafish intestines, bacterial invasion affected microbiota composition and induced immune responses (Yang et al., 2017). In grass carp (*Ctenopharyngodon idellus*), *Aeromonas hydrophila* infection induced dynamic changes in intestinal microbiota (Zhou et al., 2020). Furthermore, intestinal microbiota also has determinant roles in newly discovered diseases. In sea cucumber (*Apostichopus japonicus*), different intestinal fungal communities were observed between normal and body vesicular syndrome individuals (Zhao et al., 2021). Our recent study has indicated that TFS, a newly discovered disease, is caused mainly by Vibrio (unpublished data); however, further information on TFS is practically non-existent, however, aberrant intestinal morphology was found, therefore we speculated TFS caused changes in the gut microbiome. However, associations between TFS and the host microbiome remain unclear.

The intestinal microbiota is influenced by host and environmental factors, and responds to various internal and external stimuli, therefore, understanding the composition and structure of intestinal microbial communities is necessary to understand changes and address different situations or stimuli (Clements et al., 2014; Butt and Volkoff, 2019). Over the last decade, clone libraries, culture-based, and denaturing gradient gel electrophoresis approaches have been applied to microbiota investigations (Knapp et al., 2008; Morotomi et al., 2011; Tzuc et al., 2014). While useful, these traditional methods are limited in characterizing global diversity and complex bacterial communities

in an integrated environment. In view of this, 16S rRNA sequencing technology is widely used to study the composition of microbial communities in fish intestinal samples (Ghanbari et al., 2015; Wang et al., 2018).

In this study, the intestinal microbiota of TFS and normal *D. hystrix* was analyzed using high-throughput sequencing technology. This study investigated how TFS induced composition, diversity, and functional changes in gut microbiota in *D. hystrix*. Our research identifying gut microbiota in diseased *D. hystrix*, and provides an important clue for TFS management and control.

2 Materials and methods

2.1 Sample collection

D. hystrix were collected from aquaculture ponds in Sanya, China. The average body length of the *D. hystrix* was 10.54 ± 1.73 cm and the average weight of the *D. hystrix* was 26.33 ± 4.21g. After collection, eight TFS and eight normal *D. hystrix* fish were randomly selected and dissected immediately. Fish were anesthetized with MS-222 (Sigma-Aldrich) before sacrifice. Intestinal tracts were aseptically removed from the body cavity, and the contents gently squeezed into sterile containers. These were immediately frozen in liquid nitrogen, and maintained at −80°C until DNA extraction. This work received ethical approval from the Animal Research and Ethics Committees of South China Agriculture University.

2.2 Histopathology

Five TFS and five normal *D. hystrix* fish were selected, mid-intestinal segments removed, and fixed in Bouin's fluid. After dehydration, tissue was embedded in paraffin wax, sectioned (5 μm), stained in hematoxylin and eosin, and examined using a Nikon microscope (Nikon Eclipse E100, Japan).

2.3 DNA extraction, amplification and sequencing

Microbial DNA from intestinal contents was extracted using the QIAGEN DNA stool mini kit (QIAGEN, USA) following manufacturer's instructions. We conducted 1% agarose gel electrophoresis to assess successful DNA extraction. DNA concentrations and purity were then measured using the NanoPhotometer® Classic Launched (IMPLEN, Germany). As previously described, DNA was diluted to 1 ng/μl, and two specific barcoded primers (341F, CCTACGGGNGGCWGCAG and 806R, GGACTACHVGGGTATCTAAT; the barcode was an eight-base sequence unique to each sample) were used to amplify the V3–V4 region of the 16S rRNA gene by PCR (Zhou et al., 2020). PCR product quality was assessed by 2% agarose gel electrophoresis. Purified amplicons were pooled in equimolar amounts, and paired

ends sequenced (PE250) on an Illumina platform Hiseq™ 2500 (Gene Denovo Biotechnology Co. Ltd. Guangzhou, China).

