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Article

Dynamics of Gut Microbiome and Transcriptome in Korea Native Ricefish (*Oryzias latipes*) during Chronic Antibiotics Exposure

Ju Bin Yoon[1,2,†], Sungmin Hwang[4,†], Jun Hyeok Yang[1,3], Seungki Lee[5], Woo Young Bang[5,*] and Ki Hwan Moon[1,2,3,*]

[1] Division of Convergence on Marine Science, Korea Maritime & Ocean University, Busan 49112, Republic of Korea
[2] Ocean Science and Technology School, Korea Maritime & Ocean University, Busan 49112, Republic of Korea; fishbin12345@gmail.com (J.B.Y.)
[3] Department of Marine Biosience and Environment, Korea Maritime & Ocean University, Busan 49112, Republic of Korea; ardim4100@gmail.com (J.H.Y.)
[4] Clean Energy Research Center, Korea Institute of Science and Technology, Seoul 02792, Republic of Korea; sungminhwang@kist.re.kr (S.H.)
[5] National Institute of Biological Resources, Environmental Research Complex, Incheon 22689, Republic of Korea; metany@korea.kr (S.L.)
* Correspondence: khmoon@kmou.ac.kr (K.H.M.); wybang@korea.kr (W.Y.B.)
† These authors contributed equally to this work.

Abstract: Antibiotics have been used in various fields such as livestock farm and fish farm as well as hospital in order to treat diseases caused by bacteria. However, the antibiotics that are not completely decomposed, but remains as residue and discharge to aquatic environment, can cause an imbalance in the gut flora of host, as well as regulate abnormal host gene regulatory system. We investigated the effects of chronic exposure with the low concentrations of erythromycin and ampicillin on gut microbiome and immune and stress-related gene expression using Korea native ricefish (*Oryzias latipes*). As a result of microbiome analysis, the proportion of *Proteobacteria* was increased in the ricefish when exposed to erythromycin and ampicillin chronically, whereas the proportion of other bacterial phyla decreased. In addition, the immune and stress-related genes were significantly influenced in the ricefish under the chronic antibiotics exposure. These results show that the internal microbial flora and the host gene expression are susceptible even in the low concentration of chronic antibiotic existing environments. This study provides the importance of the appropriate use of antibiotics dose to maintain the sustainable and healthy aquaculture industry and water ecosystem.

Keywords: Ricefish; Microbiome; Ampicillin; Erythromycin; Immune and Stress-Related Genes

1. Introduction

Antibiotic is a substance used to prevent or treat the disease caused by bacteria by inhibiting the growth of bacteria with several action mechanisms such as impeding cell wall biosynthesis and translation machinery. For this reason, various antibiotics are widely used in livestock farms, hospitals, aquaculture, veterinary hospitals, agriculture, household products, research and industrial fields [1]. As growing the mass use of antibiotics, antibiotics are detected in wastewater treatment plants, freshwater and seawater, biological solids, sediments, soil, and aquatic organisms [2]. Even feces from animals that have been injected with antibiotics are excreted into the aquatic environment through drainage system and damages to aquatic environments [3,4]. Antibiotics released into the environment are diluted and affect to bacteria at low concentrations, which would not be fatal to bacteria, however, acts as selective pressure that can lead to negative consequences such as acquisition of antibiotic resistance genes and uncontrollable regulation of virulence genes [5-7]. In addition, antibiotic acts on the host as well, affecting changes in behavior, growth, development, reproduction, mortality, and essential flora [8-10].

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The gut microbiome is a community of bacteria in the gut and is known to be interactive with host, resulting in various roles such as developing host immune system, providing useful metabolites, strengthening the barrier of intestine, and regulating gene expression [11]. This gut microbiome is distinctive by species and changeable by various external stimuli as well as biological factors such as diet, hunger and age [12-17]. It is known that dysbiosis, in which the microbial diversity is rapidly reduced by certain stress conditions in the intestine and the expansion of certain bacteria is promoted, can cause metabolic disorders such as obesity, including inflammatory bowel disease [18].

Ricefish (*Oryzias latipes*) is one of the best model organisms of fish in that a completion of whole genome sequence along with zebrafish (*Danio rerio*) and a relatively short period (2-3 months) to be mature due to the absence of spawning period [19]. The Korean native ricefish used in this study is an individual caught in nature without undergoing breeding, and this is a promising viable tool to study the wild type ricefish for presenting the effects of antibiotics on the host in freshwater.

We sought to understand the effect of residual antibiotics to aquatic environment, in particular, to fish in freshwater. To verify this, ampicillin (β-lactam antibiotics) and erythromycin (macrolide antibiotics) were selected, since two representative antibiotics are widely used in various purposes and detected in environmental specimen [2,20], and were supplemented at relatively low concentrations to experimental aquarium. The Korea native ricefish that are exposed to the antibiotics were examined their gut microbial flora and monitored the alteration of gene expression. Overall, this study will provide both the potential risk and the fundamental interactions between the gut microbiome and host transcriptome at persistent low dose antibiotics.

