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Microbiome analysis of gut bacterial communities of healthy and diseased Malaysian mahseer (*Tor tambroides*) using 16S rRNA metagenomics approach

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ABSTRACT

Aims: The gut microbiota is referred to as an 'extra organ' and is critical in assisting the host in terms of nutrition and immunity. Environmental stressors could alter the gut microbial community and cause gut inflammation. This study aimed to investigate and compare the gut microbiota community between healthy and diseased *Tor tambroides*.
Methodology and results: In this study, such gut microbial alterations were explored using NGS-based 16S rDNA targeted sequencing on the Malaysian mahseer (*T. tambroides*). Three healthy adult and three diseased adult Malaysian mahseers (showing signs of exophthalmia, coelomic distension and petechial haemorrhage) were obtained from LTT Aquaculture Sdn Bhd. Our results revealed significant differences in microbial diversity, composition and function between both populations of *T. tambroides*. Alpha diversity analysis depicts lower diversity of gut microbiota composition in diseased *T. tambroides* as compared to the healthy group. In particular, Enterobacteriaceae, *Aeromonas*, *Bacteroides*, *Vibrio* and *Pseudomonas* were found within gut microbiota of the diseased fishes. In addition, cellulose-degrading bacteria and protease-producing bacteria were identified from the gut of *T. tambroides*.
Conclusion, significance and impact of study: Thus, our findings emphasized on the association between the alteration in gut microbiota composition and infectious abdominal dropsy (IAD) in *T. tambroides*. This finding is important to provide basic information for further diagnosis, prevention and treatment of intestinal diseases in fish.

Keywords: 16S rRNA gene, gut microbiota, infectious abdominal dropsy, Malaysian mahseer, metagenome

INTRODUCTION

Within the past few decades, study of microbiota within gastrointestinal tract (GIT) had been achieving remarkable progress with the discovery of more GIT microbiome communities on a host organism (Li *et al.*, 2016; Liu *et al.*, 2016; Egerton *et al.*, 2018; Tran *et al.*, 2018; Butt and Volkoff, 2019; Tan *et al.*, 2019; Burtseva *et al.*, 2021). As of today, there are two main approaches to discover on gut microbiota community: culture-dependent microbiological methods and culture-independent methods. The classical method involved seeding gut sample directly on either selective or universal media (Hovda *et al.*, 2007; Tarnecki *et al.*, 2017) while the later involved DNA barcoding. For instance, denaturing gel electrophoresis, qPCR and fluorescence *in situ* hybridization (Hovda *et al.*, 2007; Tarnecki *et al.*, 2017; Egerton *et al.*, 2018). As culture-dependent methods are time-consuming and selective, it is unable to provide the entire microbial diversity of complex environments (Hovda *et al.*, 2007). Thus, NGS-based method involving metabarcoding based on 16S rRNA gene is now a popular method among researchers to undercover more uncultured forms of microorganisms and estimate different bacterial groups within the sample as it is able to describe both cultivable and uncultivable bacteria (Tarnecki *et al.*, 2017; Egerton *et al.*, 2018).

Gut microbiota is considered as an 'extra organ' due to its important role in intestinal development, immunological protection, growth and health and homeostasis (O' Hara and Shanahan, 2006). Thus, various studies comparing the gut microbiota composition between healthy and diseased freshwater fish, including largemouth bronze gudgeon (*Coreius guichenoti*) suffering from furunculosis (Li *et al.*, 2016), Crucian carp (*Carassius auratus*) suffering from "red-operculum" disease (Li *et al.*, 2017) and grass carp (*Ctenopharyngodon idellus*) suffering from enteritis had been done to further understand on its role. Myriad diversity of mutualistics, commensal and pathogenic microbes within the intestinal tube would assist the host in terms of protection against infectious agents, nutrients

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uptake and absorption as well as synthesizing digestive enzymes (Nayak, 2010). Researchers concur that the intestine acts as the main portal of entry for pathogen invasion and disease occurrence (Dash *et al.*, 2008; Nayak, 2010; Li *et al.*, 2016). Environmental stress, such as poor water quality, exerted upon the fish would disrupt the gut microbiota community and cause gut inflammation (Liu *et al.*, 2016). An inflamed gut can compromise the immune system and eventually cause the overgrowth of invading pathogenic bacteria and existing opportunistic pathogens (Zeng *et al.*, 2017). Massive development of Enterobacteriaceae initiated by gut inflammation would induce further pathogen invasion as well (Zeng *et al.*, 2017). Intestinal diseases in fishes encompassing infectious dropsy (Aly and Ismail, 2016), furunculosis (Li *et al.*, 2016), 'red-operculum' disease (Li *et al.*, 2017), enteritis (Tran *et al.*, 2018) are reported to be associated with changes in gut microbiota community. Invasion of pathogenic species is associated with imbalance microbiota community. For instance, infectious dropsy (*Aeromonas hydrophilia* and *Pseudomonas fluorescens*) (Aly and Ismail, 2016), enteritis (*Pseudomonas* and *Flavobacterium*) (Tran *et al.*, 2018), 'red-operculum' disease (*Vibrio*, *Aeromonas* and *Shewanella*) (Li *et al.*, 2017) and furunculosis (*Aeromonas salmocida*) (Li *et al.*, 2016).

Tor tambroides is one of the most exorbitant freshwater species in Malaysia due to its unique flesh contributed by its engkabang consumption. It is still remained re-evaluated as it is classified as data deficient by International Union for Conservation of Nature (Kottelat *et al.*, 2018). Nevertheless, it is under threat due to environmental degradation caused by human activities (Pinder *et al.*, 2019). Among the *Tor* species, *T. tambroides* which is indigenous to Sarawak of East Malaysia, is one of the most valuable freshwater species. To date, there are 16 *Tor* species that can be found worldwide, with three (18.8%) found in Malaysia, which are *Tor tambroides*, *Tor tambra* and *Tor douronensis* (Ng, 2004). Studies had found out that different colours of *T. tambroides* (silver-bronze and reddish) had the possibility to associate with environmental influences (Esa *et al.*, 2006; Siraj *et al.*, 2007). However, ambivalent descriptions of these three *Tor* species inhabiting in Malaysia had caused misidentification among scientific communities in the past few years (Walton *et al.*, 2017). Furthermore, a recent study in disentangling the phylogenetic relationship among *T. tambra* and *T. tambroides* sampled from Malaysia (Sarawak, Pahang and Terengganu) and Indonesia (Java) using 13 protein coding genes and two ribosomal RNA genes had shown a monophyletic clustering based on the sampling locations (Lim *et al.*, 2021). *Tor tambroides* is among the most valuable game fish, aquaculture fish and ornamental fish, which can also serve as a superior source of protein (Ng, 2004), with an aquaculture production of mahseer in 2018 and 2017 recorded at 12.31 tonnes (RM 3.43 million) and 24.19 tonnes, respectively (RM 5.07 million) (DOF, 2017; 2018). Thus, it can be said that *Tor* genus species owns great potential in the aquaculture industry (Ingram *et al.*, 2005).

Similar to other freshwater fishes, *T. tambroides* is vulnerable to infectious diseases as well. Infectious abdominal dropsy (IAD) disease is one of the infectious diseases faced by most freshwater fish due to bacterial infection. It is described as an acute hemorrhagic disease which causes mortality and morbidity among fish (Dash *et al.*, 2008). IAD had been reported across various species of fishes, including Indian major carps (*Catla catla*, *Labeo rohita*, *Cirrhinus mrigala*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), bighead carp (*Aristichthys nobilis*), silver carp (*Hypojtjalmichthys molitrix*), sheatfish (*Silurus glanis*), crucian carp (*Cyprinus carassius*), tench (*Tinca tinca*) and rainbow trout (*Onchorhynchus mykiss*) (Dash *et al.*, 2008; Petty *et al.*, 2012). As one of the most common genera dominating freshwaters, *Aeromonas* also associated with virulence genes and hemolytic activity which allows infection when the host in under stress (Hamid *et al.*, 2016; Vatsos, 2017). Besides mahseers, it was being detected in giant freshwater prawns, tilapia and catfish as well (Chiew *et al.*, 2019).

To the best of our knowledge, there were various studies conducted to understand more on *T. tambroides*, but most of them focused more on its feed formulation and feed additives in improving its growth rate, while only a minority of them focused on the molecular biology aspect (Apun *et al.*, 1999; Ng *et al.*, 2008; Misieng *et al.*, 2011; Ng and Andin, 2011; Kamarudin *et al.*, 2012; Ramezani-Fard *et al.*, 2012; Ishak *et al.*, 2016; Lau *et al.*, 2021; Lim *et al.*, 2021). Although there is a finding highlighting on the gut microbiota comparison among wild and captive *T. tambroides* sampled from West Malaysia (Tan *et al.*, 2019), the gut microbial community of *T. tambroides* suffering from disease remains unclear. Thus, in this study, Illumina MiSeq Paired-end sequencing (Apical Scientific Sdn Bhd) was used to sequence the V3-V4 regions of 16S rRNA genes for the comparative gut bacterial identification. The healthy and diseased *T. tambroides* were sampled from an aquaculture farm in Sarawak, East Malaysia, in order to identify and compare the gut microbiota between healthy and dropsy diseased *T. tambroides* and to identify the possible pathogenic agents associated with the diseased state of the fish. V3-V4 regions were chosen in this study as it generates a higher richness and diversity while giving a more in-depth characterization of microbial composition (García-López *et al.*, 2020).

MATERIALS AND METHODS

Sample collection

In this study, both healthy and diseased sample groups were included, with three adult fish representing each group. All the samples were obtained from an aquaculture farm located at Asajaya, Sarawak, Malaysia (GPS coordinates: 1°32'53.647" N, 110°32'53.233" E). Altogether, three adult healthy *T. tambroides* (standard

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length 44.4 ± 0.43 cm, weight 1.05 ± 0.15 kg) and three diseased *T. tambroides* (standard length 57.57 ± 6.08 cm, weight 2.77 ± 0.71 kg) were obtained. The diseased *T. tambroides* fishes were examined morphologically to record on their clinical symptoms before some internal fluids were cultured.