2.4 Bioinformatics and statistical analysis

DADA2 was used to perform quality filtering on amplicons, infer exact amplicon sequencing variants (ASVs), and filter and remove chimeras (Callahan et al., 2016). Taxonomic assignment (i.e., kingdom, phylum, class, order, family, genus, and species) by a naive Bayesian model was conducted using the RDP classifier (version 2.2, Wang et al., 2007) based on the SILVA database (version 132, Quast et al., 2013). Singletons across samples and reads which mapped to mitochondria or chloroplasts were discarded.

Alpha diversity comparisons (Sob, Chao1, ACE, Shannon, Simpson, and Pielou indices) between groups were calculated using the Wilcoxon rank test. To compare microbial community differences between groups, non-metric multidimensional scaling (NMDS) ordination and analysis of similarities (ANOSIM) using the Bray-Curtis distance was conducted in the R project Vegan package (Oksanen et al., 2016). Biomarker features in each group were screened using the random forest model in the randomForest R package (Liaw and Wiener, 2002).

Kyoto Encyclopedia of Genes and Genomes pathway analysis of ASVs was inferred using PICRUSt2 (Douglas et al., 2020). Functional differences between groups were analyzed by Welch's t-test.

3 Results

3.1 Morphological TFS characteristics

D. hystrix with TFS exhibited tail stalk ulceration, resulting in tail loss (Figures 1B, D), whereas normal fish did not exhibit this phenomenon (Figures 1A, C). When we dissected TFS fish abdomen and observed viscera, ascites and white feces in intestines (Figure 1E).

3.2 Histopathology

TFS and normal fish intestines were sectioned and histologically analyzed. TFS fish intestinal villi had many cavities and gaps (Figures 2A, B). Furthermore, intestinal villi were shorter and distances between villi were larger in TFS fish intestines. Normal fish intestines are shown (Figures 2C, D).

3.3 Microbiota composition and distribution

After quality control and rarefaction, 512368 high-quality reads were obtained from 16 samples, comprising eight normal *D. hystrix* and eight TFS *D. hystrix*. The total number of ASVs was 14434,

ranging from 612–2481 ASVs per sample. ASV and taxonomy data from samples are presented (Table S1).

The top 10 taxa with the highest abundance in each group at phylum and genus levels are shown (Figure 3). At the phylum level (Figure 3A), the average abundance of Firmicutes was 37.27%; Bacteroidetes was 25.80%; Proteobacteria was 19.32%, and Actinobacteria was 4.43% in the TFS group. In the normal fish group, the average abundance of Firmicutes was 36.32%; Bacteroidetes was 21.70%; Proteobacteria was 19.04%, and Actinobacteria was 5.95% (Figure 3A). At the genus level, the average abundance of the Lachnospiraceae_NK4A136_group was 5.04% in TFS and 5.64% in normal fish, also the average abundance of Lactobacillus was 4.91% in TFS and 3.39% in normal fish (Figure 3B).

Furthermore, we identified nine genus-level indicator taxa associated with TFS by using a random forest model and Wilcoxon test (Figure 4A). Of these, *Halomonas*, Prevotellaceae_NK3B31_group and *Psychrobacter* abundances were significantly decreased in TFS fish. In contrast, *Brevibacterium*, *Mesorhizobium*, *Ochrobactrum*, *Ralstonia*, *Anaerococcus*, and *Alistipes* were significantly increased (Figure 4B).

3.4 Microbiota diversity and structural differences

For alpha diversities, all Sob, Chao1, ACE, Shannon, Shannon, and Pielou indices showed no significant differences between groups. We calculated the coefficient of variation (standard deviation/mean), which indicated larger deviations in alpha diversities in TFS fish (Figure 5; Table 1). In addition, Pielou's evenness index indicated a decreasing tendency (Wilcoxon rank test: p = 0.089) in the TFS group. These results suggested increased instability in TFS *D. hystrix* intestinal microbiota.

NMDS analysis showed that community structures were significantly altered between groups (Figure 6A). Furthermore, microbial community differences between groups were tested using ANOSIM, and showed statistically significant differences (R = 0.106, P = 0.034) between groups (Figure 6B).

