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Short communication

Immunosuppression-induced alterations in fish gut microbiota may increase the susceptibility to pathogens

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ABSTRACT

Intestinal bacteria play an important role in the health and provide a variety of beneficial effects to host. Immunosuppressant can reduce the immunity of host and increase the susceptibility to pathogens. But it is not clear whether the increased susceptibility caused by immunosuppressant is related to changes of gut microbiota. In this study, we used crucian carp administrated with dexamethasone to explore the effects of immunosuppressants on gut microbial communities and further evaluate the potential association between changes in gut microbiota and susceptibility to pathogens. The results of MANOVA based on the top 10 PCoA axis scores from unweighed/weighted UniFrac distances showed that administration of dexamethasone ($P = 0.021$) and the administration time ($P = 0.027$) had a significant impact on the gut microbial composition, regardless of pathogens infection status ($P = 0.35$). After administration with dexamethasone, the fish had higher abundance of *Cetobacterium* and lower abundance of *Bacillus* and *Lactococcus*, and the abundance of genus *Bacillus, Pseudomonas* and *Lactococcus* decreased along with prolong administration time of dexamethasone. The results may help us understand the correlation between the host susceptibility to pathogenic bacteria and gut microbial community shift, and extend our knowledge regarding the role of gut microbiota in keeping the balance between pathogenic and symbiotic bacteria.

1. Introduction

It is well known that the intestinal bacteria play an important role in the health and well-being of the host. Commensal gut bacteria provide a variety of beneficial effects to host, including metabolizing otherwise indigestible food components, producing essential nutrients, strengthening gut barrier integrity, educating the host immune system, and other function [1]. However, abnormal gut microbiota composition may cause these functional disorders [2,3], for example, metabolic, inflammatory, and immunological disease [4–6]. Not only that, microbial community imbalances even could increase the susceptibility of host to pathogen [7]. Previous research had proved that the antibiotic treatment induced substantial changes in the gut microbial community of mice, and then increased the susceptible to *Clostridium difficile* infection [7]. Allison [8] had found that western diet induces a shift in microbiota composition and enhanced susceptibility to adherent-invasive *Escherichia coli*. Zhou [9] had revealed that feed oxyteracycline decreased the intestinal microbial richness of zebrafish. Although the mechanisms underlying this phenomenon are unknown, it has been postulated that colonization resistance is the ability of the indigenous gut microbiota to prevent colonization by pathogens [10].

Dexamethasone has been routinely used as an anti-inflammatory immunosuppressive agent [11]. At this regard, different cellular and humoral components of innate [12–14] and adaptive [15,16] immune responses are down-regulated after exogenous dexamethasone treatments. Our previous work has shown that administration with dexamethasone in crucian carp had lower lysozyme activity in serum and lower expression of immune gene, and increased the susceptibility of crucian carp to *A. hydrophila* [17]. Another study reported that administration with dexamethasone significantly increased the susceptibility of rainbow trout to *Gyrodactylus derjavini* [18]. Some researchers considered that increased susceptibility may be due to the immunosuppressive effect of dexamethasone, but there is no evidence that dexamethasone can alter fish gut microbiota and further enhance the susceptibility to pathogens. In this study, the fish were administrated with dexamethasone and then challenged with *A. hydrophila*. The aim of this research was to reveal the effect of dexamethasone on the intestinal microbiota structure, and preliminarily discusses the relationship between the shift of intestinal microbiota structure and pathogen susceptibility.

