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Full Length Article

Changes in Intestinal Microflora of *Cynoglossus semilaevis* Günther Following *Shewanella algae* Infection

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Abstract

The intestinal microbial communities of *Cynoglossus semilaevis* were studied using high-throughput sequencing of 16S ribosomal RNA gene after infection with the pathogenic *Shewanella algae*. A total of 964881 effective tags were obtained and assigned to 4308 OTUs based on a 97% similarity cut-off. The group at 3 days post infection (dpi) had significantly higher number of OTUs and alpha diversity indexes than other groups. Proteobacteria, Spirochaetes, Bacteroidetes and Firmicutes were found to be dominant in the intestines of both infected and uninfected fish. There were no significant differences for the relative abundances of Proteobacteria and Bacteroidetes among all groups. The relative abundance of the Spirochaetes was higher in 1 dpi and 4 dpi than that in the control, 2 dpi, and 3 dpi. Firmicutes was more in 3 dpi than other groups. At genus level, *Shewanella, Vibrio, Brevinema, Phaeodactylibacter* and *Halomonas* were dominant taxa in all samples. *Shewanella* in 2 dpi was more than that in 3 dpi and had similar abundance compared with the control, 1 dpi, and 4 dpi. *Brevinema* had lower relative abundances in the control, 2 dpi, and 3 dpi compared with 1 dpi and 4 dpi. *Halomonas* in the control was more than that in all the infection groups. *Vibrio* and Phaeodactylibacter had similar relative abundances among all groups. The weighted unifrac principal coordinate and hierarchical clustering analysis revealed that samples of 1 dpi and 4 dpi groups formed a distinct cluster, while the samples from the control and 2 dpi groups were more similar.

Keywords: Intestinal microflora; *Cynoglossus semilaevis*; *Shewanella algae*; Infection

Introduction

Gut microbiota have many key functions for the host health, and they may be considered as an integral component of the host (Sugita *et al.*, 1997). As for the fish, gut microbiota are also important for the physiological functions (Ganguly and Prasad, 2011). However, many opportunistic pathogens exist in the gut of fish, and this indicates that gut is a potential pathway for pathogen invasion (Li *et al.*, 2016). Some studies have been performed on gut microbiota in the healthy fish. Recently, some suggested changes of microbiota in the intestine of fish can influence host immune functions (Brown *et al.*, 2012; Morgan *et al.*, 2012). Herein, it is necessary to investigate the intestinal microbial diversity and community structure of the infected fish. This may benefit to learning more about the pathogenesis. However, there are only few studies on the gut microbiota in the diseased or infected fish by using next generation sequencing (Li *et al.*, 2016).

The half-smooth tongue sole *Cynoglossus semilaevis* Günther has increasingly become a commercially important aquaculture species in China, due to its high market value (Wang *et al.*, 2018). Recently, after the industrial high-density culture mode was applied for *C. semilaevis*, infectious diseases often happened and caused a high mortality. Some bacterial species, including *Vibrio aestuarianus* (Zhang *et al.*, 2011), *Vibrio parahaemolyticus* (Hu *et al.*, 2014), *Aeromonas salmonicida* (Zhou *et al.*, 2017) have been identified as the pathogens of *C. semilaevis*. In our lab, a pathogenic *Shewanella algae* CSG-15 strain was recently reported. This pathogen caused the disease of *C. semilaevis* with classical symptoms of ascitic fluid, and could elicit the acute infection with obvious clinic and histopathological signs occurring in the intestines of the infected fish (Han *et al.*, 2017). In this study, the intestinal microbial communities of *C. semilaevis* were studied based on high-throughput sequencing of 16S ribosomal RNA gene after infection with the pathogenic *S. algae* strain.

Materials and Methods

Fish Husbandry

Healthy *C. semilaevis* (average weight: 130 ± 5 g; average length: 29 ± 2 cm) were purchased from a commercial fish farm in Tianjin, China, and acclimatized for two weeks in

20°C sea water with 22 salinity. Constant aeration was provided and commercial diets were fed twice a day at 10:00 a.m. and 17:00 p.m. at the rate of 1% body weight throughout the experiment. Five fish were randomly selected for pathogen examination, and no bacteria were detected from the livers of the fish.

Bacteria Culture and Experimental Infection

The *Shewanella algae* CSG-15 strain was cultured, and then collected according previous report of Han *et al.* (2017). The bacterial suspension with a concentration of 1.0×10^8 CFU/mL was obtained by dilution with 0.9% physiological saline.