Fish dissection and DNA extraction

Fishes were euthanized and their abdomens were dissected using sterile instruments inside the HEPA filter graded laminar flow hood. All procedures in this study were in compliance to the guidelines and permission approved by the Animal Ethics Committee of Universiti Malaysia Sarawak (UNIMAS/TNC(PI)-04.01/06-09(17)). The gut was taken from oesophagus to anus. The GI tract (gastrointestinal tract) was cut into small pieces of an approximate length of 1 cm within CTAB buffer before DNA extraction using modified CTAB-based protocol (Chung, 2018). Eluted DNA was taken to check for its concentration using a Nanodrop DS-11 Series (DeNovix; USA). Only samples with ratio absorbance at 260 nm and 280 nm ($A_{260/280}$) around 1.8 were selected for further processing.

Library preparation and sequencing

The V3-V4 hypervariable regions of 16S rRNA genes of gut microbiota was chosen to be amplified through polymerase chain reaction (PCR) using following primers: 27F_B (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3') and 519R_A (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG 3'). 27F_B is the forward primer and 519R_A is the reverse primer which were both widely used (Lane, 1991; Handl *et al.*, 2011). The reaction mixtures (25 µL) included: 2× KAPA HiFi HotStart ReadyMIx (12.5 µL) (Kapa Biosystems, USA), forward and reverse primers (1 µM and 5 µL each) and template DNA (5 ng). Amplification conditions were set as follow: 3 min of initial denaturation at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 30 sec and a final elongation at 72 °C for 5 min.

The DNA samples were sequenced on an Illumina MiSeq platform. In brief, DNA samples were subjected to library preparation prior to sequencing. The amplicons were cleaned up for the attachment of unique index adapter pairs to the amplicons using Nextera XT Index kit (Illumina, USA). The indexed DNA libraries were cleaned up with Agencourt AMPure XP (Beckman Coulter, USA). The concentrations of libraries were quantified using Qubit dsDNA HS Assay Kit and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) and size validated using Agilent 2100 Bioanalyzer (Agilent, USA). Next, the libraries were normalized and pooled for subsequent MiSeq sequencing (2 × 300 bp paired-end). All sequences were submitted to NCBI Sequence Read Archive (SRA) under accession number of PRJNA778601 (https://www.ncbi.nlm.nih.gov/sra/PRJNA778601).

Data analysis using Quantitative Insights into Microbial Ecology (QIIME)

Data was then analyzed using Quantitative Insights into Microbial Ecology (QIIME2 ver 2020.8) (Bolyen *et al.*, 2019). Adapter sequences were cleaved from both paired-ends forward and reverse reads using cutadapt command prior to trimming chimeric sequences. Divisive Amplicon Denoising Algorithm 2 (DADA 2) was used to denoise and filter chimeric sequences based on parametric model which infer true biological sequences from reads (Prodan *et al.*, 2020). Forward and reverse reads were denoised independently and merged prior to removal of chimeric sequences through 'removeChimeraDenovo', which eventually formed Amplicon Sequence Variants (ASV). DADA2 was chosen due to its high sensitivity to detect and differentiate ASVs at single-base resolution, even at high abundance ratio (Callahan *et al.*, 2017; Prodan *et al.*, 2020). ASVs-based method had demonstrated high sensitivity as well and specificity as good as or even better than Operational Taxonomic Unit (OTU) methods which gave better differentiated ecological patterns (Callahan *et al.*, 2017). Clustered ASVs were then summarized into different taxonomic levels based on GreenGenes database at 99% identity threshold (version 13_8) (DeSantis *et al.*, 2006).

Alpha rarefaction curve was plotted to determine the sequencing depth. Different alpha diversity index parameters (Chao1, Simpson and Shannon) were utilized to further elucidate on the species richness and diversity of each sample among gut microbiota of both healthy and diseased *T. tambroides.* The value of Chao1 index reflects theoretically predicted richness and abundance (Chao, 1984). Simpson index ranges from 0 to 1 with 0 indicating infinite diversity and 1 indicating zero diversity (Simpson, 1949) while Shannon index reflects species richness and evenness (Shannon, 2001). Furthermore, in order to estimate the coverage of total species represented in each sample, Good's Coverage was taken into account as well. Beta diversity analysis aims to evaluate diversity among gut microbiota of healthy and diseased *T. tambroides.* Thus, principle coordinate analysis (PCoA) was plotted for visualization of differences or likeliness of gut microbiota diversity among both healthy and diseased *T. tambroides* based on phylogenetic or count-based distance metrics. Weighted UniFrac was chosen in this study for PCoA as it is able to detect the differences in relative abundances of each taxon within the communities (Lozupone *et al.*, 2007). Permutational multivariate analysis of variance (PERMANOVA) was used to evaluate statistical differences in beta diversity while Kruskal-Wallis test was employed to evaluate differences in alpha diversity index of gut microbiota diversity among both healthy and diseased *T. tambroides*.

ANCOM was used to determine the differences of gut microbial communities among both healthy and diseased *T. tambroides* (Mandal *et al.*, 2015). ANCOM was selected over other statistical tests as it made no assumptions and performed well even involve thousands

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of taxa (Mandal *et al.*, 2015). Mandal *et al.* (2015) stated that the false discovery rate was controlled at a desired nominal level which further improved its performance.

RESULTS AND DISCUSSION

DNA quality check

The PCR product was visualized on a 1.7% TAE agarose gel at 100 V for 65 min (Figure S1). Discrete bands were observed at size ranges of 450 bp to 550 bp, indicating the presence of V3-V4 region of 16S rRNA gene in all the six samples of *T. tambroides*, with an expected size of approximately 460 bp (Figure S1). Only DNA samples with D(260)/D(280) reading more than 1.8 were subjected to 16S metagenetic sequencing (Table S1).

Clinical findings on diseased *T. tambroides*

Diseased adult *T. tambroides* were observed and identified for clinical signs. Three samples displayed some noticeable signs include exophthalmia (pop-eye), coelomic distension and petechial haemorrhage on skin, gills, tails and eyes can be observed (Figure 1). Sample D2 showed some loss of scales at its abdominal section as well. Internally, fluid was observed within the internal cavity of diseased *T. tambroides*, which might be due to organ inflammation (Figure 1). A brief microbial culture and 16S sequencing revealed the presence of *Aeromonas hydrophila* in body fluid.

In previous studies (Dash *et al.*, 2008; Aly and Ismail, 2016; Lopamudra and Nayak 2020), researchers had reported on the morphological signs of infectious dropsy in *Cyprinus carpio* (common carp), *Catla catla* (South Asian carp), *Labeo rohita* (rohu), *Cirrhinus mrigala* (Mrigal carp) and *Hypopthalmichthys molitrix* (silver carp). The typical clinical symptoms included haemorrhagic lesions presented on skin, fins, tail and eyes, coelomic distension, loss of scales and exophthalmia (Aly and Ismail, 2016). *Aeromonas hydrophilia* and *Pseudomonas fluorescens* were suggested as causative agents for the disease (Dash *et al.*, 2008; Aly and Ismail, 2016).

Studies done on fish disease exhibiting syndrome including exophthalmia, haemorrhagic lesions on fins and abdominal swelling with visceral fluid in Nile tilapia (*Oreochromis niloticus*) had included *Aeromonas veronii*, *Flavobacterium columnare*, *Plesiomonas shigeloides*, *Streptococcus agalacticae* and *Vibrio cholerae* as predominant bacteria (Dong *et al.*, 2015). Clinical signs had been mimicked by infecting the fishes with *A. veronii* and *F. columnare* (Dong *et al.*, 2015). Subsequently, Dong *et al.* (2017) had identified the bacterial isolates as *Aeromonas jandaei* and *Aeromonas veronii* in diseased Nile tilapia. They revealed a reduced 10- and 100- fold dose of both *Aeromonas* (*Aeromonas jandaeii*: 3.7×10^6 CFU/fish; *Aeromonas veronii*: 8.9×10^6 CFU/fish) had shown diseased symptoms including a significant amount of yellowish fluid built up internally. Dong *et al.* (2017) emphasized that the similar signs were exhibited with and without the treatment of *Aeromonas* strain.

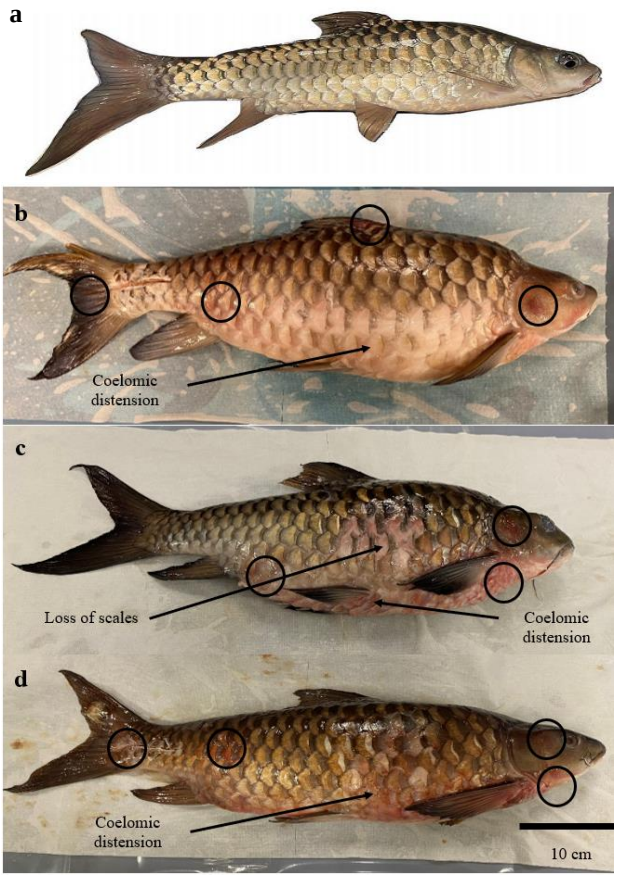

Figure 1: Healthy and diseased samples of *Tor tambroides*, including (a) healthy sample; (b) sample D1; (c) sample D2; (d) sample D3, with the clinical symptoms labelled, including loss of scales and coelomic distension pointed out by arrows while petechial haemorrhage circled on gills, skin, eyes and tails.