To identify the most important marker functions correlating with the TFS, we performed Kyoto Encyclopedia of Genes and Genomes pathway analysis of ASVs by using PICRUSt2. As shown in Figure 7, as an example, among the top nine pathways, five (biotin metabolism, steroid hormone biosynthesis, flavonoid biosynthesis, biosynthesis of type II polyketide products, and steroid biosynthesis) were classified as metabolism, one (biofilm formation - Vibrio cholera) was classified as a cellular process, one (non-homologous end-joining) was classified as genetic information processing, and two (*V. cholerae* infection and *systemic lupus erythematosus*) were classified as infectious diseases, most of which were metabolism or disease infectious related pathways, thus suggesting that these pathways (especially for "biosynthesis of type II polyketide products" and "flavonoid biosynthesis") might be the dominant direct or indirect feedback responses to TFS.
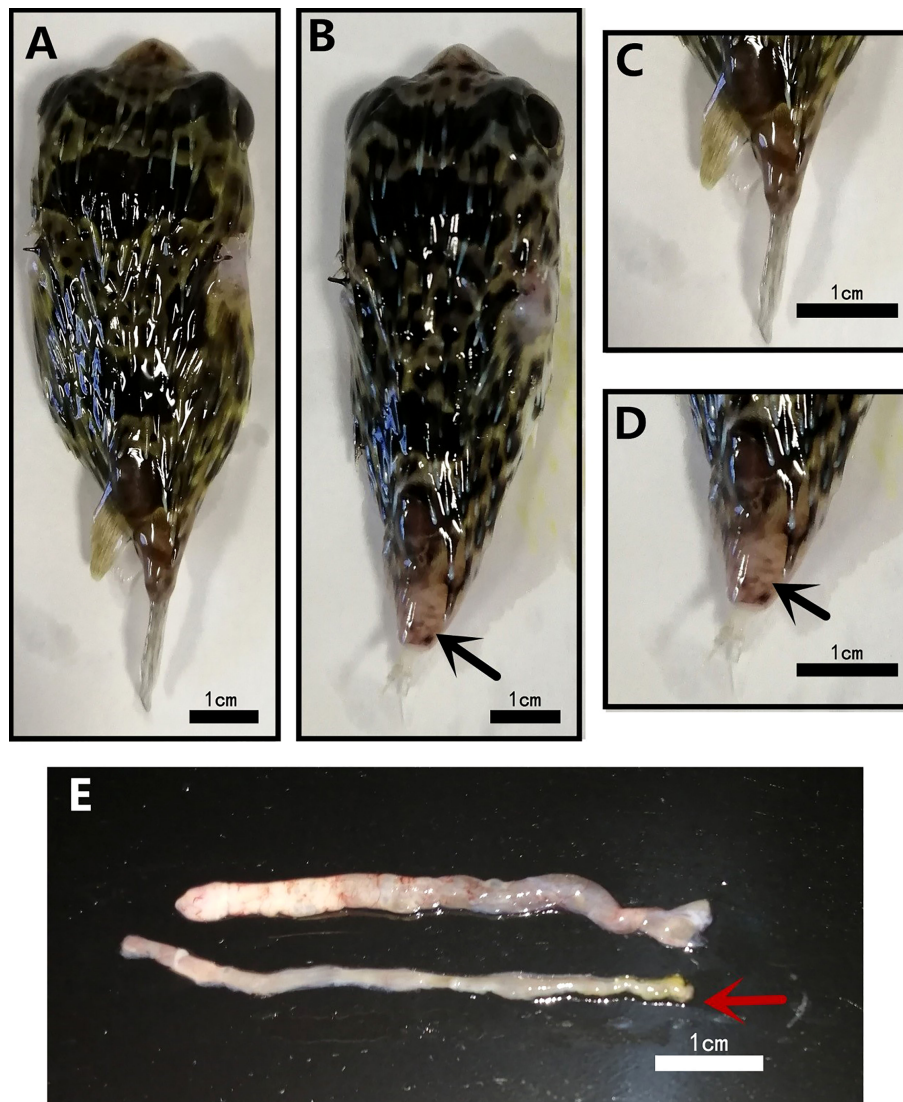
FIGURE 1
Morphological differences in normal (NOR) and tails fell syndrome (TFS) *Diodon hystrix*. **(A)** healthy *D. hystrix*. **(B)** TFS *D. hystrix*. **(C)** Closeup of the tail of health *D. hystrix*.**(D)** Closeup of the tail of TFS *D. hystrix*, the black arrows indicate the festering and broken tails. **(E)** The state of the intestines of health and TFS *D. hystrix*, the red arrows indicate the intestines of TFS *D. hystrix*.