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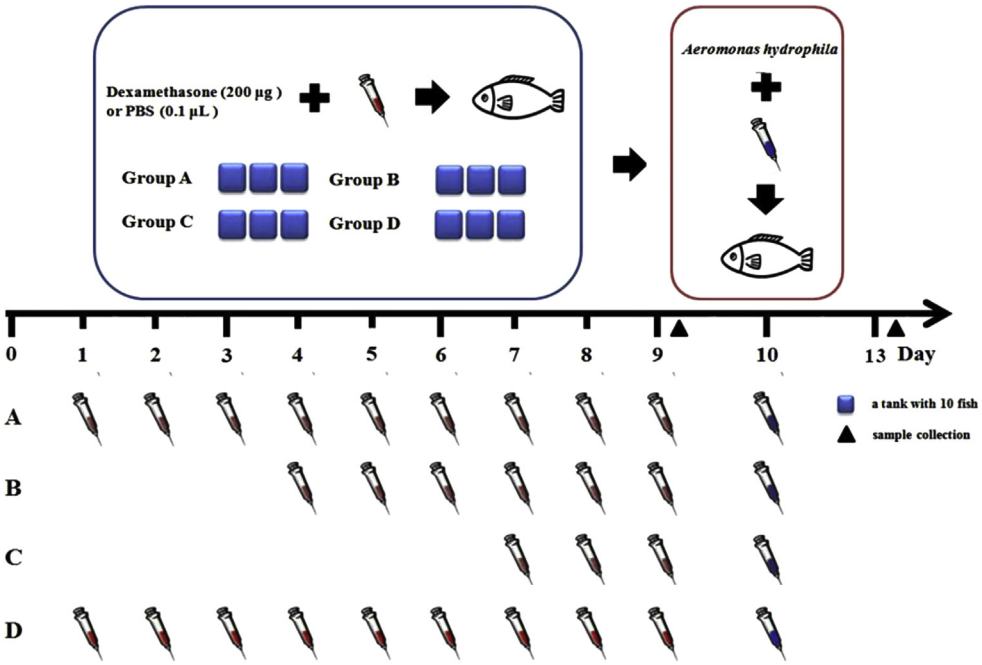

Fig. 1. Diagram of experimental design. A: the fish (n = 10 fish × 3 tanks) were intraperitoneally administrated with dexamethasone (200 μg/fish per day, 0.1 mL) for 9 days. B: the fish were intraperitoneally administrated with dexamethasone once a day for 6 days. C: the fish were intraperitoneally administrated with dexamethasone once a day for 3 days. D: control check, intraperitoneally administrated with PBS. On the 9th day of the trial, three fish were randomly selected from each group and sampled. The rest of fish were injected intraperitoneally with 0.2 ml of a 3.0×10^6 CFU mL^{-1} *Aeromonas hydrophila* on the 10th day and three fish were randomly selected from each group and sampled on the 13th day of the trial.

2. Materials and methods

2.1. Experimental design and sampling procedure

The animal protocol was approved by Northwest A&F University animal protection committee. Healthy crucian carp (20 ± 5 g and 12 ± 2 cm) purchased from a local fish farm (Xianyang, Shaanxi, China) were randomly divided into four groups (30 fish per group = 10 fish × 3 tanks) and received the following treatments: A: the fish were intraperitoneally administrated with dexamethasone (200 μg/fish day, 0.1 mL) once a day for 9 days. B: the fish were intraperitoneally administrated with dexamethasone once a day for 6 days. C: the fish were intraperitoneally administrated with dexamethasone once a day for 3 days. D: control, intraperitoneally administrated with PBS. (Fig. 1). On the 9th day of the trial, three fish were randomly selected from each group and sampled. The crucian carp were anaesthetized with 20 g/m^3 MS-222 (Geruien, China). Freshly dissected intestines were placed into filter-sterilized PBS and frozen at −20 °C until DNA extraction. Meanwhile, the rest of fish were injected intraperitoneally with 0.2 ml of a 3.0×10^6 CFU mL^{-1} *Aeromonas hydrophila* (2WCL-103) which obtained from Institute of Hydrobiology Chinese Academy of Sciences (Wuhan, China), and mortalities were recorded daily, and three fish were randomly selected from each group and sampled on the 13th day of the trial.