Our study had revealed genus *Vibrio*, *Streptococcus*, *Pseudomonas* and *Aeromonas* in the gut microbiota of *T. tambroides* through 16S rRNA gene metagenomic sequencing (Table S6). *Aeromonas* was detected through microbial culture and 16S Sanger sequencing. Nevertheless, *Flavobacterium* was only found in healthy gut microbiota while *Pleisodomonas* was found absent in both groups. As comparison, *Vibrio*, *Pseudomonas* and *Streptococcus* were found to have greater and non-significant relative abundance in diseased gut microbiota of *T. tambroides*. *Vibrio* and *Aeromonas* might be the main opportunistic bacteria which are pathogenic to fishes (Li *et al.*, 2017). They were highly-adhesive which enables them to colonize at the intestinal surface mucosa, eventually cause the gut as the primary location for stress-induced infection (Namba *et al.*, 2007). Thus, we suggested *Vibrio* and *Aeromonas* to be associated with the disease-state of *T. tambroides.* However, more evidence is needed to support this postulation.

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It is suggested that possible pathogens might be present in the intestine prior to disease occurrence and this is in accordance with previous studies done by Li *et al.* (2016) on largemouth bronze gudgeon suffering from furunculosis. The further invasion of pathogenic strain can be due to various factors, including environmental stressors (pollutants, decreased water quality) which weakens the host's immune system. This was further supported by a study on microbiota composition analysis of gut in infected Crucian carps and also its surrounding environment by Li *et al.* (2017). They emphasized on the water physiochemical factors was correlating significantly with the gut microbiota of the fish. Among the water parameters taken, Li *et al.* (2017) highlighted that the temperature and total ammonia-nitrogen (NO_3^--N) were the most essential factors in shaping the gut microbiota composition.

Healthy *T. tambroides* owns greater gut microbiota species richness

Illumina Miseq 16S sequencing at the V3-V4 region of 16S rRNA gene enabled an in-depth view and characterization of the gut microbial communities of both healthy and diseased *T. tambroides*. The sequence information of gut microbiome of both healthy and diseased *T. tambroides* were summarized in Table S2. A total of 3,093,610 reads had been generated in this study, ranges from 373,170 to 444,833 and 102,759 to 117,066 in healthy and diseased group, respectively, which were found to be higher than previous studies involving fish gut metagenomic studies (Li *et al.*, 2016; Tran *et al.*, 2018; Burtseva *et al.*, 2021). The reads were assigned to 421 ASVs were generated by QIIME 2 at 99% similarity levels, with 301 and 165 ASVs detected in healthy and diseased *T. tambroides* gut microbiota, respectively. Among the 301 ASVs detected in healthy *T. tambroides* gut, 12 ASVs (4%) were found to be present in all healthy samples while 15 ASVs (9.10%) out of total 165 ASVs can be found across all diseased samples. In this study, ASV was generated despite of OTU which can be found in other metagenomic studies. Due to the generation of exact sequence by distinguishing the sequence variants differing by one nucleotide, the total number of ASVs generated were lower than OTU which is also dependent on the denoising approach used (Callahan *et al.*, 2017; Nearing *et al.*, 2018).

Reduced microbiota diversity in dropsy diseased *T. tambroides* gut

The sequencing depth was normalized to 50,000 for both healthy and diseased *T. tambroides* samples (Figure 2) as plateau was observed when the curve flattened gradually as the sequencing depth increase, indicating the current sequencing depth is sufficient to reflect diversity in each sample and the probability of discovering more ASVs beyond the depth is negligible. To estimate completeness of bacterial diversity by obtained 16S rRNA amplicon data, it is necessary to analyze the relationship between Chao1 index and the observed taxa abundance. In Table 1, Good's coverage had confirmed the sequencing had covered up to an approximate 100% of all gut microbiota found in both healthy and diseased *T. tambroides*. Other alpha diversity parameters (Chao1, Shannon, and Simpson) had been included to evaluate on species diversity of each sample group. Shannon index showed that diseased *T. tambroides* gut microbiota had higher species richness than that of the healthy *T. tambroides.* Nevertheless, Chao1 index and Simpson index for healthy *T. tambroides* which recorded as 119.33 and 0.37, respectively, indicates a higher diversity than diseased fish at 74.00 and 0.54, respectively.

It is in agreement with the diversity resistance hypothesis that greater diversity microbial community possess greater possibility of having a species with antagonistic trait towards invading pathogens (Fargione and Tilman, 2005). Similar trend can be seen in previous studies done on Crucian carp (*Carassius auratus*) (Li *et al.*, 2017), largemouth bronze gudgeon (*Coreius guichenoti*) (Li *et al.*, 2016), Gibel carp (*Carassius gibelio*) (She *et al.*, 2017) and ayu (*Plecoglossus altivelis*) (Nie *et al.*, 2017), exerting greater protection on the host towards invading pathogen. Possible explanation on this is due to competition among invading pathogens with gut commensals, therefore reducing diversity in diseased fish (Xiong *et al.*, 2019). Thus, it can be said that lower gut microbiome diversity is closely associated with diseases (Li *et al.*, 2017; Xiong *et al.*, 2019). However, our findings contradict with previous study on grass carps with intestinal disease (Tran *et al.*, 2018), which might be due to differences in species and associated diseases.

Beta diversity analysis of healthy and diseased *T. tambroides*

The PCoA plots shown in Figure 3 portrayed clusters based on healthy and diseased samples observed at Principal Coordinate 1 vs Principal Coordinate 2 (PC1 vs PC2). Permutational multivariate analysis of variance (PERMANOVA) had shown non-significant differences among both groups of samples ($p=0.395$). However, from Figure 3, the plots of the diseased samples were dispersed widely while the healthy sample plots tend to be located in closer proximity. The overall distribution distance between gut microbiota diversity of healthy and diseased *T. tambroides* was relatively far, further emphasized on the differences among the microbial compositions.

This finding was found to be consistent with reports in grass carps (Tran *et al.*, 2018) suffering from intestinal diseases. Stress exerted upon fishes would disrupt gut microbial community, termed gut dysbiosis, and this is associated with various disorders including inflammatory bowel disease (IBD) and infection (Kamada *et al.*, 2013). Such shift in relative bacterial abundance can be contributed by antibiotic administration, poor water quality and dietary changes. Inflammation would be induced due to compromised immune system, which fosters 'bloom' of low-abundance and harmful bacteria. Among the

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Table 1: Summary of the alpha diversity of healthy and diseased *T. tambroides* gut microbiota.

Index	Healthy Group					Diseased Group				
	H1	H2	H3	Mean	S.D.	D1	D2	D3	Mean	S.D.
Chao1	89.00	215.00	54.00	119.33	69.14	86.00	75.00	61.00	74.00	10.23
Simpson	0.11	0.52	0.47	0.37	0.18	0.88	0.46	0.29	0.54	0.25
Shannon	0.56	2.47	1.63	1.56	0.78	3.78	1.41	1.18	2.12	1.18
Good's Coverage	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	0.00

S.D. stands for standard deviation.

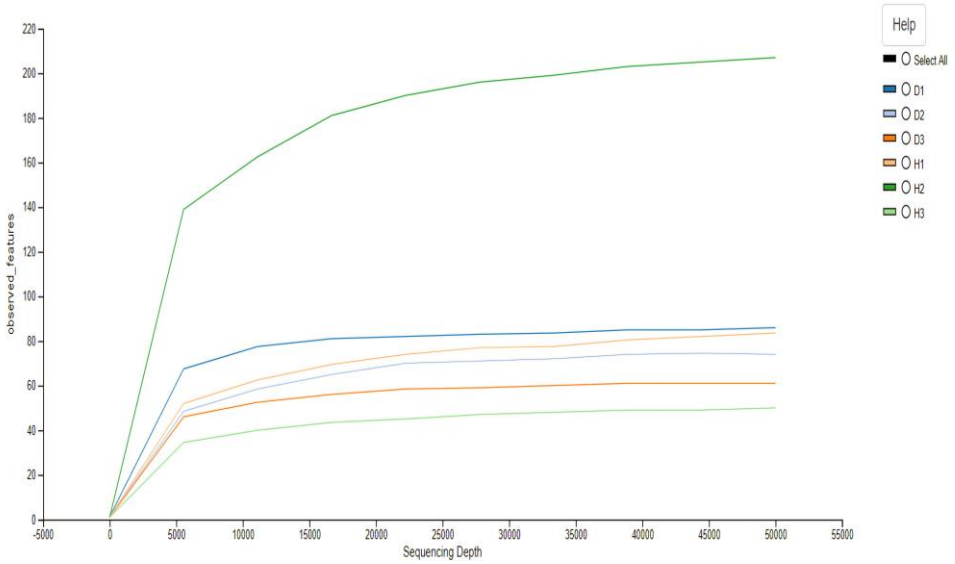

Figure 2: The alpha rarefaction curves of healthy and diseased *T. tambroides* gut microbiota. The x-axis shows the sequencing depth and the y-axis shows the observed features. (H1-H3: Biological replicates of healthy *T. tambroides*; D1-D3: Biological replicates of diseased *T. tambroides*).