4 Discussion

D. hystrix is a new aquaculture breed currently undergoing large scale cultivation in China, however, with increased aquaculture comes new diseases. TFS is the latest, with a high mortality rate, tail stalk festering, tail-loss, and ascites and white feces in the intestines. We proposed TFS may induce changes in bacteria or fungi in the gut, therefore, we investigated gut microbiota in diseased and normal fish, and observed significant differences in microbial composition and function between groups.

In recent years, an increasing number of studies have identified correlations between immune functions, disease, and intestinal microbiota in fish (Li et al., 2011; Sudheesh et al., 2012; Guijarro et al., 2015; Liu et al., 2016; Qi et al., 2017). Many fish diseases induce intestinal microbiota changes, e.g. the related taxa, *Vibrio*, *Aeromonas*, and *Shivella* were overexpressed in the intestinal flora of *Carassius auratus* 'red film' disease (Li et al., 2017). Similarly, *Aeromonas* is a biomarker of furuncles in largemouth bass (Li et al., 2016). These phenomena suggested that certain intestinal microbial characteristics were indicative of host health status, independent of disease etiology, as demonstrated by human intestinal diseases (Mancabelli et al., 2017). Thus far, few studies have investigated the relationship between disease severity and gut microbiome disruption during fish disease development; thus, the transition from healthy to diseased gut flora is unclear (Knights et al., 2014). However, intestinal microbiota characteristics may be used as factors in predicting the incidence of fish disease (Xiong et al., 2017; Xiong et al., 2018), therefore, significant compositional
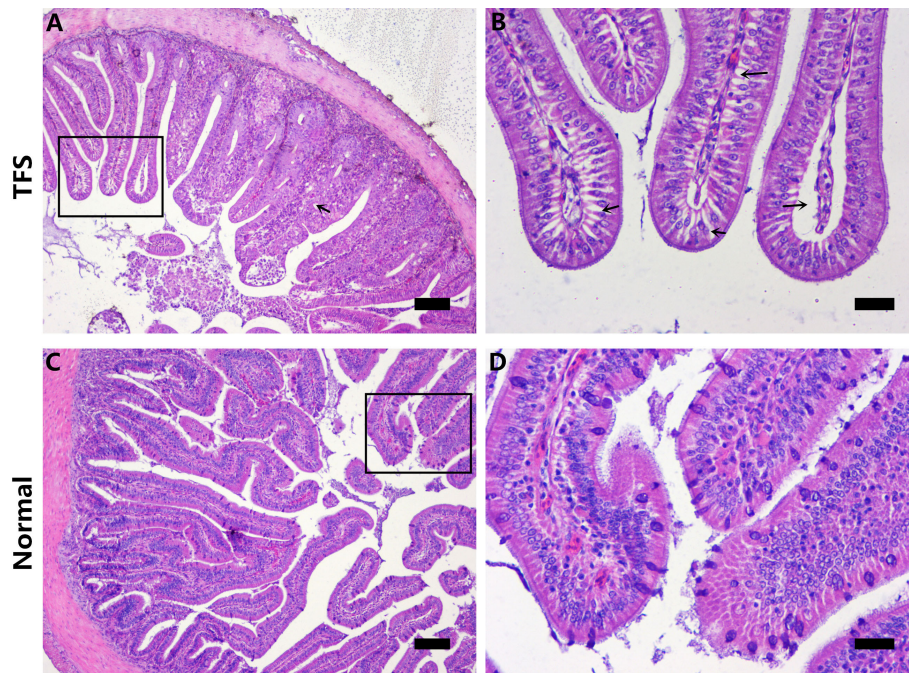
FIGURE 2