2.2. DNA extraction and 16S rDNA sequencing and bioinformatic analysis

Total genomic DNA of fish intestinal was extracted using Soil DNA Kit (OMEGA, USA) following the manufacturer's instructions. DNA yield and purity were determined spectrophotometrically using Nanodrop ND-1000. The V3-V4 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95 °C for 2 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min) using primers 341F 50- CCTAYGGGRBGCASCAG -30 and 806R 5' - GGACTACNNGGGTATCTAAT - 30, where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 mL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 mM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

Then Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor -ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina platform according to the standard protocols.

2.3. Statistical analyses

The raw reads were filtered using a combination of tools from Mothur (ver. 1.25.0; http://www.mothur.org). Preliminary quality control steps were performed according to a previous study [19,20]. Using ChimeraSlayer, chimera sequences arising from the PCR amplification were detected and excluded from the denoised sequences. The high-quality sequences were assigned to samples according to the barcodes. The high-quality reads were clustered into operational taxonomic units (OTUs) using Mothur. OTUs that reached a 97% nucleotide similarity level were used for alpha diversity. And α-diversity was calculated using the vegan and phyloseq R packages. The difference of OTU abundances for the specific phyla and genera were performed on STAMP software [21]. Unweighted and weighted UniFrac distance and PCoA scores were calculated by Phyloseq library in R [22]. Linear discriminant analysis (LDA) to PCoA 1–10 axes and multivariate analysis of variance (MANOVA) were implemented by Mass, Vegan and Base library in R. Significant difference is set at the level of $P < 0.05$.

3. Results

Using 16S hypervariable region sequencing, we characterized gut microbiotas of crucian carp in a controlled dexamethasone exposure experiment. The fish administrated with dexamethasone had a higher level of Fusobacteria phyla which increased about 13.03 ± 10.29% (mean abundance) compared with the ones in the control, whereas a lower of Firmicutes phylum which decreased about 15.38 ± 11.52% (Fig. 2). At genus level, the fish had higher level of *Cetobacterium*, and lower *Bacillius*, *Pseudomonas* and *Lactococcus* genus after the administration of dexamethasone (Fig. 3). The abundance of *Cetobacterium* had increased from 24.77% to 45.64%, while the abundance of *Bacillius*, *Pseudomonas* and *Lactococcus* decreased from 26.52% to 3.41%, 15.28%–2.14%, and 10.24%–1.70%, respectively. We further used MANOVA to determine effects of dexamethasone status on micriobioal

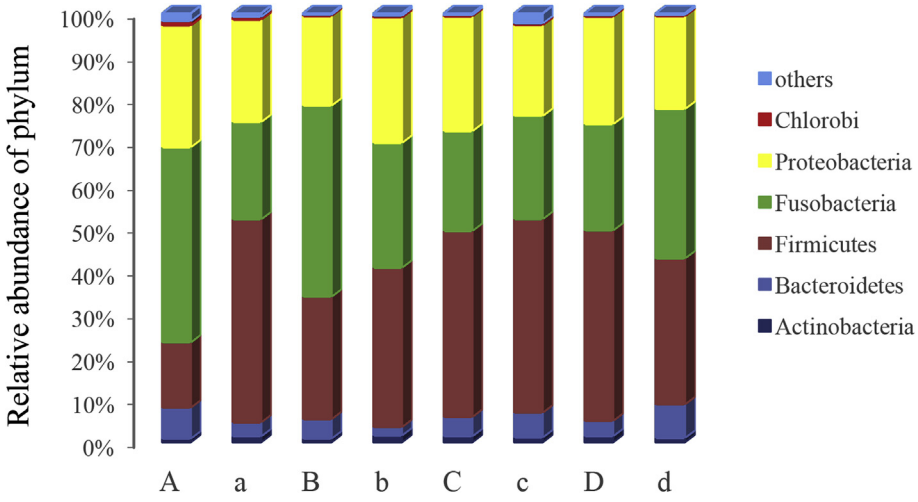

Fig. 2. Relative of intestinal microflora in crucian carp at the phylum level. A-D represented the fish in group which had sampled at 9th day. A: the fish after administration of dexamethasone once a day for 9 days; a: the fish in the group A at 4 days post-infection with *A. hydrophila*. B: the fish after administration of dexamethasone once a day for 6 days; b: the fish in the group B at 4 days post-infection with *A. hydrophila*. C: the fish after administration of dexamethasone once a day for 3 days; c: the fish in the group C at 4 days post-infection with *A. hydrophila*. D: control; d: the fish in the group D at 4 days post-infection with *A. hydrophila*.