Each fish for the pathogen infection was injected intraperitoneally with the bacterial suspension at a dose of 200 μL. Injection with equal volume physiological saline was used for the control group (CG). Feces and mucosa were collected from the fish in the infection and control groups. Thirty six fish were used for infection and 9 fish were used for the control. Feces and mucosa from three individuals at 1, 2, 3, 4 days post infection (dpi), or the CG were pooled together to avoid bias, respectively. All the samples were in triplicate and kept at -80°C until use. The samples from the infection groups and the control group were performed for DNA extraction. All the sample names were provided in Table 1. After euthanization with an overdose of MS-222 (Sigma Aldrich, St Louis, MO, USA), all fish were sacrificed for sample collection.

DNA Extraction and High-Throughput Illumina HiSeq Sequencing

QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) was used for the genome DNA extraction according to the manufacture's protocol. To analyze microbial populations of the extracted DNA samples, amplification of variable region V3-V4 of 16S ribosomal RNA gene was performed using the bacterial universal primers 515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3' (Xiong *et al.*, 2015). A 6-bp barcode sequence adjacent to the forward primer was used to uniquely distinguish each DNA sample. Each PCR reaction was performed as described before (Jia *et al.*, 2016). Agarose gel electrophoresis was used to detect the PCR products, which was then recycled with QIAquick® Gel Extraction Kit (Qiagen, Germany). TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to construct the DNA libraries. A Qubit1 2.0 Fluorometer (Invitrogen, USA) was used for quantification. Finally, all the samples were sequenced on an Illumina HiSeq platform.

Bioinformatics and Statistics Analysis

The resulting sequences were acquired and recognized as the raw tags after the barcode and primer sequences were filtered. QIIME was used to assemble these raw tags according the previous report of Caporaso *et al.* (2010). FLASH V1.2.7 was used to merge the split sequences for each sample according to the description of Magoč and Salzberg (2011). High-quality effective tags were obtained after the low-quality and chimaera sequences were excluded by using QIIME and UCHIME algorithm, respectively (Edgar *et al.*, 2011). Uparse software was used to cluster these effective tags, and tags with 97% similarity were classified in to one operational taxonomic unit (OTU) according to the method of Edgar (2013). The SILVA SSU database was used for sequence analysis. A representative sequence was selected and annotated for its taxonomic information. At phylum, class, order, family, and genus levels, the taxon abundance of each sample was acquired. Greengenes database was used to compare the sequences for the taxonomy assignment of OTUs. Chao1, Shannon and ACE indexes were included in alpha diversity analysis using QIME. Weighted unifrac metric distances were used to determine beta diversity index, and the distance tree based on Unweighted Pair-group Method with Arithmetic Mean (UPGMA) was used to examine the relationship of the community structures of the microbiota from the different samples. The differences in the samples were compared based on the principal coordinate analysis (PCoA) using weighted uniFrac metric matrices.

The data were analyzed using SPSS 16.0 (IBM; www.ibm.com). Differences in the alpha diversity indexes of the community, OTU-based analysis and taxon abundances were assessed using one-way ANOVA with Duncan's test. Significant difference was accepted if P-value was less than 0.05.

Results

OTUs and Alpha-diversity Analysis

A total of 964881 effective tags were obtained after quality control and chimaera removal for all the samples, with a mean of 64324 tags for each sample. The 1 dpi-1 sample had the minimum effective tags (32107), while the 3 dpi-3 sample had the maximum (93044) (Table 1). These sequences were classed into 4308 OTUs based on a 97% similarity cut-off. The average of OTUs for the control, 1 dpi, 2 dpi, 3 dpi, and 4 dpi groups was 1169, 1633, 1942, 3099 and 1780, respectively, and 133, 233, 350, 930 and 157 OUTs were unique to each group, respectively. While total 461 OUTs were shared by all groups (Fig. 1). The average of Shannon index was 3.3, 4.0, 4.2, 6.9 and 4.2 for the control, 1 dpi, 2 dpi, 3 dpi, and 4 dpi group, respectively. The average of ACE was 933.0, 1197, 1329.9, 2436.7 and 1403.4, while the average of Chao1 was 879.5, 1155, 1256.1, 2319.3 and 1308.5 for each group respectively. The number of OTUs, Shannon, ACE and Chao1 indexes in the 3 dpi group were higher than other groups ($P<0.05$; Table 1).