2. Materials and Methods

2.1. Experimental animals

Korean native ricefish (*Oryzias latipes*) were supplied from the National Institute of Biological Resources (NIBR) (Incheon, Republic of Korea). Fish were caught from the Naerincheon, Hongcheon, Gangwon-do in Republic of Korea (37.9536, 128.3121) and stabilized in the NIBR laboratory for 4 weeks. The individuals survived were procured for subsequent experiments including microbiome and transcriptome under our laboratory at the Korea Maritime & Ocean University. The procured fish from NIBR were acclimatized for 2 weeks in approximately 15 L (25 cm long, 25 cm wide, 25 cm deep) water tanks with aerated dechlorinated tap water and a natural photoperiod of 14 h light and 10 h dark before the experiment. The water temperature was kept at 27 ± 2℃. During the acclimatization, fish were fed with a commercial diet (PROPAC, Italy) once per day.

2.2. Gut microbe isolation and 16S rDNA sequencing

Three ricefish were randomly selected and anesthetized using tricaine methanesulfonate (Sigma-Aldrich, Burlington, MA, USA). Fish were washed with 70% ethanol to remove surface bacteria and washed once more with 1X phosphate buffered saline (PBS), then guts were dissected and homogenized with 10 ml of 1X PBS. After diluting tenfold with 1X PBS, the homogenates were spread by 100 μl on Nutrient agar (NA), Luria-Bertani (LB) agar, Tryptic Soy agar (TSA), Brain Heart Infusion (BHI) agar and Lactobacilli MRS (MRS) agar, respectively. Separate media were cultured at 27℃ for a maximum of 2 days. Twenty five strains of bacteria were isolated from separate media, then finally six strains were isolated by comparing the colony phenotypes of the isolates. Six isolates gDNA were extracted using HiGene™ Genomic DNA Prep Kit (BIOFACT, Daejeon, Republic of Korea) according to manufactures instructions and quantified them using the Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, Vermont, USA). 16S rDNA sequencing were conducted at DNALINK, Inc. (Seoul, Republic of Korea). Six isolates were stored at –80℃.

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2.3. Minimum inhibitory concentration (MIC) test against Oryzias latipes gut isolates

To set up antibiotics concentration for chronic antibiotics exposure assay, we conducted MIC test against six *Oryzias latipes* gut isolates. Before the experiment, all isolate strains were streaked on TSA and incubated at 27℃ for 18 hr. Then cultured single colonies were pre-cultured on 2 ml Tryptic Soy broth (TSB) at 27℃ for 18 hr. Stock solutions were prepared by dissolving erythromycin (Ery) (Sigma Aldrich, Burlington, MA, USA) in absolute Ethyl alcohol and ampicillin (Amp) (Generay biotech, Shanghai, China) in ddH_2O. To perform MIC test, antibiotics-treated TSB was prepared by using two-fold serial dilutions from maximum concentration: Ery 62.5 μg/ml and Amp 100 μg/ml. All diluted medium was aliquoted into 96-well plate in 150 μl, and the pre-cultured strains were inoculated by 1% (v/v) each. The 96-well plate was incubated at 27℃ within an Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, Vermont, USA) for 18 hr and optical density (OD) at 600 nm was used to measure cell growth every 20 min.

2.4. Chronic antibiotics exposure assay

Four liters of aerated dechlorinated tap water was added to three glass aquarium (approximately, 5 L volume at 15 cm long, 22 cm wide and 17 cm deep). Using antibiotics stock, three water conditions were prepared: Non-treated, Ery (3.9 μg/ml) and Amp (3.125 μg/ml). Ricefish were exposed to three condition (Control, Ery, Amp) of antibiotics during 30 days under a semi-static condition. The tank water was refreshed twice a week by discarding 2 L of the old breeding water and adding 2 L of the new breeding water with same antibiotics concentration as the old one. And every two weeks, all breeding water was refreshed. During the exposure, fish were fed with a commercial diet (PROPAC, Italy) once per day.

2.5. Oryzias latipes gut microbiome analysis

Three ricefish exposed to chronic antibiotics were randomly selected from each group and anesthetized using tricaine methanesulfonate. To extract bacterial genomic DNA from fish gut dissected by using HiGene™ Genomic DNA Prep Kit (BIOFACT, Daejeon, Republic of Korea) according to manufacture instruc-tions. Specific primer pairs (Bakt_341F 5′- CCTACGGGNGGCWGCAG -3′ and Bakt_805R 5′- GACT- ACHVGGGTATCTAATCC -3′) that recognize the hypervariable V3–V4 region were used to prepare for a library construction. High-throughput sequencing was performed using the MiSeq platform (Illumina, San Diego, CA, USA), followed by quality check including removal of adapter and low sequence score (< 20) via FastQC v.0.11.7 and Cutadapt v.1.18. The preprocessed sequence data was clustered by using CD-HIT-OTU with cutoff value at 0.03 which is equivalent to over 97% sequence similarity score for operational taxonomic units (OTUs) [21], followed by the further analysis for the alpha diversity included Shannon and Simpson (relative dominance of some species) indices in each sample, as well as for the rarefaction curves and PCoA (principle coordinate analysis) plots in OTU-based groups under the QIIME2 platform [22]. SILVA reference database version 138 was used to classify the bacterial taxonomy from phylum to species levels [23].