community composition summarized by top 10 PCoA axes scores from unweighed/weighted UniFrac distances. The results showed that administration of dexamethasone had a significant impact on the gut microbial composition (F = 5.90, $P = 0.021$ for weighted PCoA axis scores). But, comparison of indexes of alpha diversity, as measured by the Shannon diversity index showed no significant differences among the A-D groups (Fig. 4). Similarly, there was no remarkable difference in the number of OTUs among the groups (Table 1).

Furthermore, we investigated if administration time could influence relative abundance of intestinal microbiota and its composition. At phyla level, the abundance of Fusobacteria showed an increasing trend along with extension of administration time, while the abundance of Firmicutes had an opposite trend (Fig. 2). The level of genus *Bacillus, Pseudomonas* and *Lactococcus* decreased along with prolong administration time (Fig. 3). Notably, the abundance of *Bacillus* and *Pseudomonas* were decreased about 7-fold after the fish were treatment with dexamethasone once a day for 9 days, and *Lactococcus* reduced almost 5-fold. The abundance of *Cetobacterium* showed an increasing trend along with prolong administration time, and its level in group A was fifth as high as that in the control group. STAMP results showed that the abundance of *Bacillus, Pseudomonas* and *Lactococcus* had a significant change after the administration of dexamethasone ($P < 0.05$) (Fig. 6). MANOVA results showed the administration time exhibited a significant effect on the intestinal microbial structure summarized both top 10 weighted and unweight PCoA scores (F = 2.77, $P = 0.027$ and F = 1.60, $P = 0.028$, respectively). Moreover, the results of linear discriminant analysis (LDA) to the first 10 unweighted PCoA axes demonstrated that the first leading LDA axis (87.46%) separated the fish

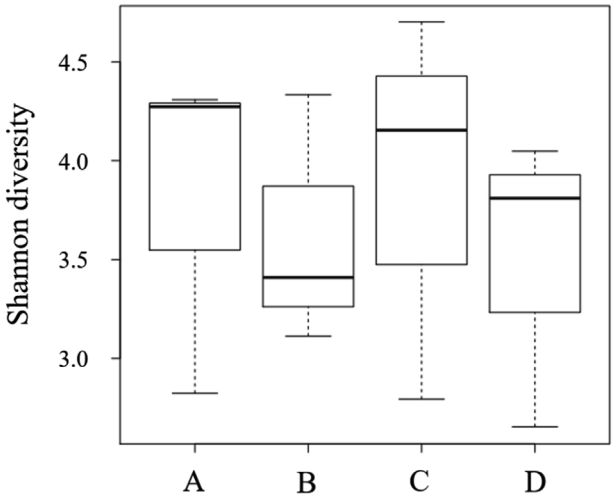

Fig. 4. Alpha diversity pattern with dexamethasone exposure. Shannon diversity measures are plotted as interquartile range with median. A: the fish were intraperitoneally administrated with dexamethasone once a day for 9 days. B: the fish were intraperitoneally administrated with dexamethasone once a day for 6 days. C: the fish were intraperitoneally administrated with dexamethasone once a day for 3 days. D: control, intraperitoneally administrated with PBS.