Table 1: Alpha-diversity indexes, the numbers of OTUs and effective tags for the bacterial community

Sample name	Group name	Effective tags	OTUs	Number of OTUs	ACE	Average of ACE	Chao1	Average of Chao1	Shannon	Average of Shannon
CG-1	CG	68073	868	1169[a]	861.0	933.0[a]	796.8	879.5[a]	2.8	3.3[a]
CG-2		65289	954		976.6		948.8		3.3	
CG-3		66537	948		961.4		892.7		3.9	
1 dpi -1	1 dpi	58903	1456	1633[a]	1634.5	1197[a]	1576.2	1155.5[a]	5.1	4.0[a]
1 dpi -2		63632	855		838.2		774.0		3.0	
1 dpi -3		62098	991		1118.4		1116.4		3.8	
2 dpi -1	2 dpi	70060	1154	1942[a]	1126.2	1329.9[a]	1035.8	1256.1[a]	2.8	4.2[a]
2 dpi -2		56931	1339		1557.8		1485.2		5.1	
2 dpi -3		65579	1312		1305.7		1247.3		4.7	
3 dpi -1	3 dpi	32107	2036	3099[b]	1924.7	2436.7[b]	1846.3	2319.3[b]	8.2	6.9[b]
3 dpi -2		90599	2633		2952.5		2860.1		5.8	
3 dpi -3		45503	2303		2433.0		2251.6		6.7	
4 dpi -1	4 dpi	67903	1293	1780[a]	1237.4	1403.4[a]	1165.9	1308.5[a]	4.7	4.2[a]
4 dpi -2		58623	699		811.6		718		3.0	
4 dpi -3		93044	1940		2161.2		2041.6		4.9	

Note: Different lower case superscript letters in the same column indicated significant difference among the different groups ($P < 0.05$)

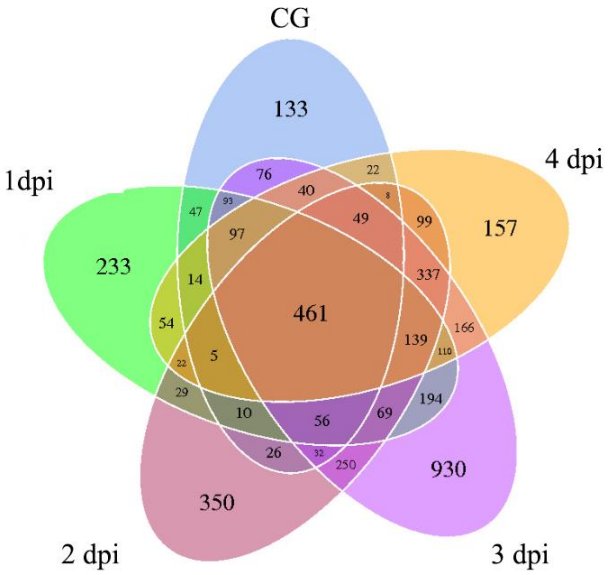

Fig. 1: Venn Diagram of OTUs from all the groups. Total 4308 OTUs were identified in 5 group; 1169, 1633, 1942, 3099, 1780 OTUs were found in CG, 1 dpi, 2 dpi, 3 dpi and 4 dpi, respectively; 461 OUTs were shared by all groups and 133, 233, 350, 930 and 157 were unique in CG, 1 dpi, 2 dpi, 3 dpi and 4 dpi, respectively