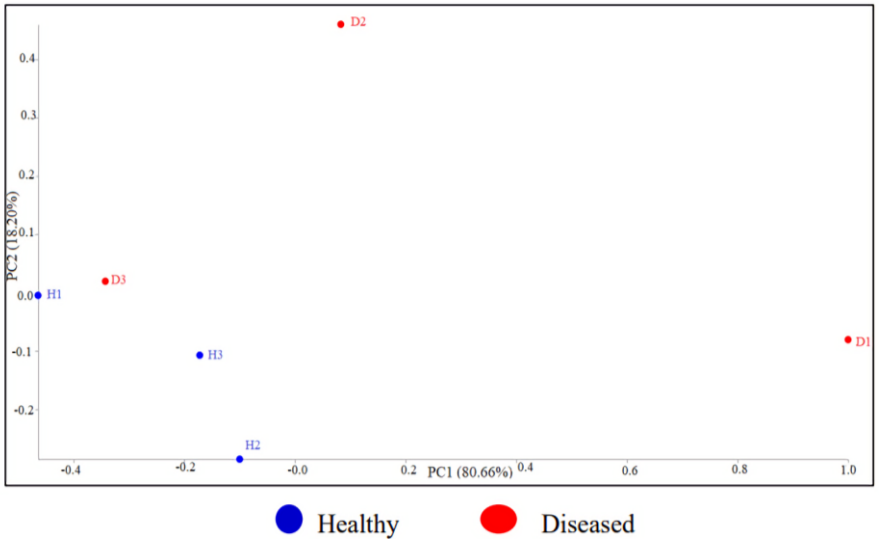

Figure 3: Principal Coordinate Analysis (PCoA) plots based on weighted UniFrac distance metric, representing PC1 vs PC2 on biological replicates of *T. tambroides*.

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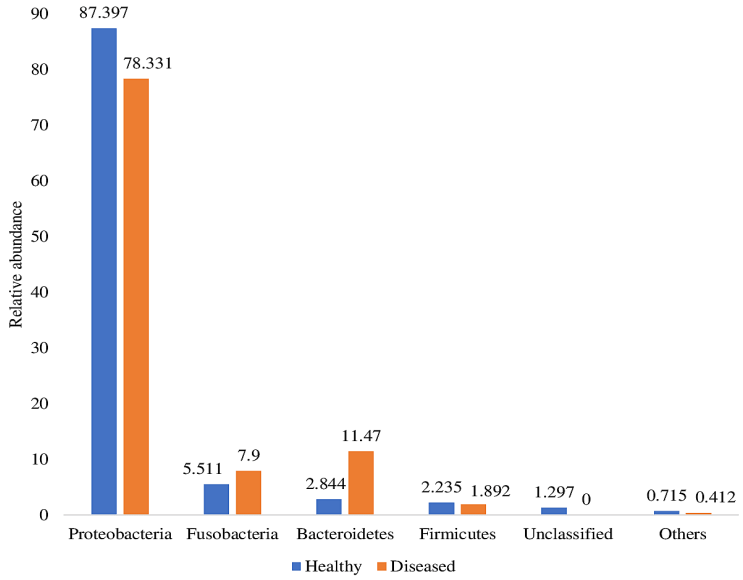

Figure 4: Relative abundance of dominant phyla (%) (mean relative abundance >0.4%) in gut microbiota of healthy group and diseased group of *T. tambroides*. Phyla with low abundance (<0.4%) were combined and categorized under 'others'.

diseased samples, sample D3 tend to lie closer to healthy group samples (Figure 3) than other two diseased samples, suggesting on the less severity in its disease state as compared to D1 and D2.

Microbiota taxonomy of healthy and diseased *T. tambroides* (Phylum level)

The gut microbiota of healthy *T. tambroides* was dominated mainly by Proteobacteria (87.397%), followed by Fusobacteria (5.511%), Bacteroidetes (2.844%), Firmicutes (2.235%), unclassified phyla from kingdom bacteria (1.297%) and others (0.715%) (Figure 4). On the other hand, gut microbiota of diseased *T. tambroides* was found to be mainly dominated by Proteobacteria (78.331%), Bacteroidetes (11.470%), Fusobacteria (7.900%), Firmicutes (1.892%) and others (0.412%) (Figure 4). Table S3 had summarized on relative abundance and frequency of each phylum between all samples while Table S4 summarized on the mean and percentage on each phylum on both groups. Proteobacteria was found to be the most abundant phyla in gut microbiota of both datasets. It was due to Proteobacteria are predominant in fish gut across all known metagenomic studies (Tan *et al.*, 2019; Burtseva *et al.*, 2021). The most abundant phylum observed in both datasets were in consistent with the findings by Tan *et al.* (2019) on wild and captive bred *T. tambroides* sampled from Kenyir Lake.

This study had highlighted an increase in relative abundance of phylum Proteobacteria, Firmicutes and unclassified bacteria which was found in relative higher abundance in healthy gut of *T. tambroides*. In contrast, phylum Fusobacteria and Bacteroidetes in the diseased gut microbiota of *T. tambroides* were observed to be in greater relative abundance. Such changes, especially with regards to the increased level of the bacterial species within the phyla, suggested that the overgrowth of opportunistic bacteria might reduce the relative abundance of other taxa colonizing fish intestine prior to pathogen invasion (Tran *et al.*, 2018). Furthermore, reduction in relative abundance of members within phyla in diseased fish might be related to dietary nutrients accessibility in the fish intestine during diseased state (Tran *et al.*, 2018).

However, the distribution of main classes (Alphaprotebacteria, Betaproteobacteria, Gammaproteobacteria) of phylum Proteobacteria differed among both healthy and diseased group. Alphaproteobacteria (91.77%) had the most counts in healthy gut microbiota of *T. tambroides*, followed by Gammaproteobacteria (8.17%) and lastly Betaproteobacteria (0.06%). In contrast, the dominant class found in diseased gut microbiota is Gammaproteobacteria which accounted for 52.15% of the counts, followed by Alphaproteobacteria (46.93%) and Betaproteobacteria (0.93%). Such phenomenon is due to Gammaproteobacteria consist of a wider range of pathogens as compared to Alphaproteobacteria which associates with host metabolism (Austin and Austin, 2012). Gammaproteobacteria in diseased *T. tambroides* was mostly comprises of possible disease causative agents, such as *Aeromonas*, *Enterobacteriaceae*, *Vibrio*, *Pseudomonas* and *Clostridium,* which is responsible for the diseased state of the fish. ANCOM was performed to determine significant differences among gut microbiota of healthy and diseased *T. tambroides* at phylum level, however there is no significant differences detected.

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Table 2: Top ten families found in gut microbiota of healthy and diseased *Tor tambroides*.

Healthy Group	Relative Abundance (%)	Diseased Group	Relative Abundance (%)
Sphingomonadaceae	79.021	Sphingomonadaceae	36.689
Aeromonadaceae	5.878	Enterobacteriaceae	25.728
Fusobacteriaceae	5.511	Bacteroidaceae	9.446
Bacteroidaceae	2.131	Pseudoalteromonadaceae	9.178
Ruminococcaceae	1.099	Fusobacteriaceae	7.900
Methylobacteriaceae	1.120	Aeromonadaceae	5.871
Pseudoalteromonadaceae	0.597	Peptostreptococcaceae	0.669
Peptostreptococcaceae	0.504	Lachnospiraceae	0.674
Lachnospiraceae	0.173	Campylobacteraceae	0.593
Others	1.402	Others	1.221

Not including families with lower abundance <0.40%.

Unclassified bacteria had been identified in a high relative abundance in healthy gut microbiota of *T. tambroides* as compared to the diseased group. It is in consistence with the previous study done on largemouth bronze gudgeon (*Coreius guichenoti*) suffering from furunculosis (Li *et al.*, 2017). In this study, an interesting taxon had been identified, CK-1C4-19, which can be found in healthy *T. tambroides* only, was classified under phylum Tenericutes. It had been reported by previous studies on zebrafish (*Danio rerio*) (Roeselers *et al.*, 2011), fathead minnow (*Pimephales promelas*) (Narrowe *et al.*, 2015) and anjak (*Schizocypris altidorsalis*) (Ghanbari *et al.*, 2019) as small relative abundance.

Microbiota taxonomy of healthy and diseased *T. tambroides* (Family level)

There were 72 families identified from both diseased and healthy gut of *T. tambroides* with frequency and relative abundance reported for each sample in Table S5. Out of the total of 72 families, 61 and 50 families were reported within healthy and diseased group respectively. Table 2 showed the relative abundance of top ten most abundant family found in gut of both healthy and diseased *T. tambroides*. Sphingomonadaceae was found to be the dominant family for both datasets, covering up to 79.021% and 36.689% in healthy and diseased group, respectively. In previous studies, Sphingomonadaceae had been observed in healthy human gut and milk as one of the core members of Proteobacteria and elucidated on its role in host immunity (D'Auria *et al.*, 2013; Corona-Cervantes *et al.*, 2020). Sphingomonadaceae was found to be modulating and maintaining immune response (D'Auria *et al.*, 2013) and as a potent stimulators of natural killer T cells (Long *et al.*, 2007). It further stimulates innate immune system to control growth of microbial and as a continuous surveillance of invading pathogens (Long *et al.*, 2007).

Enterobacteriaceae (25.728%) were found to be in high abundance, followed by Bacteroidaceae (9.446%) in diseased gut group. In contrast, Aeromondaceae (5.878 %) and Fusobacteriaceae (5.511%) were found in high abundance in healthy gut of *T. tambroides*. Enterobacteriaceae are the most common overgrown symbionts and can be found in inflammation related infection, such as IBD, obesity and antibiotic treatment (Zeng *et al.*, 2017). This alteration in microbe community compromises colonization resistance which lead to further colonization and growth of pathogens. High relative abundance of Enterobacteriaceae found in diseased gut of *T. tambroides* as compared to the healthy group can be further elucidated through elevation of gut luminal oxygen level due to increased blood flow. Under inflammatory condition, invading microbes were destroyed by transmigrated neutrophils (PMN) accumulating at apical epithelium and gut lumen due to deposition of autoantibodies. NAPDH oxidase were activated to destroy invading pathogens further leads to excessive release of oxygen (Chin and Parkos, 2006). Moreover, both epithelial cells and transmigrated PMNs would produce nitrate species as well. Nitrate respiration favour Enterobacteriaceae bloom at certain nitrate-rich tissue environment, as gene encode for nitrate reductase were found within the family. Amount of nitrate would rise significantly when a superoxide reacts with nitric oxide (NO) and eventually converted into nitrate (NO_3^-) that enable nitrate respiration (Winter *et al.,* 2013). Such PMN transmigration were shown in an inflammed zebrafish model with expression of green fluorescence protein (GFP) by neutrophils under control of myeloperoxidase promoter (Mathias *et al.*, 2006).