2.6. Differentially expressed genes (DEG) analysis

Three ricefish were randomly selected from each group, anesthetized using tricaine methanesulfonate, and homogenized to extract total RNA with TRIzol™ Reagent. The purified total RNA was further processed to construct mRNA sequencing library according to the manufacturer's instructions (Illumina Truseq stranded mRNA library prep kit, USA). mRNA was purified and fragmented from total RNA (1 μg) using poly-T oligo-attached magnetic beads, then reverse transcribed into cDNA with adapters. The Illumina Novaseq 6000 sequencing system was used to perform high-throughput sequencing that generated average 70 million reads with 2 x 100 bp read length in each sample. Tophat

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v.2.0.13 [24] was used to map reads to the reference genome of *Oryzias latipes*, followed by the identification of differentially expressed genes (DEGs) with a default options of Cuffdiff v.2.2.0 [25]. For ontology analysis, DEGs (log2 fold change larger than 1 and a false discovery rate less than 0.05) were applied to DAVID as an input to get a comprehensive set of functional annotation [26].

3. Results

3.1. Determination of exposure concentration of ampicillin and erythromycin in Oryzias latipes gut isolates

We expected that the gut microbes of ricefish would have different antibiotic susceptibility depending on the species. Therefore, we tried to determine the exposure concentration of antibiotics based on the antibiotic susceptibility of representative bacteria isolated from the gut of ricefish. We confirmed that six gut microorganisms separated from the intestines of Korean native ricefish were *Shewanella xiamenensis*, *Flavobacterium* sp., *Microbacterium* sp., *Aeromonas hydrophila*, *Aeromonas* sp., and *Bacterium* strain BS2147, respectively, through 16S rDNA sequencing. In addition, the minimum inhibitory concentration (MIC) test for ampicillin and erythromycin was performed in the six identified strains, respectively. Based on the results of the MIC test and the concentration of antibiotics used in the aquaculture (data not shown) [27], we determined the final exposure concentration of antibiotics as Ery 3.9 μg/ml and Amp 3.125 μg/ml.

3.2. Effects of chronic antibiotics exposure on the richness and diversity of the gut microbiome

We evaluated α-diversity to determine how the richness and diversity of gut microbiome of ricefish was altered by chronic exposure to antibiotics. When comparing with control group in Figure 1, we confirmed the decrease of average Operational Taxonomic Units (OTUs) in both ampicillin and erythromycin treatment group. The rarefaction curve created on the species observed in Figure 2 was gradually reached a plateau in all groups, indicating that the amount of sequence data to identify all species included in the sample was adequate. In addition, species richness decreased in the ampicillin and erythromycin treatment group because the curve was less steep than the control group. As shown in Table 1, the Good's Coverage index of all the groups was observed bigger than 99.9%, indicating the sequencing result is reliable. The Inverse Simpson was confirmed to have no significant difference in all the groups ($P > 0.05$). Chao1 was confirmed to significantly decrease in the ampicillin treatment group when comparing the control group ($P < 0.01$). Shannon was confirmed to significantly decrease in the erythromycin treatment group when comparing the control group ($P < 0.05$) (Table 1). These results show the chronic exposure of all the selected antibiotic decrease both richness and diversity of intestinal microbial community.

Table 1. Effects of chronic exposure to antibiotics on gut bacterial diversity indexes.

Treatment	Control	Ampicillin	*P* value	Erythromycin	*P* value
Chao1	60.33 ± 5.83	35.42 ± 7.66	0.001	43.52 ± 22.95	0.051
Shannon	2.42 ± 0.3	2.2 ± 0.03	0.087	1.78 ± 0.1	0.0001
Inverse Simpson	0.67 ± 0.08	0.68 ± 0.01	0.887	0.62 ± 0.02	0.096
Good's Coverage	0.99 ± 0	0.99 ± 0	0.0001	0.99 ± 0	0.0001

*Note: Values are presented as means ± SD. Differences between each treatment group and control group were analyzed by t-tests, significant difference at $P < 0.05$.

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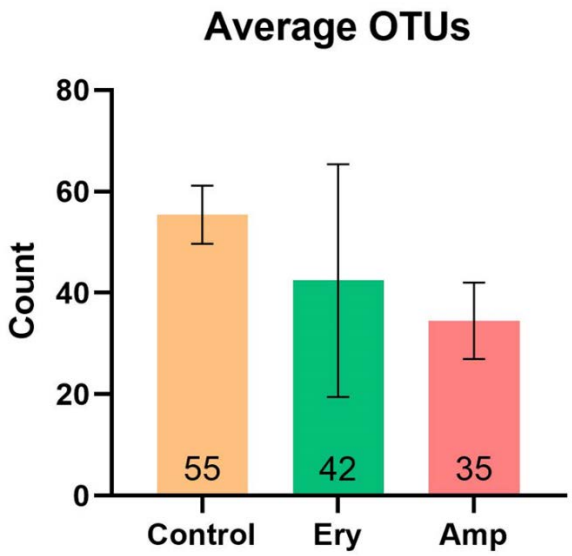

Figure 1. Effects of chronic antibiotics exposure on gut bacterial community shown as average OTUs.