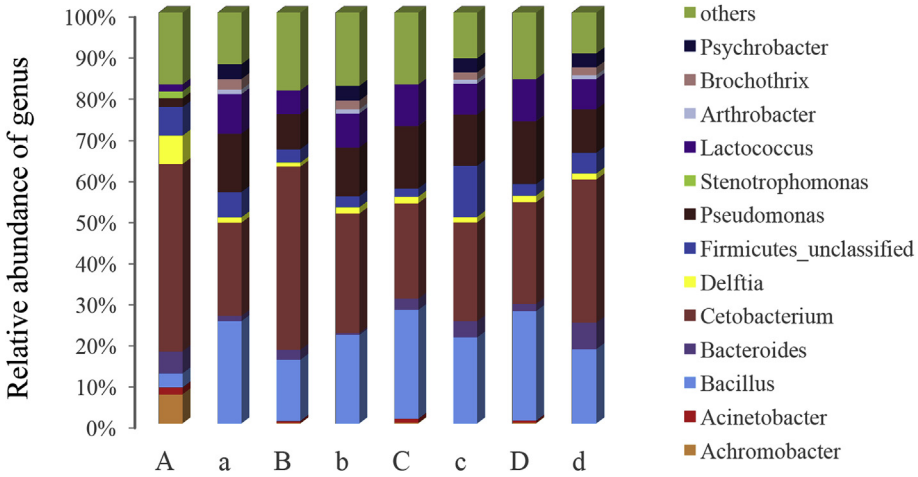

Fig. 3. Relative of intestinal microflora in crucian carp at the genus level. A-D represented the fish in group which had sampled at 9th day. A: the fish after administration of dexamethasone once a day for 9 days; a: the fish in the group A at 4 days post-infection with *A. hydrophila*. B: the fish after administration of dexamethasone once a day for 6 days; b: the fish in the group B at 4 days post-infection with *A. hydrophila*. C: the fish after administration of dexamethasone once a day for 3 days; c: the fish in the group C at 4 days post-infection with *A. hydrophila*. D: control; d: the fish in the group D at 4 days post-infection with *A. hydrophila*.

Table 1
Operational taxonomic unit analysis of intestinal flora in crucian carp. A-D represented the fish in group which had sampled at 9th day. A: the fish after administration of dexamethasone once a day for 9 days; a: the fish in the group A at 4 days post-infection with *A. hydrophila*. B: the fish after administration of dexamethasone once a day for 6 days; b: the fish in the group B at 4 days post-infection with *A. hydrophila*. C: the fish after administration of dexamethasone once a day for 3 days; c: the fish in the group C at 4 days post-infection with *A. hydrophila*. D: control; d: the fish in the group D at 4 days post-infection with *A. hydrophila*.

sample	A	a	B	b	C	c	D	d
OTU	1784 ± 453	1333 ± 9	1578 ± 334	1443 ± 634	1763 ± 487	1456 ± 446	1592 ± 243	1600 ± 435