Histological analysis of the intestinal sections of normal (NOR) and TFS *D. hystrix*. **(A)** The histological of TFS *D. hystrix*. **(B)** High magnification of the boxed areas in A, black arrows indicate the cavitation and voids. **(C)** The histological of normal *D. hystrix*. **(D)** High magnification of the boxed areas in **(C)** Scale bars **(A, C)**: 100μm, Scale bars **(B, D)**: 25μm.

differences in intestinal microbial communities observed here may be used to predict TFS occurrence in *D. hystrix* stocks. Our results help to elucidate differences in intestinal microorganisms between normal and diseased fish.

Bacterial diversity is closely associated with the functional stability of the intestinal microbiota; the more diverse the gut microbial community, the more likely it is to have species with antagonistic properties against invading pathogens; e.g., this is often observed for *Coreius guichenoti* (Li et al., 2016), *C. auratus* (Li et al., 2017), and *Plecoglossus altivelis* (Nie et al., 2017). In this study, we observed no significant differences in intestine microbiota species diversity and richness between groups. However, NMDS and ANOSIM strategies demonstrated differential microbial communities between normal and TFS fish. Also, intestinal
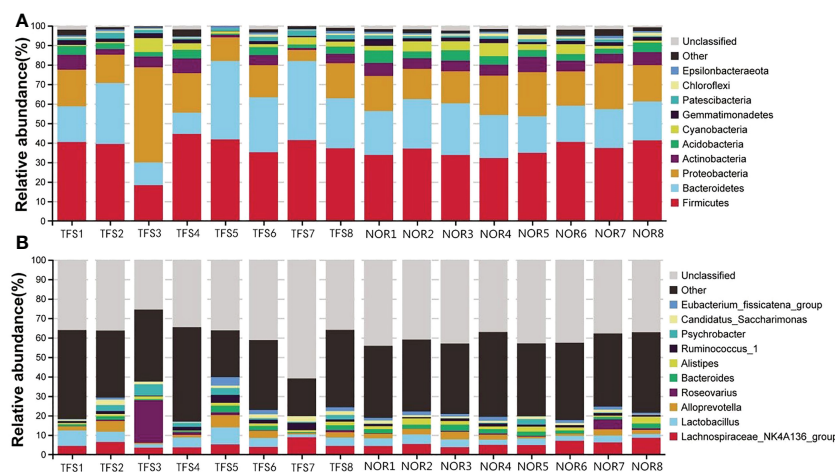

FIGURE 3

Taxonomy stack distributions of different levels of classification for normal (NOR) and TFS *D. hystrix*. The top 10 phylum **(A)** and genus **(B)** were selected, and the rest were unified to the other category.
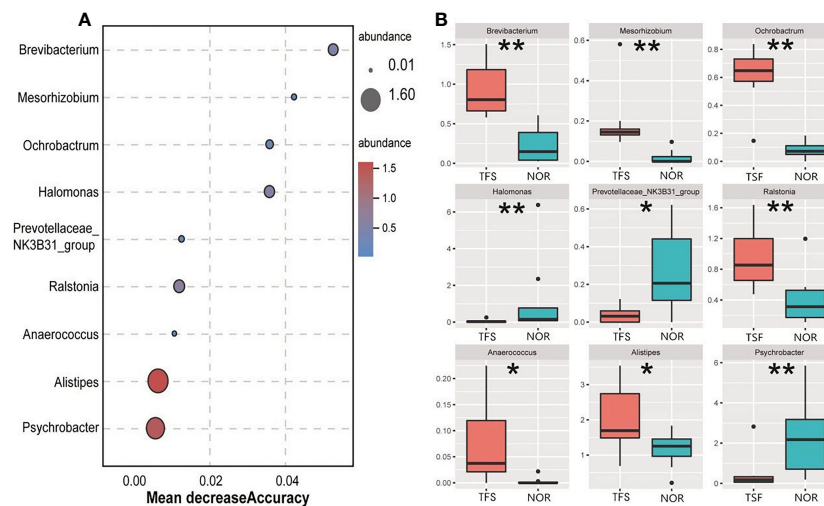
FIGURE 4
Identified differentially abundant genera between the normal (NOR) and TFS *D. hystrix.* by random forest model **(A)** and wilcox test **(B)**. A mean decrease in accuracy measures the increase in misclassification produced on average by removing the given predictor. * P < 0.05, ** P < 0.01.