In addition, previous studies had emphasized on bloom of Enterobacteriaceae which disrupt gut microbiota balance, lead to gut inflammation and further foster disease occurrence (Zeng *et al.*, 2017). Enterobacterial blooms which involved gram-negative facultative bacteria within class Gammaproteobacteria would compromise host immune system, which increases its vulnerability towards incoming pathogens. This can be elucidated through the lipopolysaccharides which act as potent inflammatory pathogen-associated molecular pattern (PAMP) in Enterobacteriaceae which worsen intestinal injury (Wallace *et al.*, 2011). Enterobacteriaceae bloom act as a key factor for potential pathogenesis development of various diseases. For instance, it would cause the host to be more vulnerable to colitis disease induced by *Clostridium* in mice (Buffie *et al.*, 2012). This is consistent with the increase in relative abundance of *Clostridium* found in diseased gut microbiota of *T. tambroides* as compared to healthy gut.

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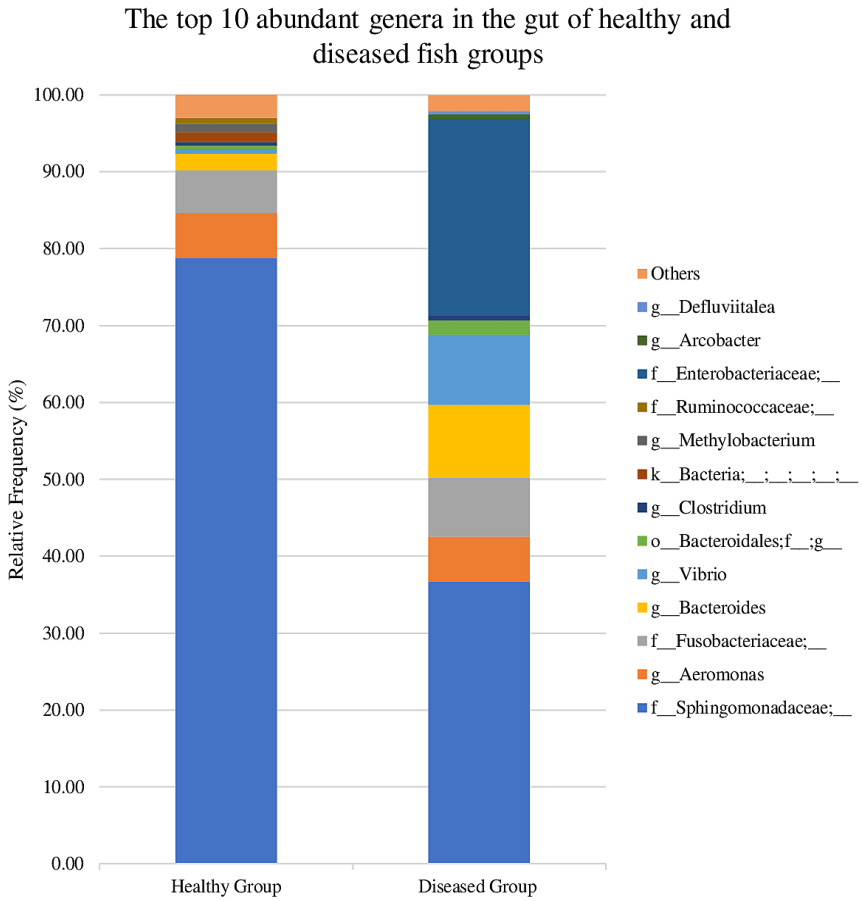

Figure 5: Histogram showing the relative abundance of the top 10 most abundant genera in gut microbiota of both healthy and diseased groups of *Tor tambroides.* Genera with lower abundance (<0.40%) were combined and clustered under 'others'.

Microbiota taxonomy of healthy and diseased *T. tambroides* (Genera level)

Table S6 had reported on the relative abundance of genera on each samples of both groups. A total of 88 genera was found with 70 found within healthy group and 49 found within diseased group. Both groups shared 30 genera, with 40 and 18 genera uniquely identified from gut microbiota of healthy and diseased *T. tambroides* respectively (Table S7). Figure 5 showed the top ten most abundant genera observed in gut microbiota community of both healthy and diseased *T. tambroides*. *Aeromonas* (Healthy: 5.878%; Diseased: 5.871%) and *Bacteroides* (Healthy: 2.131%; Diseased: 9.446%) were the top 3 highest abundance in healthy and diseased gut of *T. tambroides*. *Bacteroides* were found to be increase in abundance in diseased gut as compared to the healthy gut but *Aeromonas* reported on an approximate similar relative abundance in both groups. Furthermore, *Vibrio* (Healthy: 0.600%; Diseased: 9.178%) is reported to be increased in diseased gut microbiota of *T. tambroides*, suggesting it might be associated with the disease-state of the fish. Table 3 showed the top ten most abundant unique observed bacteria among the gut microbiota of both groups. Among them, *Arcobacter* showed the highest relative abundance (0.593%) in diseased gut of *T. tambroides* but it was absent in healthy fish gut.

In this study, a few highlighted genera have contributed towards the relative abundance in diseased gut as compared to healthy gut of *T. tambroides*, namely *Bacteroides*, *Vibrio*, *Pseudomonas* and *Clostridium.* Thus, it can be suggested that these genera may play an essential role in disease progression of *T. tambroides*. Nevertheless, the association between the high relative abundance of these genera and intestinal inflammation remained unclear until further investigation is done (Tran *et al.*, 2018). For instance, an experimental infection with these genera on healthy fish to undercover its association with intestinal inflammation. With the presence of these species in healthy gut as well, it is widely deemed that the fish digestive tract is a reservoir for opportunistic pathogens (Wu *et al.*, 2012).

Probiotics successfully detected in guts of both healthy and diseased *T. tambroides*, include *Streptococcus*, *Lactococcus* and from order Lactobacillales, which they can be utilized as candidate

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Table 3: Top ten most abundant unique observed bacteria found in gut microbiota of either healthy or diseased *Tor tambroides*.

Healthy group	Percentage (%)	Disease group	Percentage (%)
Papillibacter	0.344	*Arcobacter*	0.593
Gammaproteobacteria	0.249	Fusobacteriaceae	0.233
Sphingomonas	0.223	Lactobacillales	0.106
Bacteroidetes	0.122	*Enterobacter*	0.104
Gemmatimonadales	0.104	*Parasegitibacter*	0.032
CK-1C4-19	0.078	*Prevotella*	0.027
Firmicutes	0.065	Microbacteriaceae	0.016
Geodermatophilaceae	0.044	*Succinivibrio*	0.016
Verrucomicrobiaceae	0.041	*Mycoplasma*	0.014
Methylobacteriaceae	0.035	*Methanobrevibacter*	0.011

probiotics. Another possible pathogenic pathogen, *Aeromonas* spp. was found in gut microbiota of both healthy and diseased *T. tambroides*, with a similar relative abundance (Healthy: 5.878%; Diseased: 5.871%). Previous studies had revealed that *Lactococcus* could suppress the microbial activity of seven *Aeromonas* strains (Hagi and Hoshino, 2009). The higher relative abundance of the lactic acid bacteria (LAB) in diseased *T. tambroides* may suggest the presence of higher suppression activity by *Aeromonas* spp. Nevertheless, there were studies suggesting *Aeromonas* play essential role in fish biology rather than as an opportunistic pathogen (Wu *et al.*, 2012). Further *Aeromonas* colonization may be mostly due to stress-induced infection (Wu *et al.*, 2012). Aside from *Lactococcus*, relative abundance of *Streptococcus* is relatively higher in diseased *T. tambroides* and this is consistent with the findings by Tran *et al.* (2018). It might be involved in controlling the intestinal inflammation in *T. tambroides*. The underlying mechanisms need further investigations.

Elevated production and release of mucin is a hallmark of intestinal inflammation mostly triggered by enteric infection, which act to expulse pathogens and maintain the integrity of mucus layer as the first line of host defence (Frank *et al.*, 2007). Significant increase of *Bacteroides*, as a mucin-degrading bacteria, was detected in diseased gut microbiota of *T. tambroides*, which mediated release of less complex sugar from mucin, such as lactose, raffinose, melibiose and galactinol. *Bacteroides* arbitrated release of sialic acid from mucin in inflamed gut which can be taken up by bacteria for sugar production, further enhance Enterobacteriaceae bloom (Stecher, 2015).

Arcobacter was found uniquely in gut microbiota of diseased *T. tambroides* as compared to the healthy group. *Arcobacter* which previously known as 'Aerotolerant *Campylobacter*" was found as a zoonotic pathogen infecting both animals and human (Ho *et al.*, 2006). It is usually associated with gastrointestinal diseases, with high degree of similarity of clinical signs as compared to the *T. tambroides* in this study. The clinical signs include inflammatory gut, fallen scales and rotted fins (Açik *et al.*, 2016). *Arcobacter* had been isolated from intestinal tracts from zebrafish and human, yet its pathogenicity mechanisms were still in vague (Karadas *et al.*, 2013; Açik *et al.*, 2016). Studies had shown on its ability to adhere onto the wall of gut and invade as other bacteria such as *Campylobacter* (Açik *et al.*, 2016).

Cellulose-degrading bacteria including *Actinomycetospora*, *Clostridium*, *Ruminococcus* and *Enterobacter* were found within gut of *T. tambroides*, which is consistent with the finding by Wu *et al.* (2012). Possible protease-producing bacteria found were *Flavobacterium* and Gammaproteobacteria (Skrodenytė-Arbačiauskienė, 2000). *Flavobacterium* was identified from healthy guts of *T. tambroides* in lower relative abundance but was absent in the diseased group. It was suggested to be associated with various digestive processes by producing enzymes such as amylase, chitinase and protease (Skrodenytė-Arbačiauskienė, 2000).