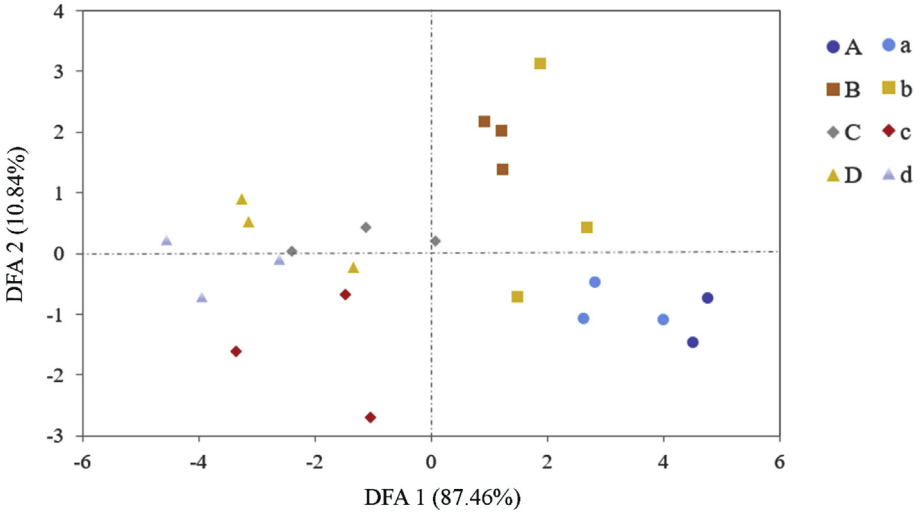

Fig. 5. PCoA analysis of intestinal microbiota samples of crucian carp. Here we plot discriminant function axis scores of individual crucian carp, based on a discriminant function analysis of microbial PCoA scores (first 10 axes of weighted UniFrac distances) as a function of fish dexamethasone status, infection status and administration of time. The administration of dexamethasone ($P = 0.0207$) and time ($P = 0.0271$) had significant effect on gut microbial structure. A-D represented the fish in group which had sampled at 9th day. A: the fish after administration of dexamethasone once a day for 9 days; a: the fish in the group A at 4 days post-infection with *A. hydrophila*. B: the fish after administration of dexamethasone once a day for 6 days; b: the fish in the group B at 4 days post-infection with *A. hydrophila*. C: the fish after administration of dexamethasone once a day for 3 days; c: the fish in the group C at 4 days post-infection with *A. hydrophila*. D: control; d: the fish in the group D at 4 days post-infection with *A. hydrophila*.

administrated with dexamethasone from the controls (Fig. 5). The above results indicated that dexamethasone alter the gut microbial community, and importantly longer administration time can change more strongly fish gut microbial composition.

Next, we analyzed the composition of gut microbiota in survived fish after infection with *A. hydrophila*. MANOVA results showed that no significant effect of infection status on the intestinal microbial composition using the top 10 neither weighted or unweight PCoA scores. Interestingly, infection with *A. hydrophila* can't alter the effect of dexamethasone (including administration time) on fish gut microbiota (Fig. 5), and there is no interaction between infection status and other variables (Table 2). However, infection with *A. hydrophila* changed the relative abundance of some phyla/genera in the fish gut. For example, for the fish were administrated with dexamethasone once a day for 9 days, the abundance of Firmicutes had increased from 15.11% to 47.24% (Fig. 2); and at genus level, *Bacillus, Pseudomonas* and *Lactococcus* had increased a lot. Particularly in group A, the abundance of *Bacillus* and *Pseudomonas* had a significant increase after *A. hydrophila* infection (3.41%–24.79%, 2.14%–14.24%, respectively) (Fig. 3). Contrariwise, *Cetobacterium* had a significant decline (decreased about 22.93%) in group a (Fig. 3).

Our previous research [17] showed the connection between *A. hydrophila* infecting fish and mortality. The highest mortality was observed in fish infected by *A. hydrophila* in group A. Fish in group D (control) had lower mortality rate than others. In this study, *Lactococcus* and *Bacillus* hold the lower abundance in fish which had the highest mortality, the abundance of *Lactococcus* and *Bacillus* decreased along with the along with prolong administration time of dexamethasone and the mortality also increased. but *Cetobacterium* and *Bacteroide* hold higher abundance in these fish (Fig. 3).

4. Discussion

This study showed that administration of dexamethasone ($P = 0.021$) and the administration time ($P = 0.027$) had a significant impact on the gut microbial composition, regardless of pathogens infection status ($P = 0.35$). After administration with dexamethasone, the fish had higher abundance of *Cetobacterium* and lower abundance of *Bacillus* and *Lactococcus*, and the abundance of genus *Bacillus, Pseudomonas* and *Lactococcus* decreased along with prolong administration time of dexamethasone.