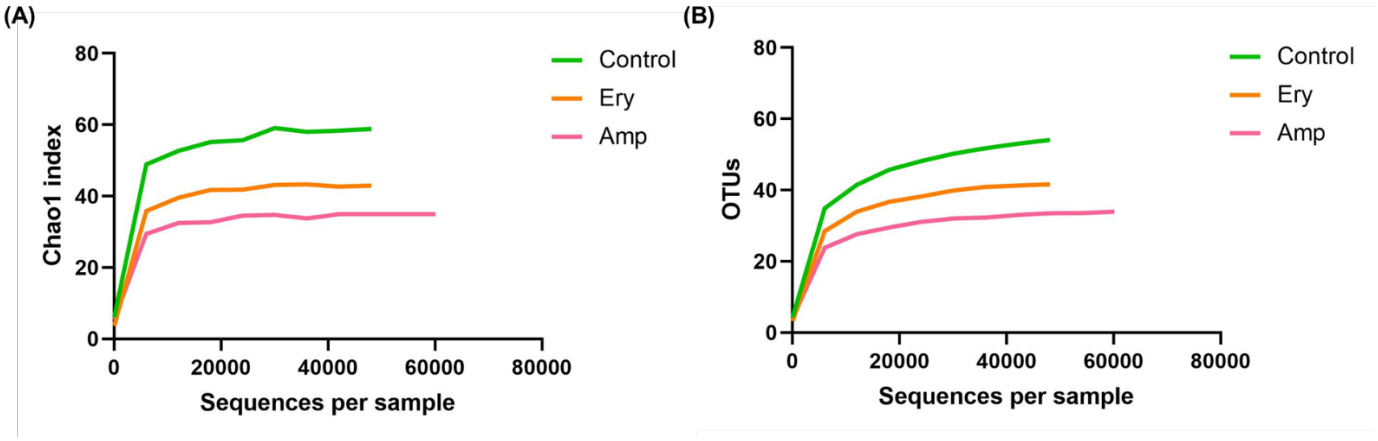

Figure 2. Effect of chronic antibiotics exposure on rarefaction curve. (A) chao1 index (B) OTUs.

3.3. Effect of chronic exposure to antibiotics on the structure of gut microbial community

In order to compare the entire structure of intestinal microbial community, β-diversity was evaluated with PCoA. As shown in Figure 3, each group was confirmed to have separate microbiome cluster. This indicates the structure of microbiome was significantly modified by the antibiotic chronic exposure because clusters of ampicillin and erythromycin treatment group were remote from the cluster of control groups.

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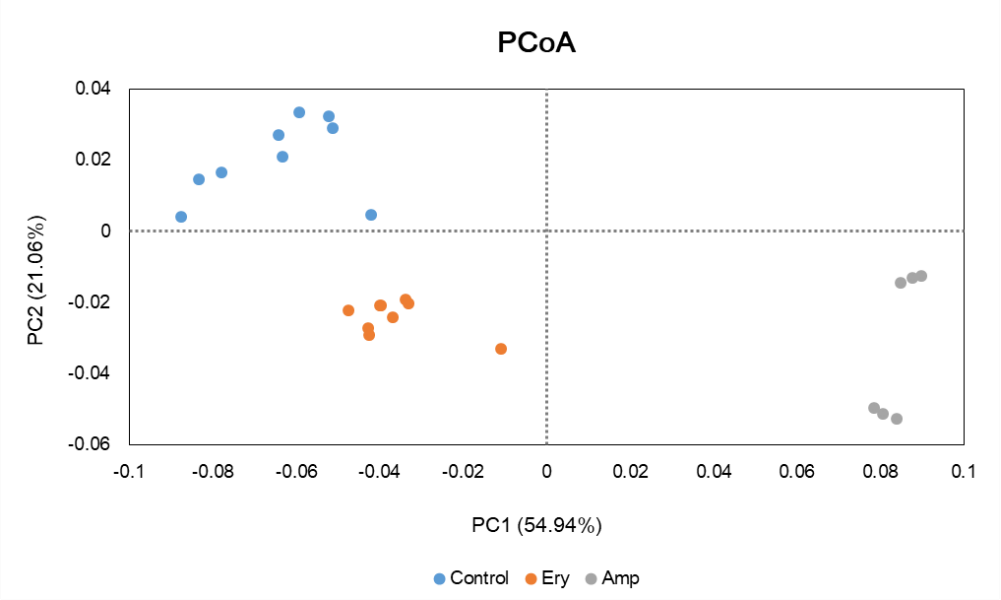

Figure 3. Effect of chronic antibiotics exposure on β-diversity based on the principle coordinate analysis (PCoA) in the gut microbiota. Each point represents a sample with colors representing different groups.