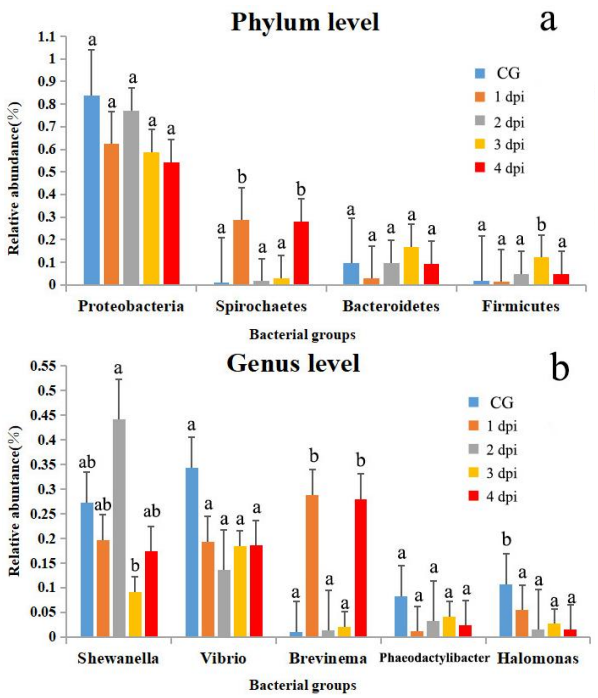

Fig. 2: Relative abundance of the dominant bacteria in the intestine of *Cynoglossus semilaevis*. (a): At phylum level, Proteobacteria, Spirochaetes, Bacteroidetes and Firmicutes were the dominant phyla in all groups; (b): At genus level, *Shewanella*, *Vibrio*, *Brevinema*, *Phaeodactylibacter*, *Halomonas* were identified as the major bacterial taxa in all groups. The difference letters on the bar chart indicated significant difference among the different groups at P< 0.05 level

Microbial Community Composition

To investigate bacterial community structure succession after infection, all valid sequences were analyzed. At phylum level (Fig. 2a), more than 90% of the sequences in all the samples were found dominated by four bacterial phyla, which were identified as Proteobacteria, Spirochaetes, Bacteroidetes and Firmicutes. In all the samples, Proteobacteria was the most predominant phylum. There were no significant differences for the relative abundances of Proteobacteria and Bacteroidetes among 1, 2, 3, 4 dpi and CG ($P>0.05$). The relative abundance of the Spirochaetes was higher in 1 dpi and 4 dpi than that in CG, 2 dpi, and 3 dpi ($P<0.05$). The relative abundance of Firmicutes was significantly higher in 3 dpi than other groups ($P<0.05$).

At genus level (Fig. 2b), *Shewanella*, *Vibrio*, *Brevinema*, *Phaeodactylibacter* and *Halomonas* were found to be the dominated bacterial taxa in all samples.

Shewanella in 2 dpi was more than that in 3 dpi (P<0.05) and had similar abundance compared with CG, 1 dpi, and 4 dpi (P>0.05). *Brevinema* had lower relative abundances in CG, 2 dpi, and 3 dpi compared with 1 dpi and 4 dpi (P<0.05). *Halomonas* in CG was more than that in all the infection groups (P<0.05). *Vibrio* and *Phaeodactylibacter* had similar relative abundances among the five groups (P>0.05).

Species taxonomy tree was structured on a basic of genera with the top 10 relative abundance (Fig. 3). Total 17 species of *Bacteroides* were detected, *B. eggerthii, B. fragilis, B. graminisolvens, B. intestinalis, B. pyogenes*, B. sp. C13EG172, and *B. propionicifaciens* were enriched in 2 dpi; *B. barnesiae, B. caccae, B. coprocola, B. coprophilus, B. ovatus, B. plebeius, B. stercoris, B. uniformis,* and *B. vulgatus* were found in 4 dpi; *B. paurosaccharolyticus* was only detected in 3 dpi. Both P. sp._GYP20 and bacterium_KD52 of *Phaeodactylibacter* had similar relative abundance among the five groups. At the same time, *A. butzleri* and *A. skirrowii* of *Arcobacter* were observed only in 3 dpi. In addition, *Candidatus Halomonas_phosphatis* belonging to *Halomonas* was enriched in 1 dpi. Only *A. ursingii* of *Acinetobacter* was observed in all groups with similar abundance. Total eight species of *Pseudomonas* were detected, Pseudomonadaceae_bacterium_X2, *P. caeni*, *P. pertucinogena* and *P. psychrotolerans* were only found in 3 dpi; *P. balearica* and *P. peli* were enriched in 3 dpi; *P. fulva* was detected in all samples and *P. pachastrella* was not identified only in CG. *Shewanella*, *Brachymonas*, *Vibrio* and *Brevinema* were not identified to the species level.

Beta-diversity Analysis

According to weighted unifrac PCoA (Fig. 4), the samples of CG and 2 dpi, the samples of 1 dpi and 4 dpi and the samples of 3 dpi were respectively grouped into three distinct clusters based on PC1 and PC2 analysis (40.12% and 27.66% of variance, respectively). The samples of 3 dpi separated from the other samples. The hierarchical clustering of all the samples was analyzed by using UPGMA (Fig. 5). Similar results were obtained with those of the weighted unifrac PCoA. The samples of 1 dpi and 4 dpi formed a distinct cluster, while the samples of CG and 2 dpi were more similar.