Analysis of composition of microbiomes (ANCOM)

Analysis of composition of microbiomes (ANCOM) was used to determine the differences of gut microbial communities among both healthy and diseased *T. tambroides* (Mandal *et al.*, 2015) (Table S8). Both healthy and diseased gut microbiota of *T. tambroides* showed the highest abundance from family Sphingomonadaceae (Figure 6), but there are no significant differences shown in both sample groups. As comparison of gut microbiota of healthy group, some observed bacteria experienced a significant increment in their relative abundance of gut microbiota in diseased *T. tambroides*, including Fusobacteriaceae, *Bacteroides*, *Vibrio*, Bacteroidales and *Clostridium*. These changes found among gut microbiota suggested that occurrence of diseases would cause changes in gut microbiota composition and diversity. In Figure 6, among the top 15 abundant observed bacteria, only Enterobacteriaceae showed significant differences in gut microbiota between diseased and healthy *T. tambroides*.

CONCLUSION

To our knowledge, this is the first report that delineated the changes of composition, diversity and function of gut microbiota in Malaysian mahseer (*T. tambroides*) suffering from diseases. We compared the healthy and

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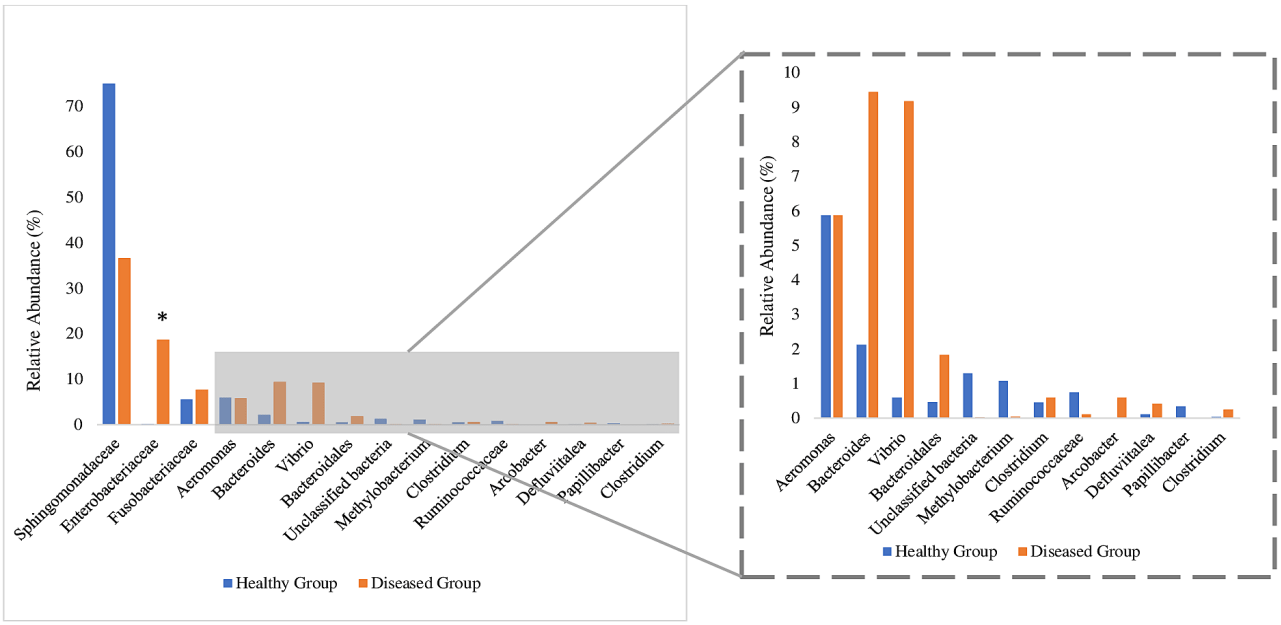

Figure 6: Comparison of top 15 most abundant observed bacteria in gut microbiota of both healthy and diseased *T. tambroides* (Bars with * indicated significant difference between healthy and diseased *T. tambroides* samples through ANCOM.

diseased *T. tambroides* gut microbiota and revealed more on the diversity of microbial communities. Based on the beta diversity analysis plotted on PCoA, gut microbiota of healthy *T. tambroides* were generally distantly spaced from the diseased *T. tambroides*. Furthermore, our findings unearthed exclusive genera namely *Vibrio*, *Bacteroides*, Enterobacteriaceae, *Pseudomonas*, Fusobacteriaceae and *Clostridium* in diseased gut microbiota of *T. tambroides*. Metagenetic sequencing had revealed on the gut microbiota of both healthy and diseased fish from an aquaculture farm, highlighting its role in further diagnosis, prevention and developing an ideal approach for better health management of Malaysian mahseer. Besides, we had also provided an insight into etiology of IAD and help to control strategies in intensive aquaculture practice and early detection. Yet, further studies are needed to elucidate more on the gut microbiota existing within captive and wild *T. tambroides* to further understand on the physiological functions, such as growth and disease resistance. Studies on reciprocal interactions between intestinal microbiota and host-produced enzymes in response to microbial alterations are recommended for future studies.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS' CONTRIBUTION

Chung, Lihan and Apun conceived of the study conception and experimental design. Lau, Kho and Sia were responsible for acquiring data while Lau, Leonard and Kho analyzed and interpreted the data. Lau, Leonard and Kho drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY INFORMATION

Table S1: DNA Quantification using Nanodrop DS-11 Series (DeNovix; USA).

Sample	DNA concentration (ng/µL)	Volume (µL)	Total amount (ug)	D(260)/D(280)
H1	117.80	13	1.53	2.002
H2	69.25	25	1.73	1.887
H3	3564.00	20	71.28	2.123
D1	2237.20	25	55.93	2.098
D2	786.50	20	15.73	2.043
D3	2326.30	25	58.16	2.100

H1-H3 stand for the gut samples of healthy fish, while D1-D3 stand for the gut samples of diseased fish.

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Table S2: Summary of gut microbiome in healthy and diseased *T. tambroides*.

Sample		Sequences after merging forward and reverse read	Sequences after QC filter	Sequences after chimera filter	ASVs
Healthy group	H1	398,247	277,904	274,546	89
	H2	373,170	257,861	250,598	215
	H3	444,833	304,346	277,385	54
Diseased group	D1	117,066	87,079	79,097	86
	D2	102,759	78,046	72,957	75
	D3	110,730	84,549	78,366	61

Table S3: Frequency and relative abundance of each phylum for each of the sample.

Phylum	H1	%	H2	%	H3	%	D1	%	D2	%	D3	%
Proteobacteria	269262	98.075	189278	75.531	242850	87.55	33434	42.27	69777	95.641	77280	98.614
Fusobacteria	224	0.082	14620	5.834	29384	10.593	17304	21.877	623	0.854	276	0.352
Bacteroidetes	502	0.183	22143	8.836	179	0.065	26088	32.982	163	0.223	167	0.213
Firmicutes	947	0.345	16859	6.728	134	0.048	1825	2.307	2107	2.888	427	0.545
Unclassified	403	0.147	5461	2.179	4544	1.638	25	0.032	17	0.023	13	0.017
Actinobacteria	2046	0.745	499	0.199	174	0.063	320	0.405	208	0.285	189	0.241
Gemmatimonadetes	833	0.303	0	0	0	0	0	0	0	0	0	0
Planctomycetes	133	0.048	575	0.229	0	0	0	0	36	0.049	0	0
Tenericutes	0	0	544	0.217	85	0.031	32	0.04	0	0	0	0
Cyanobacteria	183	0.067	161	0.064	35	0.013	0	0	3	0.004	14	0.018
Verrucomicrobia	0	0	327	0.13	0	0	0	0	0	0	0	0
Lentisphaerae	0	0	120	0.048	0	0	0	0	0	0	0	0
TM7	13	0.005	5	0.002	0	0	0	0	0	0	0	0
Chloroflexi	0	0	6	0.002	0	0	0	0	0	0	0	0
Chlamydiae	0	0	0	0	0	0	27	0.034	13	0.018	0	0
Euryarchaeota	0	0	0	0	0	0	24	0.023	2	0	0	0
Spirochaetes	0	0	0	0	0	0	18	0.023	0	0	0	0
OD1	0	0	0	0	0	0	0	0	4	0.005	0	0
Acidobacteria	0	0	0	0	0	0	0	0	4	0.005	0	0

H1, H2 and H3 stands for healthy *T. tambroides* while D1, D2 and D3 stands for diseased *T. tambroides*.

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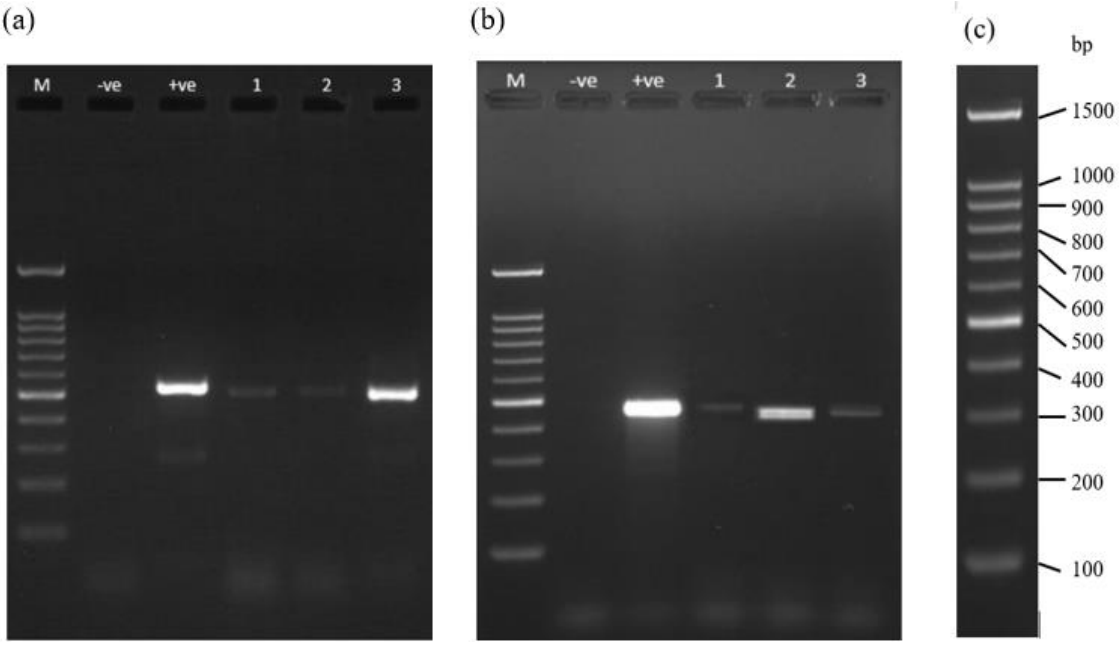

Figure S1: Visualization of PCR products (Forward primer: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3') and reverse primer: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG 3') under UV on 1.7% TAE agarose gel after running on 100 V for 65 min. Lane M is the DNA marker; "-ve" is the no-template control; "+ve" is the positive control with 10 ng of gDNA template for both Figure 1a and 1b. Figure 1a showing the PCR result of healthy fish: Lane 1, H1; Lane 2, H2; Lane 3, H3. Figure 1b showing the PCR result of diseased fish: Lane 1, D1; Lane 2, D2; Lane 3, D3. Figure 1c is the DNA marker used is ExactMark 100 bp (C/No. BIO-5130).