Our previous study had proved that crucian carp administrated with dexamethasone had lower expression of immune gene and higher mortality [17]. In this study, we found that dexamethasone could alter the gut microbial community of crucian carp. The reduction of *Lactococcus* and *Bacillus* in the gut of fish after the administration of dexamethasone may be responsible for the susceptibility. Lactic acid bacteria are very efficient at metabolizing a large variety of small fractions of lactic acid, various enzymes, lipids, and proteins [23,24]. These metabolic products can stimulate immune responses in humans and animals, and improve the immune system [25]. Lactic acid bacteria could produce lactic acid, which inhibits the survival of pathogenic bacteria in the intestine [26,27]. *Lactococcus* is a kind of Lactic acid bacteria, and a common probiotic that have been widely studied for their probiotic properties in aquaculture [28,29]. It is well known for improvement of digestion, and immune modulatory effects in animals [25,30,31]. Some studies have showed that feeding *Lactococcus lactis* could improve the fish's growth rate, innate immunity, and survival rate of host infected with pathogenic bacteria [32–36]. *Bacillus* is a genus of gram-positive, rod-shaped bacteria and a member of the phylum Firmicutes. It is a widely used probiotic in aquaculture. Sun et al. [37] reported that two probiotics of *Bacillus pummilus* and *Bacillus clausii*, dominant gut *Bacillus* strains with antagonistic ability from the fast growing *Epinephelus. coioides* were able to improve host' growth and health. It has been shown that *Bacillus* strains supplementation in diet could increase disease resistance in fish through the stimulation of both the cellular and humoral immune function, such as phagocytic activity, lysozyme activity and complement activity [38,39]. But in this study, the abundance of *Lactococcus* and *Bacillus* was decreased along with prolong administration time of dexamethasone, and this is one of the main factors caused the susceptibility of crucian carp to *A. hydrophila*. The result showed that different administration time of dexamethasone