Discussion

The microflora of fish gut have been investigated by many researchers, but unfortunately, most was known about the intestinal flora of healthy individuals, and little was known regarding continuous changes in the intestinal flora induced by a bacterial pathogen (Han *et al.*, 2010; Huang *et al.*, 2016). In this study, the community succession of the intestinal microbiota from *C. semilaevis* after artificial injection with the pathogenic *S. algae* was first reported

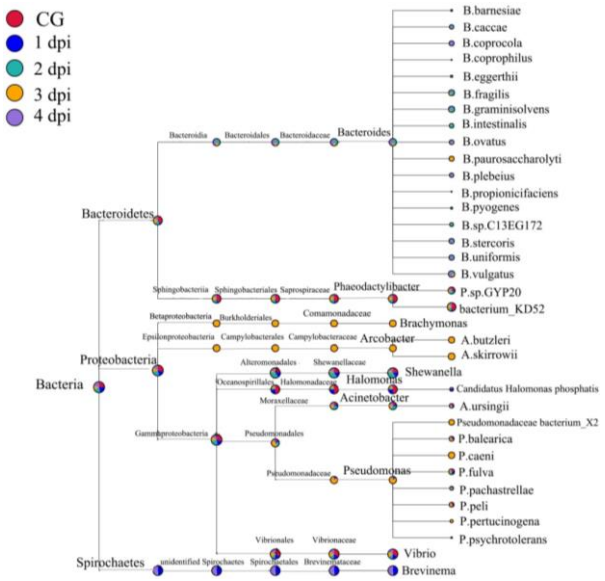

Fig. 3: Species taxonomy tree was structured on a basic of genera with top 10 relative abundances. Total 31 species of *Bacteroides, Phaeodactylibacter, Arcobacter, Halomonas, Acinetobacter* and *Pseudomonas* were detected; *Shewanella, Brachymonas, Vibrio, Brevinema* were not identified to the species level

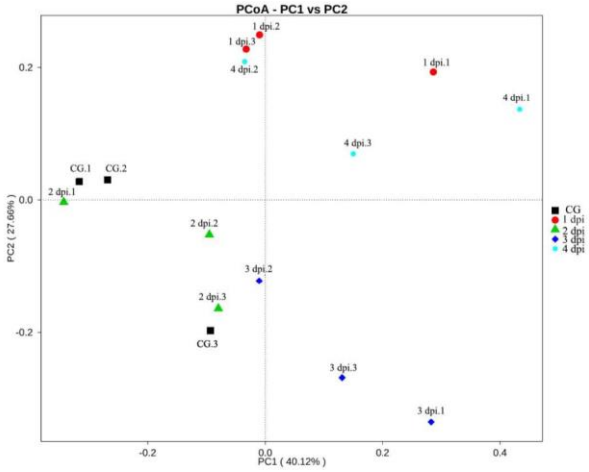

Fig. 4: Two-dimensional principal coordinates analysis (PCoA) plot of weighted unifrac distance matrices for intestinal microflora of *Cynoglossus semilaevis.* The percentage of variation indicated by the principal coordinates is shown on the axes; Black, red, green, dark blue and blue represent samples of CG, 1 dpi, 2 dpi, 3 dpi and 4 dpi, respectively; The samples of CG and 2 dpi, the samples of 1 dpi and 4 dpi and the samples of 3 dpi were respectively grouped into three distinct clusters

based on the high-throughput sequencing of 16S ribosomal RNA gene.

In the previous study, a significantly higher diversity of bacterial community was observed in gut microbiota

from healthy aquatic animals, compared to those from diseased ones, such as largemouth bronze gudgeon (*Coreius guichenoti*) (Li *et al.*, 2016). In this study, the alpha-diversity indexes of intestinal microbiota community in the healthy *C. semilaevis* were found not higher than those in the infected fish. Moreover, the alpha-diversity indexes in 3 dpi had significantly higher than those in other groups. Herein, the injection dosage 1.5×10^5 CFU/g of the pathogenic *S. algae* was used for the infection experiment. This dosage allowed the majority of fish alive (Han *et al.*, 2017) and provided the convenience for collecting the intestinal microbiota from fish at a special time. Only minority of the fish presented the mild ascites symptom throughout infection experiment period. The increasement or reduction of the diversity of fish gut microbiota was usually affected by the disease progress, disease severity, and infection intensity of pathogens. This could account for the inconsistence regarding the changes of the diversity of the gut microbiota compared with the previous study. And it can be speculated that infection with a bacterial pathogen did not always lead to reduction of the diversity of the gut microbiota in fish.