microbiota compositional structures were changed in TFS fish. Combined, these data suggested diseased fish intestinal microbial community homeostasis was disrupted.

Previous studies have demonstrated that bacteria are responsible for fish diseases (Senderovich et al., 2010; Rønneseth et al., 2017; Castillo et al., 2019). At the phylum level, we observed Bacteroidetes levels were increased in TFS fish intestines. Many bacteria in the Bacteroidetes phylum cause inflammation, abscesses, and deep abscesses in the abdominal cavity and anus of both animals and humans (Louis and Flint, 2009), thus, TFS appeared to cause intestinal ulceration which may have been associated with these bacteria however, more analyses are required. At the genus

level, we observed significantly increased bacteria in diseased fish, including *Anaerococcus, Brevibacterium* and *Alistipes. Anaerococcus* are considered enteropathogenic in mammals (Cobo and Navarro-Marí, 2020), *Brevibacterium* is becoming increasingly pathogenic to humans (Magi et al., 2018), and *Alistipes* are associated with gut inflammation (Parker et al., 2020). In contrast, *Halomonas* and *Prevotellaceae_NK3B31* groups were decreased in TFS fish guts. The fish digestive tract has a stable ecosystem just like the nature. Normal fish contain huge bacterial numbers and proportions in the gastrointestinal tract; however, different factors can elicit a vast variety of changes. Normally, these changes fluctuate within certain limits, thus maintaining a relatively stable equilibrium. However,
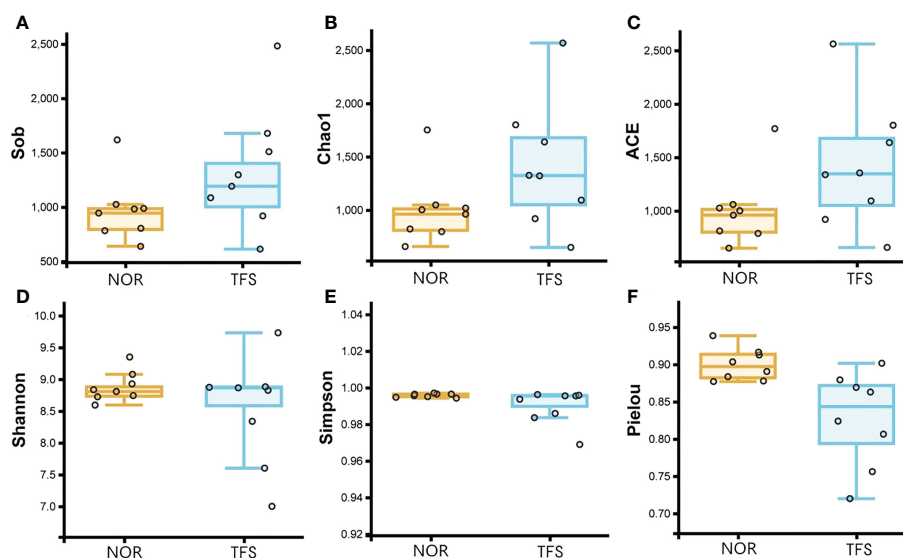

FIGURE 5
Comparisons of intestinal microbiota alpha diversity between normal (NOR) and TFS *D. hystrix.* Sob **(A)**, Chao1 **(B)**, ACE **(C)**, Shannon **(D)**, and Simpson **(E)** and Pielou indexes **(F)**.