3.4. Changes in the expression of stress and immune-related genes in ricefish chronically exposed to antibiotics

To determine whether dysbiosis is induced by the chronic exposure to antibiotics in the gut of ricefish, we investigated the proportion of altered gut microbiota. As an analysis result of gut microbiome of ricefish which have been exposed chronically to erythromycin, it was confirmed that *Proteobacteria* proportion (16.55% increase) was increased the most at phylum level compared to the control group (Figure 4A). Conversely, it was confirmed remaining phylum including *Fusobacteria* (4.03% decrease) decrease, showing the imbalance that the variety of intestinal flora decreases and only a few floras have dominance. (Figure 4A). In addition, when compared to the details at the species level, it was confirmed the proportion of *Aeromonas veronii* increases (21.29% increase), the proportion of *Cetobacterium somerae* decreases (4.03% decrease) and almost other species decrease (Figure 4B). As a result of the gut microbiome analysis of ricefish chronically exposed to ampicillin, the proportion of *Proteobactria* (53.95% increase) increases almost twice at phylum level compared to the control group, which takes up almost (Figure 4A). However, the remaining phylum including *Fusobacteria* (45.95% decrease) show the trend of decrease, confirming the imbalance of gut flora like ricefish which are exposed chronically to erythromycin (Figure 4A). In addition, the proportion of *Aeromonas veronii* and *Vibrio parahaemolyticus* were increased 28.6% and 25.92%, respectively, whereas the proportion of *Cetobacterium somerae* was decreased 41.92% (Figure 4B).

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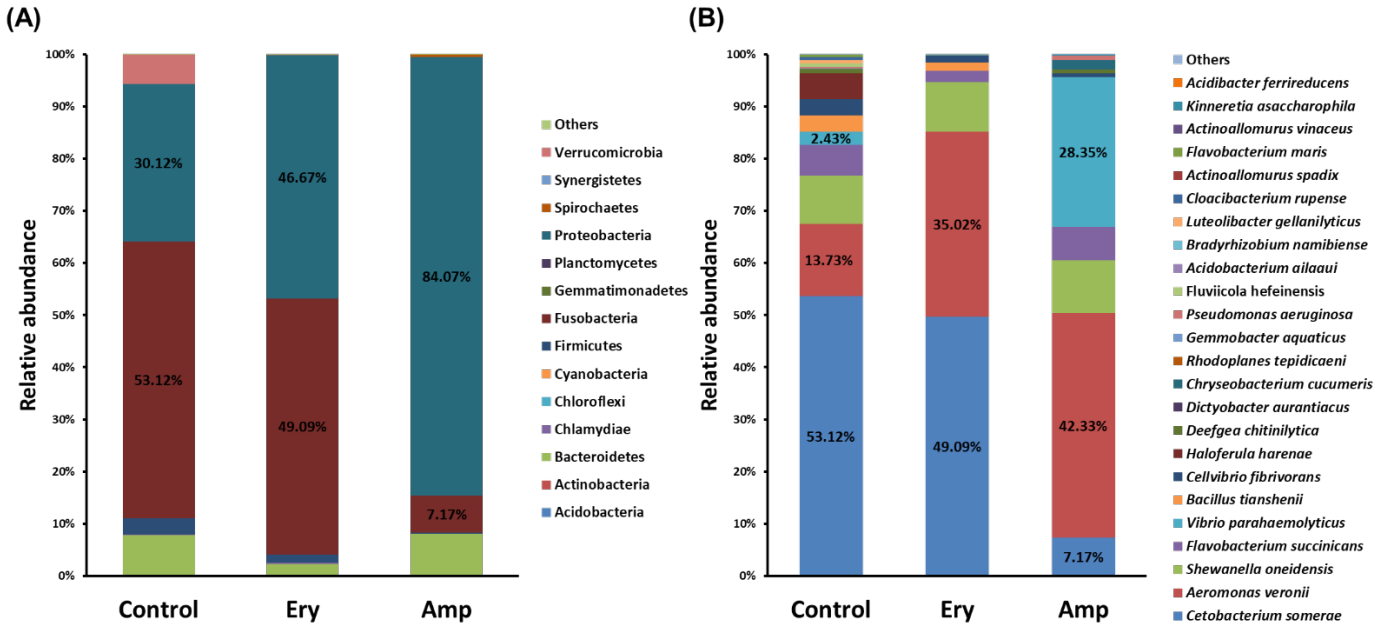

Figure 4. Relative abundance of gut microbiome in richfish chronically exposed to antibiotics. (A) phylum level (B) species level. Each bar represents the average relative abundance of each bacterial taxa for a treatment group.