In this study, Proteobacteria, Spirochaetes, Bacteroidetes and Firmicutes accounted for more than 90% of the intestinal microbiota in *C. semilaevis*, which was similar with the previous research on *Paralichthys olivaceus* and *C. semilaevis* (Zhang *et al.*, 2014; Li *et al.*, 2015). These findings were indicative that these four taxa could be regarded as a core intestinal microbiome of *C. semilaevis*.

In addition, our results showed *Shewanella* and *Vibrio* were the major dominant genera in both uninfected and infected *C. semilaevis*. Previous researches revealed that *Vibrio* were the most common organisms in the fish guts (Jensen *et al.*, 2004; Hovda *et al.*, 2007). Some strains of these bacteria often caused fish diseases, such as *V. Parahaemolyticus*, *V. harveyi*, *V. alginolyticus* (Yeh and Chen, 2008). Furthermore, some reported that some species of *Shewanella* were pathogenic to aquatic animals, especially the two species, *S. algae* and *S. putrefaciens* (Pękala *et al.*, 2014).

Investigation on microbial diversity at the species level showed that *A. ursingii* was *identified* in all groups and *A. butzleri* and *A. skirrowii* were identified only at 72 h after infection. These findings in this study were indicative that some pathogenic bacteria co-exist in fish intestines, which was in agreement with the viewpoint of Pond *et al*. (2006). Previous studies showed that some species belonging to *Acinetobacter* genus were found frequently in water or diseased fish, which had increasingly been recognized as opportunistic pathogens, such as *A. lwoffii*, *A. baumannii* and *A. ursingii* (Dortet *et al.*, 2006). Moreover, *A. butzleri* and *A. skirrowii* were frequently isolated from coastal environment, fish and patients with food-borne illness. They could be the potential sources of animals' infections (Prouzet-Mauléon *et al.*, 2006; Cervenka *et al.*, 2008).

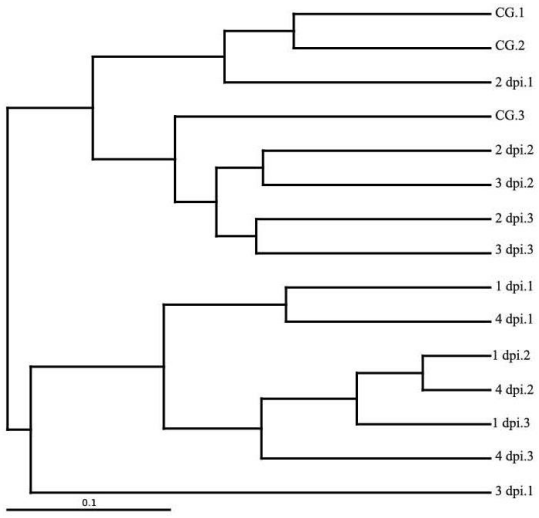

Fig. 5: Hierarchical clustering of microbiota from intestine of *Cynoglossus semilaevis*. Samples of 1 dpi and 4 dpi formed a distinct cluster, while the samples of CG and 2 dpi were more similar

In this study, most of the identified species belonged to the genus of *Bacteroides*, which were especially enriched in 2 dpi and 4 dpi. It was previously reported that *Bacteroides* was the predominant bacteria in the intestines of fish (McLain *et al.*, 2009; Ahmed *et al.*, 2013). *B. uniformis*, *B. ovatus* and *B. stercoris* were ever identified from feces of grass carp (*Ctenopharyngodon idella*), channel catfish (*Ictalurus punctatus*), and blue catfish (*Ictalurus furcatus*) (Kabiri *et al.*, 2013). Some reported that *Bacteroides* may have an important anti-inflammatory function, such as *B. fragilis* (Winglee *et al.*, 2014); Not only that, *B. graminisolvens* and *B. plebeius* were considered capable of utilizing the carbohydrates (Nishiyama *et al.*, 2009). Whereas, some species of Bacteriodes, such as *B. pyogenes*, *B. ovalus* and *B. vulgatus* can cause infectious diseases if they are introduced to parts of the body other than the gastrointestinal area (Lau *et al.*, 2016). Therefore, further studies are still required to explore the relationship of specific *Bacteroides* species to the immune system and infection mechanism.