TABLE 1 Coefficient of Variation in alpha diversities in the intestinal microbiota of normal (NOR) and TFS *D. hystrix*.

group	Sobs	Chao1	ACE	Pd	shannon	simpson	pielou
NOR	0.300645	0.327363	0.335534	0.250939	0.02671	0.000994	0.024154
TFS	0.420653	0.421154	0.416582	0.32911	0.100461	0.009706	0.076838

once fish get sick, this balance is upset, and intestinal flora becomes imbalanced. Thus, TFS may disrupt intestinal microbiota homeostasis in *D. hystrix*.

Investigating bacterial functional diversity highlighted the importance of the intestinal microbiome in host immunity. In

this study, the gut bacteria of healthy and TFS fish were associated with multiple pathways. The most predominant included steroid biosynthesis and flavonoid biosynthesis. In organisms, steroids regulate innate immune responses and participate in immune regulation. As observed in Drosophila,
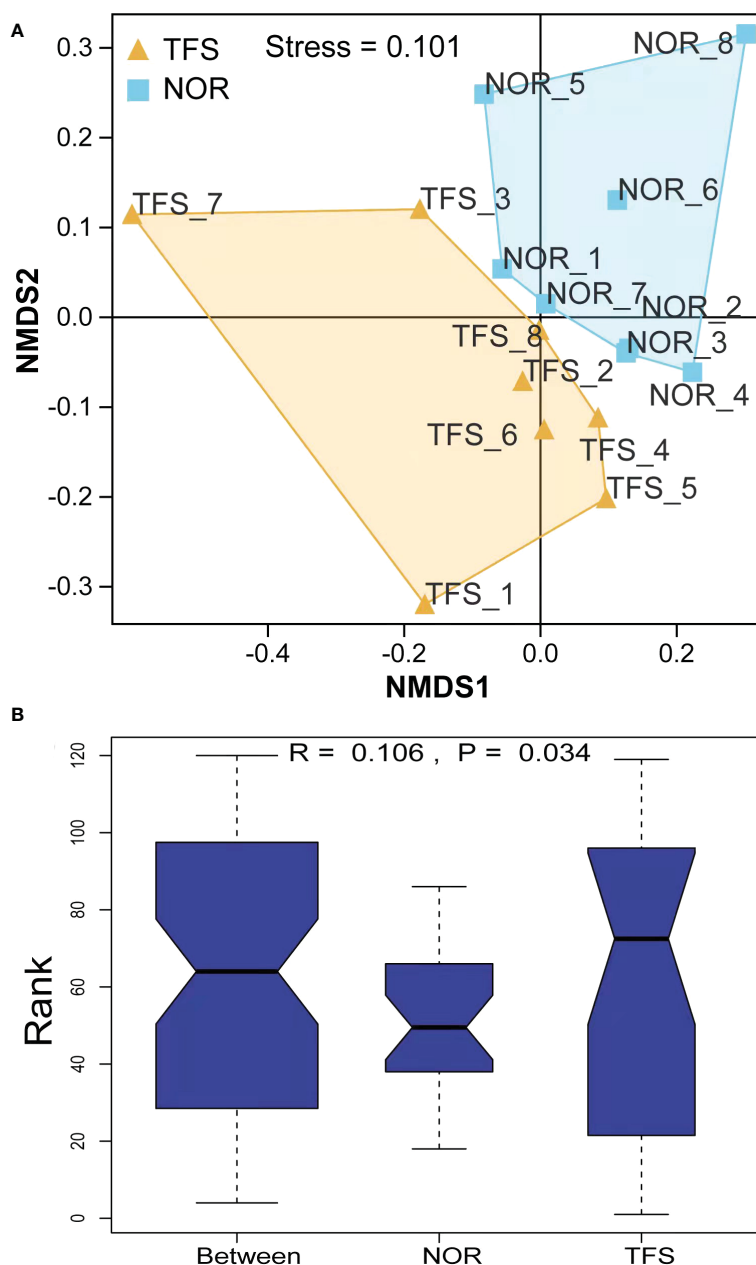