3.5. Changes in the expression of stress and immune-related genes in ricefish chronically exposed to antibiotics

To determine whether a specific pathogen becomes a dominant species in the gut of richfish by chronic exposure to antibiotics and increases infection in ricefish, transcription level of genes involved in host stress and immune-related were identified. We investigated any change in gene expression of ricefish exposed chronically to ampicillin and erythromycin by RNA-seq as well as the gene ontology analysis. The number of genes showing significant expression changes in three aspects of molecular function (MF), cellular component (GC), and biological process (BP) was shown in Figure S1, and it was confirmed that the expression of various stress and immune-related genes were changed from the ricefish when ampicillin and erythromycin were present chronically (Figure 5). Among them, gene expression for genes responsive to TNF, IL-1, and IFN-γ, and for lymphocyte and monocyte chemotaxis (BP), and chemokine receptor binding (MF) was increased compared to control group. Furthermore, the number of genes whose expression was changed and the width of expression change in the erythromycin-treated group are bigger than that of the ampicillin-treated group. The upregulated genes indicate the chronical exposure to both ampicillin and erythromycin causes the inflammatory response in ricefish. Specifically, in ricefish chronically exposed to erythromycin, it was found chemokine-mediated signaling pathway, neutrophil chemotaxis, antigen processing and presentation, response to oxidative stress, inflammatory response, defense response-related gene expression (BP), chemokine activity, and peroxidase activity related gene expression (MF) and the expression of MHC class II protein complex-related genes (CC) are further increased, resulting in a stronger inflammatory response. In ricefish chronically exposed to ampicillin, tissue regeneration, cytokine-mediated signaling pathway related gene expression (BP) increased additionally. On the contrary, it was confirmed blood coagulation, myeloid dendritic cell differentiation, response to tumor necrosis factor, response to lipopolysaccharide, complement activation (BP) related gene expression and serine-type endopeptidase activity (MF) gene expression, membrane attack complex, fibrinogen complex (CC) rather than decreased. This indicates that other inflammatory response types are caused from the host depending on the antibiotic type.

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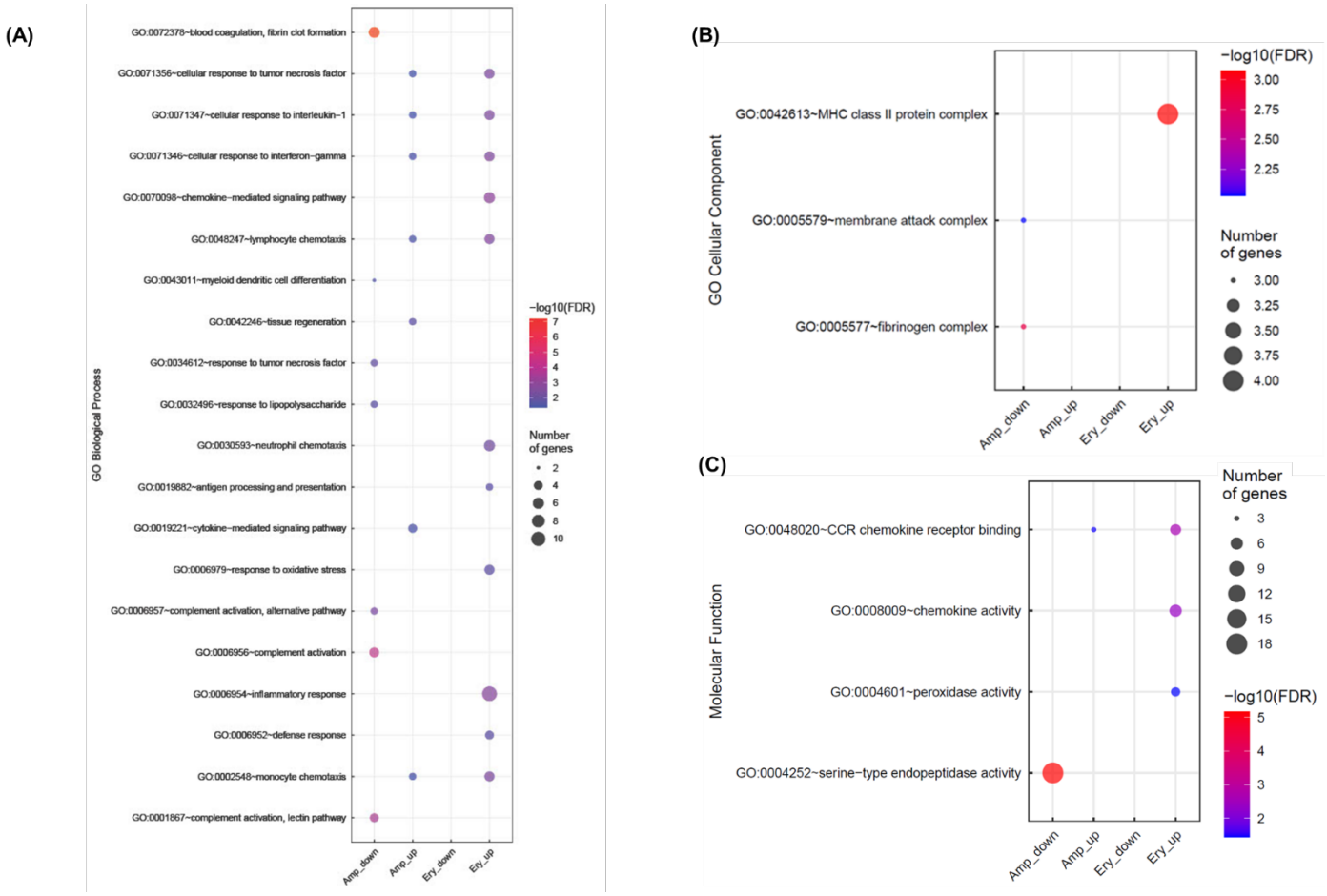