Conclusion

Injection with lower concentration of the pathogenic *S. algae* altered the intestinal microbiota community of *C. semilaevis*, including some species of opportunistic pathogenic bacteria. The variety in intestinal microbial community may be related to the health status of the host. However, the results did not address the relationships between the intestinal microbial community imbalances and different injection dosages of the pathogenic *S. algae*. The influence of different injection dosages of pathogenic *S. algae* on the intestinal microbial community of *C. semilaevis* should be further studied.

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References

Ahmed, W., T. Sritharan, A. Palmer, J.P.S. Sidhu and S. Toze, 2013. Evaluation of bovine feces-associated microbial source tracking markers and their correlations with fecal indicators and zoonotic pathogens in a Brisbane, Australia, Reservoir. *Appl. Environ. Microbiol.*, 79: 2682–2691

Brown, K., D. DeCoffe, E. Molcan and D.L. Gibson, 2012. Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. *Nutrients*, 4: 1095−1119

Caporaso, J.G., J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Peña, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld and R. Knight, 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*, 7: 335–336

Cervenka, L., I. Peskova, M. Pejchalova and J. Vytrasova, 2008. Inhibition of *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* by plant oil aromatics. *J. Food Prot.*, 71: 165–169

Dortet. L., P. Legrand, C.J. Soussy and V. Cattoir, 2006. Bacterial identification, clinical significance, and antimicrobial susceptibilities of *Acinetobacter ursingii* and *Acinetobacter schindleri*, two frequently misidentified opportunistic pathogens. *J. Clin. Microbiol.*, 44: 4471–4478

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Meth.*, 10: 996–998

Edgar, R.C., B.J. Haas, J.C. Clemente, C. Quince and R. Knight, 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, 27: 2194–2200

Ganguly, S. and A. Prasad, 2011. Microflora in fish digestive tract plays significant role in digestion and metabolism. *Rev. Fish Biol. Fisher*, 22: 11–16

Han, S., Y. Liu, Z. Zhou, S. He, Y. Cao, P. Shi, B. Yao and E. Ringø, 2010. Analysis of bacterial diversity in the intestine of grass carp (*Ctenopharyngodon idellus*) based on 16S rDNA gene sequences. *Aquac. Res.*, 42: 47–56

Han, Z., J. Sun, A. Lv, Y.Y. Sung, H. Shi, X. Hu and K.Z. Xing, 2017. Isolation, identification and characterization of *Shewanella algae* from reared tongue sole, *Cynoglossus semilaevis* Günther. *Aquaculture*, 468: 356–362

Hovda, M.B., B.T. Lunestad, R. Fontanillas and J.T. Rosnes, 2007. Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 272: 581–588

Hu, X., J.F. Sun, C.X. Chen and K.Z. Xing, 2014. Isolation and identification of pathogen of ascites disease from *Cynoglossus semilaevis* Günther and its antibiotic sensitivity analysis. *J. Tianjin. Agric. Coll.*, 21: 12–16

Huang, Z., X. Li, L. Wang and Z. Shao, 2016. Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei*. *Aquac. Res.*, 47: 1737–1746

Jia, H.R., L.L. Geng, Y.H. Li, Q. Wang, Q.Y. Diao, T. Zhou and P.L. Dai, 2016. The effects of Bt Cry1Ie toxin on bacterial diversity in the midgut of Apis *mellifera ligustica* (Hymenoptera: Apidae). *Sci. Rep.*, 6: 24664

Jensen, S., L. Øvreås, Ø. Bergh and V. Torsvik, 2004. Phylogenetic analysis of bacterial communities associated with larvae of the *Atlantic halibut* propose succession from a uniform normal flora. *Syst. Appl. Microbiol.*, 27: 728–736

Kabiri, L., A. Alum, C. Rock, J.E. Mclain and M. Abbaszadegan, 2013. Isolation of *bacteroides* from fish and human fecal samples for identification of unique molecular markers. *Can. J. Microbiol.*, 59: 771

Lau, J.S., T.M. Korman, A. Yeung, R. Streitberg, M.J. Francis and M. Graham, 2016. *Bacteroides pyogenes* causing serious human wound infection from animal bites. *Anaerobe*, 42: 172–175

Li, S., J. Zhang, D. Qiu, S. Yang and Z. Huang, 2015. Biological characteristics and pathogenicities of *Shewanella algae* and *Shewanella abalone* from Babylonia. *Agric. Sci. Technol.*, 16: 1845–1859