FIGURE 6
Beta diversity difference in the intestinal microbiota of normal (NOR) and TFS *D. hystrix*. **(A)** Nonmetric multidimensional scaling (NMDS) analysis base on Bray-Curtis dissimilarity index. **(B)** Statistical significance *via* Analysis of Similarity (ANOSIM) test with 999 permutations.
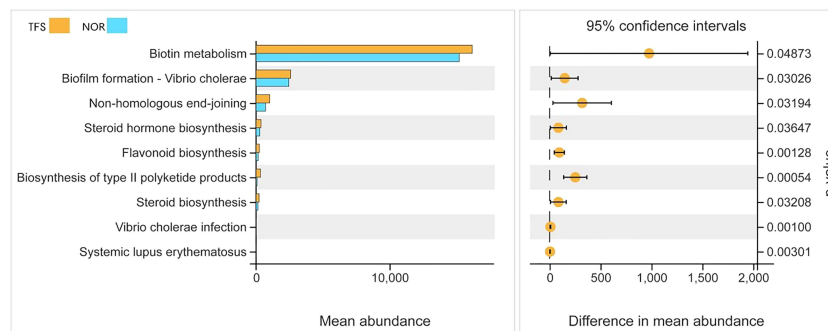
FIGURE 7

Functional differences in intestinal microbial communities between normal (NOR) and TFS *D. hystrix*. Functions were predicted by PICRUSt2 analysis of bacterial sequences. Significantly different functions (P < 0.05) of KEGG pathway categories (level 3) are show.

steroid hormone signaling is essential to regulate innate immune cells and fight against bacterial infection (Regan et al., 2013). The steroid-signaling pathway can also help Caenorhabditis defend against bacteria (Fischer et al., 2013). Moreover, several studies have shown that steroid hormones exert excellent anti-inflammatory properties; e.g., 2-methoxyestradiol and corticosteroids elicit wide ranging anti-inflammatory effects (Shand et al., 2011; Doycheva et al., 2018). Steroid hormones play important roles in enhancing biological immunity; however, our results showed that steroid biosynthesis and flavonoid biosynthesis were down-regulated in the intestinal microbiota in diseased fish, and this downregulation can decrease sterol synthesis, weaken fish immunity, and increase susceptibility to the invasion of pathogens, thus resulting in TFS. However, these functional profiles were predicted by 16S data, therefore, more experimental studies are required.

5 Conclusions

In this study, the changes in the intestinal tissue and microbiota of TFS and healthy *D. hystrix* were explored through histopathological sectioning and 16S rRNA sequencing. Histological analysis indicated that diseased guts were highly disrupted. Analysis of the community diversity of the intestinal microbiota showed that TFS decreased microbiota community stability and induced intestinal microbiota community structural change in *D. hystrix*. Furthermore, functional comparison predictions between normal and TFS *D. hystrix* revealed that the significantly altered pathways were associated primarily with metabolism, thus leading to the alteration of a number of pathways related to sterol synthesis. Overall, our data indicate that TFS affects intestinal microbiota composition and function in *D. hystrix*, thus expanding knowledge of diseases associated with porcupine pufferfish aquaculture.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA913412.

Ethics statement

The animal study was reviewed and approved by The Animal Research and Ethics Committees of South China Agriculture University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LZ, XZ, and QW designed the experiments. XJ, KL, and FH conducted the DNA extraction, amplification and sequencing. FH, XD and ZL extraction, amplification and sequencing of DNA. KL, XD, and ZL conducted the Bioinformatics and statistical analysis. LZ, SS and QW wrote the manuscript with feedback from all authors. All authors contributed to the article and approved the submitted version.

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Conflict of 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1108737/full#supplementary-material

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