Figure 5. Changes in stress and immune-related gene expression from ricefish by the chronic exposure to antibiotics. (A) biological process (B) cellular component (C) molecular function. The size and color of the dots indicate the number and expression level of DEGs whose expression is changed in each treatment group, respectively.

4. Discussion

The balance of microbiome in the gut of the exposed host as well as the underwater microbiome is being damaged by the antibiotic emitted to the aquatic environment. Numerous studies have suggested that intestinal microbiome can be changed quickly and significantly due to environmental changes, and that the changed intestinal microbiome can have various effects on the host [28-32]. However, studies that reveal the correlation between altered gut microbiota and host specific gene expression are lacking. This study investigated the relevant effect by exposing Korean native ricefish chronically to the low concentration antibiotics. We predicted that the low concentration antibiotics can cause the dysbiosis to the gut microbiome of host, and effecting the expression of stress and immune related gene of host.

We selected ampicillin and erythromycin, antibiotics that are used in various fields such as livestock industry, aquaculture, and hospitals and are continuously detected in the environment, as representative antibiotics [2,20]. In addition, after selecting and confirming 6 representative bacteria isolated from the intestine of Korean native ricefish, the exposure concentrations of ampicillin and erythromycin were determined by conducting MIC test. For the concentration of selected antibiotics, the host is thought to be continuously exposed to the selected antibiotic concentration sufficiently when considering it is detected at 1mg/L or more in the industrial waste water and some rivers [33,34]. As a result of α-diversity analysis in ricefish chronically exposed to the selected concentration of antibiotics, it was confirmed Chao1 index reduced significantly in ampicillin treatment

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group than control group, while Shannon index reduced significantly in erythromycin treatment group (table 1). In addition, when comparing it with control group, it was confirmed average OTUs reduced in both ampicillin and erythromycin treatment group (Figure 1), and the same trend was also observed in rarefaction curve (Figure 2). As a result of β-diversity analysis, it was confirmed the cluster distance was remote in both ampicillin and erythromycin treatment group than control group (Figure 3). These results indicate the chronic exposure to antibiotics causes the change of gut microbiome of ricefish, reducing the diversity, and breaks the balance of gut microbial community which used to be maintained.

Putting together the results of relative abundance analysis of gut microbiota, in richfish, where *Proteobacteria* and *Fusobacteria* were dominant, both *Proteobacteria* became more dominant when chronic exposure to erythromycin and ampicillin, whereas most other phyla including *Fusobacteria* were decreased. (Figure 4). It is known that the gut microbiota of a healthy host remains stable over time, allowing for symbiotic interactions with the host, such as maintaining gut homeostasis and developing the immune system, whereas in dysbiosis, in which the *Proteobactria* dominate, it can lead to intestinal inflammation [35]. Although altered microbial diversity tends to partially recover over time, it is also known that opportunistic pathogens can significantly increase under certain environmental conditions [36]. In addition, *Proteobactria* increased by chronic exposure to antibiotics is a phylum containing various opportunistic pathogens and pathogens, including *Vibrio parahaemolyticus* and *Aeromonas veronii*, which we have focused on, and it is generally known that there are many cases of antibiotic resistance in the environment [37,38]. Therefore, if *Proteobactria* survive through resistance and become dominant in the host's guts chronically exposed to antibiotics, it can cause diseases by being able to invade inside of host through increased intestinal permeability without barrier action through competition with other bacteria [39]. From this point of view, it was confirmed that the proportion of *Cetobacterium somerae*, a non-pathogenic bacterium which used to be dominant in essential flora of both erythromycin and ampicillin chronically exposed ricefish, decreased, and on the contrary, the proportion of *Aeromonas veronii*, an opportunistic infectious bacterium, increased significantly (Figure 4). In addition, in the gut flora of ricefish chronically exposed to ampicillin, the pathogen *Vibrio parahaemolyticus* was also increased (Figure 4). It is known that *Aeromonas veronii* and *Vibrio parahaemolyticus* can kill the host by causing disease in the infected fish [40,41], and it is estimated to cause the disease through infection because of intestinal permeability of host when the opportunistic infectious bacterium and the pathogen become the dominant species in the intestine.