Li, T., L. Meng, J. Cheng, Z. Shen, F. Gatesoupe, X. Zhang, Q. Zhang, L. Zhang, Y. Zhao, X. Liu and A. Li, 2016. Alterations of the gut microbiome of largemouth bronze gudgeon (*Coreius guichenoti*) suffering from furunculosis. *Sci. Rep.*, 6: 1–9

Magoč, T. and S.L. Salzberg, 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27: 2957–2963

McLain, J.E., H. Ryu, L. Kabiri-Badr, C.M. Rock and M. Abbaszadegan, 2009. Lack of specificity for PCR assays targeting human *Bacteroides* 16S rRNA gene: cross-amplification with fish feces. *FEMS Microbiol. Lett.*, 299: 38–43

Morgan, X.C., T.L. Tickle, H. Sokol, D. Gevers, K.L. Devaney, D.V. Ward, J.A. Reyes, S.A. Shah, N. LeLeiko, S.B. Snapper, A. Bousvaros, J. Korzenik, B.E. Sands, R.J. Xavier and C. Huttenhower, 2012. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genom. Biol.*, 13: 1–18

Nishiyama, T., A. Ueki, N. Kaku, K. Watanabe and K. Ueki, 2009. *Bacteroides graminisolvens* sp. nov. a xylanolytic anaerobe isolated from a methanogenic reactor treating cattle waste. *Int. J. Syst. Evol. Microbiol.*, 59: 1901

Pękala, A., A. Kozińska, E. Paździor and H. Głowacka, 2014. Phenotypical and genotypical characterization of *Shewanella putrefaciens*, strains isolated from diseased freshwater fish. *J. Fish Dis.*, 38: 283–293

Pond, M.J., D.M. Stone and D.J. Alderman, 2006. Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 261: 194–203

Prouzet-Mauléon, V., L. Labadi, N. Bouges, A. Ménard and F. Mégraud, 2006. *Arcobacter butzleri*: underestimated enteropathogen. *Emerg. Infect. Dis.*, 12: 307

Sugita, H., J. Kawasahi and Y. Deguchi, 1997. Production of amylase by the intestinal microflora in cultured freshwater fish. *Lett. Appl. Microbiol.*, 24: 105–108

Wang, Y.Z., J.F. Sun, A.J. Lv, S.L. Zhang, Y.Y. Sung, H.Y. Shi, X.C. Hu, S.J. Chen and K.Z. Xing, 2018. Histochemical distribution of four types of enzymes and mucous cells in the gastrointestinal tract of reared half- smooth tongue sole *Cynoglossus semilaevis*. *J. Fish Biol.*, 92: 3–16

Winglee, K., E. Eloefadrosh, S. Gupta, H. Guo, C. Fraser and W. Bishai, 2014. *Aerosol mycobacterium tuberculosis* infection causes rapid loss of diversity in gut microbiota. *Plos One*, 9: e97048

Xiong, J., K. Wang, J. Wu, L. Qiuqian, K. Yang, Y. Qian and D. Zhang, 2015. Changes in intestinal bacterial communities are closely associated with shrimp disease severity. *Appl. Microbiol. Biotechnol.*, 99: 6911–6919

Yeh, S.T. and J.C. Chen, 2008. Immunomodulation by carrageenans in the white shrimp *Litopenaeus vannamei*, and its resistance against *Vibrio alginolyticus*. *Aquaculture*, 276: 22–28

Zhang, X.J., G.M. Qin, X.W. Bing, B.L. Yan and L.G. Liang, 2011. Molecular and phenotypic characterization of *Vibrio aestuarianus*, a pathogen of the cultured tongue sole, *Cynoglossus semilaevis* Gunther. *J. Fish Dis.*, 34: 57–64

Zhang, Z., M.J. Liao, B. Li, Y.G. Wang, L. Wang, X.J. Rong and G.P. Chen, 2014. Study on cultured half-smooth tongue sole (*Cynoglossus semilaevis* Günther) intestinal microflora changes affected by different disease occurrence. *J. Fish. Chin.*, 38: 1565–1572

Zhou, H.X., J.J. Yao, W.H. Fang, X.C. Li, S. Zhao, Y. Wang, Y.X. Wu and J.F. Zhou, 2017. Isolation and identification & antimicrobial susceptibility test of *Aeromonas salmonicida* associated with the skin ulceration disease of *Cynoglossus semilaevis* Günther. *Mar. Fish.*, 39: 322–330

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