Table S4: Mean and its percentage for each phylum detected in both healthy and diseased *T. tambroides*.

Phylum	Healthy		Diseased	
	Mean	Percentage (%)	Mean	Percentage (%)
Proteobacteria	233796.667	87.397	60163.667	78.331
Fusobacteria	14742.667	5.511	6067.667	7.900
Bacteroidetes	7608.000	2.844	8806.000	11.465
Firmicutes	5980.000	2.235	1453.000	1.892
Unclassified	3469.333	1.297	18.333	0.024
Actinobacteria	906.333	0.339	239.000	0.311
Gemmatimonadetes	277.667	0.104	0.000	0.000
Planctomycetes	236.000	0.088	12.000	0.016
Tenericutes	209.667	0.078	10.667	0.014
Cyanobacteria	126.333	0.047	5.667	0.007
Verrucomicrobia	109.000	0.041	0.000	0.000
Lentisphaerae	40.000	0.015	0.000	0.000
TM7	6.000	0.002	0.000	0.000
Chloroflexi	2.000	0.001	0.000	0.000
Chlamydiae	0.000	0.000	13.333	0.017
Euryarchaeota	0.000	0.000	8.667	0.011
Spirochaetes	0.000	0.000	6.000	0.008
OD1	0.000	0.000	1.333	0.002
Acidobacteria	0.000	0.000	1.333	0.002

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Table S5: Frequency and relative abundance of each family found in each sample of both healthy and diseased *T. tamrboides*.

Family	H1	%	H2	%	H3	%	D1	%	D2	%	D3	%
Sphingomonadaceae	262037	95.443	173331	69.167	198796	71.668	862	1.09	17716	24.283	65961	84.17
Enterobacteriaceae	110	0.04	298	0.119	152	0.055	4248	5.371	51580	70.7	3454	4.408
Bacteroidaceae	0	0	17005	6.786	93	0.034	21719	27.459	33	0.045	14	0.018
Pseudoalteromonadaceae	168	0.061	4108	1.639	519	0.187	21029	26.586	0	0	120	0.153
Fusobacteriaceae	224	0.082	14620	5.834	29384	10.593	17304	21.877	623	0.854	276	0.352
Aeromonadaceae	245	0.089	5196	2.073	41732	15.045	5732	7.247	248	0.34	7547	9.63
Peptostreptococcaceae	546	0.2	3501	1.397	0	0	80	0.101	1366	1.872	95	0.121
Lachnospiraceae	137	0.05	1249	0.498	2	0.001	974	1.231	548	0.751	30	0.038
Campylobacteraceae	0	0	12	0.005	0	0	1366	1.727	0	0	0	0
Propionibacteriaceae	5	0.002	56	0.022	5	0.002	219	0.277	177	0.243	167	0.213
Ruminococcaceae	98	0.036	8723	3.48	0	0	352	0.445	31	0.042	60	0.077
Streptococcaceae	32	0.012	166	0.066	0	0	346	0.437	7	0.01	42	0.054
Oxalobacteraceae	3	0.001	67	0.027	0	0	111	0.14	58	0.079	87	0.111
Chitinophagaceae	461	0.168	37	0.015	0	0	34	0.043	114	0.156	28	0.036
Prevotellaceae	2	0.001	86	0.034	0	0	0	0	0	0	121	0.154
Methylobacteriaceae	5066	1.845	3920	1.564	0	0	22	0.028	52	0.071	31	0.04
Pseudomonadaceae	0	0	124	0.049	0	0	22	0.028	49	0.067	7	0.009
Planococcaceae	99	0.036	832	0.332	0	0	52	0.066	26	0.036	0	0
Paraprevotellaceae	0	0	0	0	0	0	63	0.08	0	0	0	0
Micrococcaceae	308	0.112	113	0.045	0	0	33	0.042	17	0.023	0	0
Porphyromonadaceae	0	0	6	0.002	0	0	35	0.044	6	0.008	4	0.005
Moraxellaceae	409	0.149	544	0.217	357	0.129	0	0	31	0.042	10	0.013
Microbacteriaceae	729	0.266	165	0.066	0	0	25	0.032	6	0.008	11	0.014
Succinivibrionaceae	0	0	0	0	0	0	18	0.023	0	0	19	0.024
Mycoplasmataceae	0	0	0	0	0	0	32	0.04	0	0	0	0
Erysipelotrichaceae	0	0	244	0.097	0	0	15	0.019	11	0.015	0	0
Methanobacteriaceae	0	0	0	0	0	0	24	0.03	2	0.003	0	0
Chlamydiaceae	0	0	0	0	0	0	22	0.028	0	0	0	0
Rhodobacteraceae	66	0.024	20	0.008	0	0	10	0.013	10	0.014	0	0
Comamonadaceae	974	0.355	223	0.089	7	0.003	0	0	0	0	19	0.024
Gemmataceae	133	0.048	250	0.1	0	0	0	0	17	0.023	0	0
Neisseriaceae	0	0	24	0.01	15	0.005	0	0	0	0	18	0.023
Brevinemataceae	0	0	0	0	0	0	18	0.023	0	0	0	0
Bacillaceae	26	0.009	798	0.318	0	0	0	0	4	0.005	10	0.013
Simkaniaceae	0	0	0	0	0	0	0	0	13	0.018	0	0
Acetobacteraceae	7	0.003	36	0.014	0	0	0	0	12	0.016	0	0

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(Continued)

Family	H1	%	H2	%	H3	%	D1	%	D2	%	D3	%
Pirellulaceae	0	0	31	0.012	0	0	0	0	12	0.016	0	0
Intrasporangiaceae	0	0	0	0	0	0	7	0.009	0	0	5	0.006
Veillonellaceae	0	0	181	0.072	0	0	0	0	9	0.012	0	0
Caulobacteraceae	67	0.024	51	0.02	0	0	0	0	7	0.01	0	0
Xanthobacteraceae	0	0	0	0	0	0	8	0.01	0	0	0	0
Bradyrhizobiaceae	91	0.033	105	0.042	0	0	0	0	2	0.003	5	0.006
Pseudonocardiaceae	11	0.004	0	0	0	0	0	0	5	0.007	0	0
Corynebacteriaceae	420	0.153	0	0	0	0	5	0.006	0	0	0	0
Eubacteriaceae	0	0	90	0.036	0	0	0	0	4	0.005	0	0
Aerococcaceae	0	0	7	0.003	0	0	0	0	0	0	4	0.005
Desulfovibrionaceae	0	0	25	0.01	5	0.002	3	0.004	0	0	0	0
Methylophilaceae	0	0	0	0	0	0	3	0.004	0	0	0	0
Rhodocyclaceae	0	0	0	0	0	0	0	0	0	0	2	0.003
Geodermatophilaceae	469	0.171	0	0	0	0	0	0	0	0	0	0
Verrucomicrobiaceae	0	0	327	0.13	0	0	0	0	0	0	0	0
Clostridiaceae	0	0	264	0.105	0	0	0	0	0	0	0	0
Isosphaeraceae	0	0	143	0.057	0	0	0	0	0	0	0	0
Rikenellaceae	0	0	127	0.051	0	0	0	0	0	0	0	0
Planctomycetaceae	0	0	121	0.048	0	0	0	0	0	0	0	0
Listeriaceae	0	0	118	0.047	0	0	0	0	0	0	0	0
Coriobacteriaceae	0	0	111	0.044	0	0	0	0	0	0	0	0
Paenibacillaceae	0	0	107	0.043	0	0	0	0	0	0	0	0
Flavobacteriaceae	0	0	103	0.041	0	0	0	0	0	0	0	0
Xanthomonadaceae	0	0	74	0.03	0	0	0	0	0	0	0	0
Actinosynnemataceae	12	0.004	0	0	46	0.017	0	0	0	0	0	0
Beijerinckiaceae	0	0	39	0.016	0	0	0	0	0	0	0	0
Nocardiaceae	0	0	32	0.013	0	0	0	0	0	0	0	0
Marinilabiaceae	0	0	18	0.007	0	0	0	0	0	0	0	0
Psychromonadaceae	0	0	17	0.007	0	0	0	0	0	0	0	0
Tissierellaceae	8	0.003	0	0	0	0	0	0	0	0	0	0
Weeksellaceae	4	0.001	5	0.002	0	0	0	0	0	0	0	0
Alcaligenaceae	0	0	7	0.003	0	0	0	0	0	0	0	0
Rickettsiaceae	0	0	0	0	7	0.003	0	0	0	0	0	0
Mitochondria	0	0	4	0.002	0	0	0	0	0	0	0	0
Thermogemmatisporaceae	0	0	6	0.002	0	0	0	0	0	0	0	0

H1, H2 and H3 stands for healthy *T. tambroides* while D1, D2 and D3 stands for diseased *T. tambroides*.

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Table S6: Frequency and relative abundance of each genera found in each sample of both healthy and diseased *T. tamrboides*.