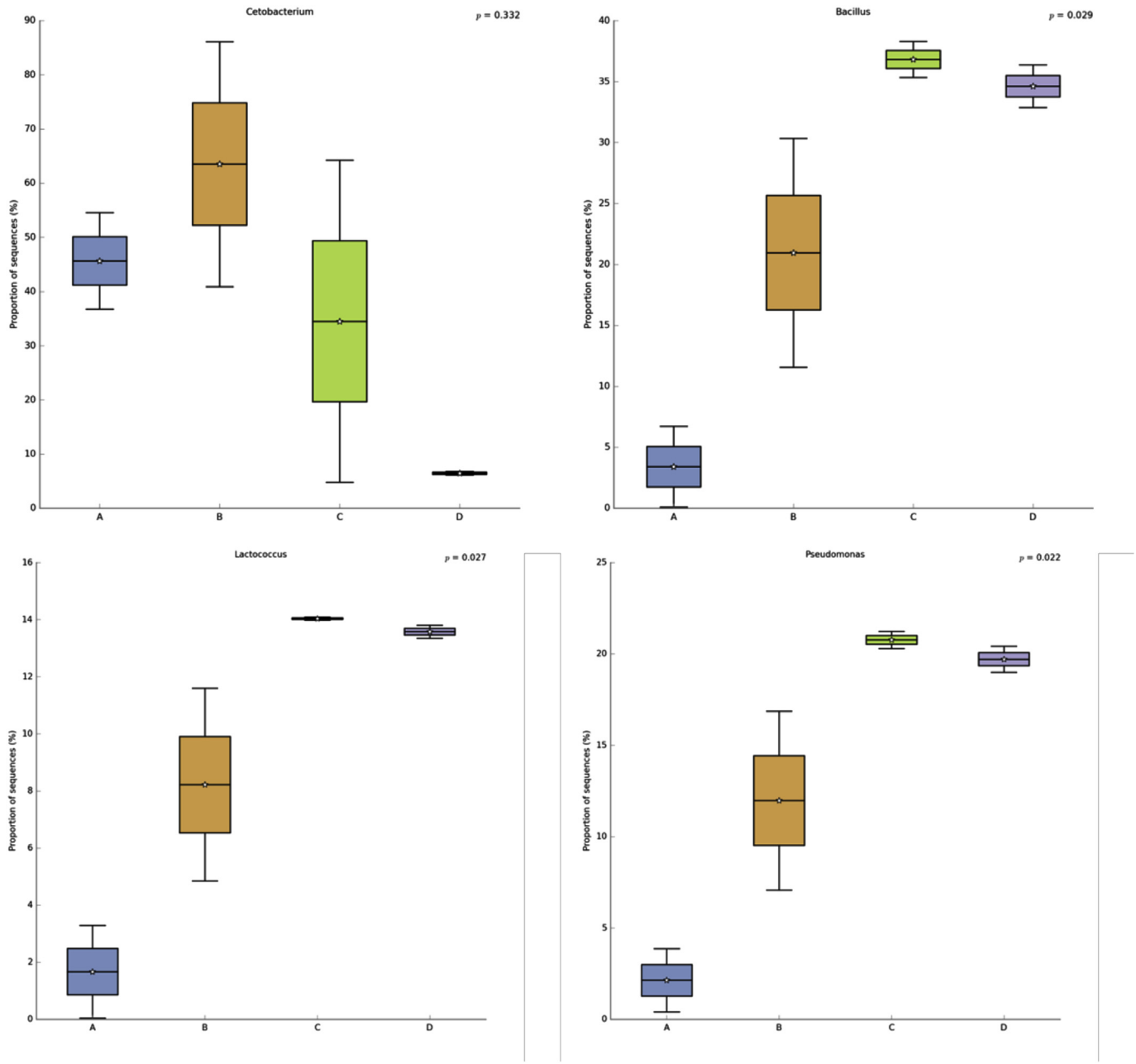

Fig. 6. Box plot showing the change of the abundance of the specific genera within the gut microbiota of crucian carp after the administration of dexamethasone. A: the fish after administration of dexamethasone once a day for 9 days; B: the fish after administration of dexamethasone once a day for 6 days; C: the fish after administration of dexamethasone once a day for 3 days; D: control.

Table 2
Results of a MANOVA testing effects of dexamethasone status, administration time and infection status, and interactions among these variables on microbial community composition (weighted or unweighted PCoA axes 1–10), **significance of bold** as significantly difference ($P < 0.05$).

Model effect	Effect Df	Unweighted PCoA 1-10			*P*	Weighted PCoA 1-10		
		Pillai's trace	F	F Df		Pillai's trace	F	*P*
Dexamethasone	1	0.70	1.40	10;6	0.35	0.91	5.90	**0.021**
Infection	1	0.70	1.40	10;6	0.35	0.70	1.41	0.35
Time	2	1.60	2.77	20;14	**0.028**	1.60	2.80	**0.027**
Dexamethasone * Infection	1	0.52	0.66	10;6	0.73	0.70	1.35	0.37
Infection * Time	2	1.27	1.23	20;14	0.35	1.32	1.35	0.29
Residuals	15							

would affect the abundance of *Lactococcus* and *Bacillus* in fish intestine. Furthermore, we found that the abundance of *Lactococcus* and *Bacillus* in fish intestine had a negative correlation with the pathogen susceptibility of host. It had higher mortality when the abundance of *Lactococcus* and *Bacillus* were low in the gut of fish which infected *A. hydrophila*. We speculate that this may because the strain is potential probiotic and could improve the fish immune-system [17,25]. But it needs further testing to verify that the relationship between the strain with the immune system. We also had sequenced the gut microbiota flora of crucian carp which survived after infection with *A. hydrophila*. We found that the survived fish had higher abundance of *Bacillus*, the result showed that higher level of *Bacillus* contributed to the resistance of *A. hydrophila* of host.

In conclusion, the present data indicated that the gut microbiota of crucian carp is diverse, but dominated by three phyla, Firmicutes, Proteobacteria and Fusobacteria. There was a clear shift in the bacterial community after administration with dexamethasone. Fish had high level of *Cetobacterium* and lower *Bacillus* and *Lactococcus* genus after injected with dexamethasone, and the level of genus *Bacillus, Pseudomonas* and *Lactococcus* decreased along with prolong administration time. The abundance of *Lactococcus* and *Bacillus* in fish intestine had a negative correlation with the pathogen susceptibility of host. The results may help us understand the correlations between the host susceptibility to pathogenic bacteria and gut microbial community shift under dexamethasone exposure and extend our knowledge regarding the roles of gut microbial community in pathogenicity of pathogenic bacteria.

Acknowledgments

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