Genera	H1	%	H2	%	H3	%	D1	%	D2	%	D3	%
Bacteroides	0	0	17005	6.786	93	0.034	21719	27.459	33	0.045	14	0.018
Vibrio	168	0.061	4108	1.639	519	0.187	21029	26.586	0	0	120	0.153
Aeromonas	245	0.089	5196	2.073	41732	15.045	5732	7.247	248	0.34	7547	9.63
Clostridium	443	0.161	3264	1.302	0	0	0	0	1366	1.872	0	0
Arcobacter	0	0	0	0	0	0	1366	1.727	0	0	0	0
Defluviitalea	82	0.03	837	0.334	2	0.001	871	1.101	60	0.082	23	0.029
Clostridium	56	0.02	250	0.1	0	0	97	0.123	473	0.648	0	0
Propionibacterium	5	0.002	56	0.022	5	0.002	219	0.277	177	0.243	167	0.213
Enterobacter	0	0	0	0	0	0	222	0.281	17	0.023	0	0
Lactococcus	32	0.012	0	0	0	0	179	0.226	0	0	31	0.04
Streptococcus	0	0	166	0.066	0	0	167	0.211	7	0.01	11	0.014
Butyricicoccus	0	0	42	0.017	0	0	147	0.186	0	0	0	0
Proteocatella	103	0.038	237	0.095	0	0	40	0.051	0	0	95	0.121
Prevotella	2	0.001	86	0.034	0	0	0	0	0	0	121	0.154
Methylobacterium	4947	1.802	3775	1.506	0	0	22	0.028	52	0.071	31	0.04
Pseudomonas	0	0	124	0.049	0	0	22	0.028	49	0.067	7	0.009
Staphylococcus	99	0.036	832	0.332	0	0	52	0.066	26	0.036	0	0
Parasegitibacter	0	0	0	0	0	0	26	0.033	25	0.034	23	0.029
Prevotella	0	0	0	0	0	0	63	0.08	0	0	0	0
Macellibacteroides	0	0	6	0.002	0	0	35	0.044	6	0.008	4	0.005
Acinetobacter	295	0.107	536	0.214	357	0.129	0	0	31	0.042	7	0.009
Succinivibrio	0	0	0	0	0	0	18	0.023	0	0	19	0.024
Micrococcus	158	0.058	9	0.004	0	0	16	0.02	17	0.023	0	0
Mycoplasma	0	0	0	0	0	0	32	0.04	0	0	0	0
Methanobrevibacter	0	0	0	0	0	0	24	0.03	2	0.003	0	0
Clostridium	0	0	0	0	0	0	0	0	23	0.032	0	0
Faecalibacterium	0	0	0	0	0	0	0	0	0	0	23	0.029
Chlamydia	0	0	0	0	0	0	22	0.028	0	0	0	0
Albidovulum	59	0.021	0	0	0	0	10	0.013	10	0.014	0	0
Gemmata	133	0.048	0	0	0	0	0	0	17	0.023	0	0
Brevinema	0	0	0	0	0	0	18	0.023	0	0	0	0
Arthrobacter	15	0.005	0	0	0	0	17	0.021	0	0	0	0
Clostridium	0	0	0	0	0	0	15	0.019	0	0	0	0
Simkania	0	0	0	0	0	0	0	0	13	0.018	0	0
Anaerorhabdus	0	0	244	0.097	0	0	0	0	11	0.015	0	0
Succinispira	0	0	22	0.009	0	0	0	0	9	0.012	0	0

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(Continued)

Genera	H1	%	H2	%	H3	%	D1	%	D2	%	D3	%
Ruminococcus	0	0	56	0.022	0	0	0	0	0	0	7	0.009
Leifsonia	729	0.266	165	0.066	0	0	0	0	6	0.008	0	0
Bergeriella	0	0	0	0	0	0	0	0	0	0	6	0.008
Prauserella	0	0	0	0	0	0	0	0	5	0.007	0	0
Corynebacterium	420	0.153	0	0	0	0	5	0.006	0	0	0	0
Ornithinimicrobium	0	0	0	0	0	0	0	0	0	0	5	0.006
Pseudoramibacter	0	0	90	0.036	0	0	0	0	4	0.005	0	0
Lacticigenium	0	0	0	0	0	0	0	0	0	0	4	0.005
Enhydrobacter	91	0.033	0	0	0	0	0	0	0	0	3	0.004
Lawsonia	0	0	0	0	5	0.002	3	0.004	0	0	0	0
Methylotenera	0	0	0	0	0	0	3	0.004	0	0	0	0
Herbaspirillum	0	0	43	0.017	0	0	2	0.003	0	0	0	0
Papillibacter	4	0.001	2759	1.101	0	0	0	0	0	0	0	0
Sphingomonas	1541	0.561	252	0.101	0	0	0	0	0	0	0	0
Clostridium	0	0	264	0.105	0	0	0	0	0	0	0	0
Alistipes	0	0	127	0.051	0	0	0	0	0	0	0	0
Planctomyces	0	0	121	0.048	0	0	0	0	0	0	0	0
Brochothrix	0	0	118	0.047	0	0	0	0	0	0	0	0
Oceanicella	0	0	13	0.005	0	0	0	0	0	0	0	0
Actinomycetospora	11	0.004	0	0	0	0	0	0	0	0	0	0
Anaerococcus	8	0.003	0	0	0	0	0	0	0	0	0	0
Salipiger	7	0.003	0	0	0	0	0	0	0	0	0	0
Abiotrophia	0	0	7	0.003	0	0	0	0	0	0	0	0
Bordetella	0	0	7	0.003	0	0	0	0	0	0	0	0
Psychrobacter	0	0	8	0.003	0	0	0	0	0	0	0	0
Wolbachia	0	0	0	0	7	0.003	0	0	0	0	0	0
Chitinibacter	0	0	0	0	5	0.002	0	0	0	0	0	0
Dorea	0	0	4	0.002	0	0	0	0	0	0	0	0
Elizabethkingia	0	0	5	0.002	0	0	0	0	0	0	0	0
Mycobacterium	4	0.001	0	0	0	0	0	0	0	0	0	0

H1, H2 and H3 stands for healthy *T. tambroides* while D1, D2 and D3 stands for diseased *T. tambroides*.

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Table S7: List of genera identified exclusively. (a) 40 genera unique to healthy gut of *T. tambroides*; (b) 30 common genera found in both healthy and diseased gut of *T. tambroides*; (c) 18 genera unique to diseased gut of *T. tambroides*.

(a) 40 genera identified exclusively in healthy group			
Papillibacter	*Rothia*	*Neisseria*	*Abiotrophia*
Sphingomonas	*Pantoea*	*Desulfovibrio*	*Anaerococcus*
Clostridium	*Lysobacter*	*Alkanindiges*	*Salipiger*
Alistipes	*Oribacterium*	*Cytophaga*	*Wolbachia*
Brochothrix	*Salinarimonas*	*Oceanicella*	*Bordetella*
Planctomyces	*Kocuria*	*Psychromonas*	*Chitinibacter*
Actinotelluria	*Rhodococcus*	*Proteus*	*Campylobacter*
Eggerthella	*Belnapia*	*Xanthomonas*	*Psychrobacter*
Brevibacillus	*Flavobacterium*	*Actinomycetospora*	*Mycobacterium*
Nostocoida	*Zhouia*	*Elizabethkingia*	*Dorea*
(b) 30 common genera in both healthy and diseased groups			
Aeromonas	*Proteocatella*	*Propionibacterium*	
Bacteroides	*Clostridium*	*Ruminococcus*	
Methylobacterium	*Anaerorhabdus*	*Butyricicoccus*	
Vibrio	*Micrococcus*	*Albidovulum*	
Clostridium	*Streptococcus*	*Herbaspirillum*	
Acinetobacter	*Gemmata*	*Lactococcus*	
Staphylococcus	*Pseudomonas*	*Succinispira*	
Defluviitalea	*Prevotella*	*Arthrobacter*	
Leifsonia	*Pseudoramibacter_Eubacterium*	*Macellibacteroides*	
Corynebacterium	*Enhydrobacter*	*Lawsonia*	
(c) 18 genera identified exclusively in diseased group			
Methanobrevibacter	*Simkania*	*Bergeriella*	
Ornithinimicrobium	*Lacticigenium*	*Arcobacter*	
Prauserella	*Clostridium*	*Succinivibrio*	
Prevotella	*Faecalibacterium*	*Enterobacter*	
Parasegitibacter	*Clostridium*	*Brevinema*	
Chlamydia	*Methylotenera*	*Mycoplasma*	

Table S8: List of observed bacteria that was found to be significant different among both healthy and diseased gut of *T. tambroides* through ANCOM analysis at genus level.

Taxon	Healthy group (%)	Diseased group (%)	W-statistic value	Taxon	Healthy group (%)	Diseased group (%)	W-statistic value
Propionibacterium	0.008	0.244	19	*Proteus*	0.002	0.000	1
Lactobacillales	0.000	0.106	5	*Acinetobacter*	0.148	0.016	1
Enterobacteriaceae	0.061	25.624	2	*Zhouia*	0.003	0.000	1
Flavobacterium	0.003	0.000	1	*Psychrobacter*	0.001	0.000	1
Rhodobacteraceae	0.001	0.000	1	Thermogemmatisporaceae	0.001	0.000	1
Rhodocyclaceae	0.000	0.001	1	Xanthomonadaceae	0.001	0.000	1
Campylobacter	0.001	0.000	1	*Ornithinimicrobium*	0.000	0.002	1
Dorea	0.000	0.000	1	Streptophyta	0.013	0.007	1
Cytophaga	0.002	0.000	1	*Xanthomonas*	0.002	0.000	1
Elizabethkingia	0.001	0.000	1	*Lacticigenium*	0.000	0.002	1
Mitochondria	0.000	0.000	1	SC3	0.001	0.000	1
Oceanicella	0.002	0.000	1	*Bordetella*	0.001	0.000	1
Psychromonas	0.002	0.000	1	Betaproteobacteria	0.015	0.003	1
Bergeriella	0.000	0.003	1	*Abiotrophia*	0.001	0.000	1

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