To confirm this, gene ontology analysis was performed based on RNA-sequencing in ricefish, which had dysbiosis in the intestinal microbiome due to exposure to ampicillin and erythromycin. As a result, it was confirmed that the expression of various stress and immune-related genes was changed in both ricefish exposed to ampicillin and erythromycin (Figure 5). Genes with increased expression in both ampicillin and erythromycin chronically exposed ricefish were identified as cellular responses to TNF, IL-1, and IFN-γ, lymphocyte and monocyte chemotaxis (BP), and chemokine receptor binding (MF) (Figure 5). Cytokine is a major regulator of the immune system and is known to induce inflammatory signals that regulate the ability of macrophages to destroy pathogens that are infected with hosts [42]. Among them, IL-1 is known to be involved in the regulation of acute and chronic inflammation by microbial infection [43], and IFN-γ is produced by activated T cells and NK cells, which play a role in immune enhancement and regulation [44]. In addition, TNF is known to play various roles related to inflammation, apoptosis, and stimulation of the immune system [45]. Therefore, the increase in the expression of cellular responses to these cytokines in the fish host by chronic exposure to antibiotics supports our hypothesis that an inflammatory response is induced by infection with specific bacteria in a host with dysbiosis. In addition, because it was confirmed that the expression of genes related to the chemotaxis of lymphocytes, which are immune cells, and monocytes, which are phagocytes, and genes related to chemokine receptor binding also

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increase in common, confirming overall immune system is activated if dysbiosis occurs due to chronic antibiotics exposure. The expression of other stress and immune-related genes showed different patterns in ricefish exposed to ampicillin and erythromycin (Figure 5), confirming that different types of inflammatory reactions were induced in ricefish depending on the type of antibiotic. Specifically, it is observed a significant increase in the expression of more stress and immune-related genes compared to ampicillin in ricefish exposed to erythromycin, and in particular, it was confirmed that the expression of MHC class II complex increased strongly (Figure 5). MHC class II is a cell surface glycoprotein that plays a central role in the immune system through exogenous pathways by presenting peptides to antigen receptors in $CD4^+$ T cells [46]. The function of MHC class II in bony fishes and mammals is similar, and the expression of MHC class II is known to be upregulated after immune stimulation [47]. Therefore, an increase in MHC class II expression also implies an increase in infection with the host of extracellular pathogens. Additionally, the expression of genes related to the response to oxidative stress was also increased in ricefish chronically exposed to erythromycin. It has been known that the production of reactive oxygen species (ROS) is induced by infection with viruses, bacteria, and parasites [48], and the increase in the expression of the host response to oxidative stress induced by ROS can predict the increase in infection in the host as well.

This study confirmed the result that checks microbiome's diversity (OTUs, α-diversity, β-diversity) and the relative abundance in intestine, and the ampicillin treatment group was found to have bigger influence on the intestinal microbiome of fish than the erythromycin treatment group. However, gene ontology result showed the erythromycin treatment group has stronger immune response than ampicillin treatment group. These results are expected because the pattern of dysbiosis in the intestinal microflora is different depending on the type of antibiotic due to chronic exposure. Therefore, we confirmed that the influence on the host can be different depending which flora becomes the dominant species and competing species that is being reduced. In addition, we could additionally confirm that the expression of various genes related to metabolism, reproduction, DNA replication, RNA transcription, mitosis, hormones, etc. was also changed in ricefish, where dysbiosis occurred due to chronic exposure to antibiotics (Figure S1), which indicates that imbalance in the gut microbiome has a variety of negative effects on host homeostasis and health.

Ricefish used as host in this study, is an environment-captured entity, and if the effects of antibiotic chronic exposure are reproduced in the host of the actual environment, it can cause enormous loss of fishery and aquaculture due to disease induction, and additional side effects are expected to occur from humans consuming fish. Further studies can visually confirm the inflammatory response in the intestines of fish chronically exposed to antibiotic, the change in host sensitivity through pathogen infection in fish with dysbiosis by antibiotic, and verify the expression of immune related factors from fish chronically exposed to antibiotic and it is considered to be possible to establish more diverse pathological effects of intestinal microbiological imbalance on the host caused by chronic exposure to antibiotics.

In this study, we suggested the fact that the antibiotic chronic exposure to the low concentration remained in aqueous environment can not only induce dysbiosis of intestinal microbiome in the host and cause the sensitivity change on the disease but also have the negative effects on host homeostasis and entire health. Therefore, low concentrations of antibiotics remaining in the environment have various negative effects not only on bacteria but also on the host, providing scientific evidence for the importance of controlling the release of antibiotics. Furthermore, the interpretation of our findings can't be definitive because the interactions between the gut microbiota and the host are very complex, but our study provides additional insight into the host-microbiome interactions.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Gene ontology based on RNA-sequencing. (A) biological process

(B) cellular component (C) molecular function. The size and color of the dots indicate the numbers and expression level of DEGs whose expression is changed in each treatment group, respectively.

Author Contributions: Conceptualization, K.H.M.; investigation, S.H., J.H.Y., and J.B.Y.; data curation, S.H., J.B.Y.; writing—original draft preparation, S.H., J.H.Y., and J.B.Y.; writing—review and editing, S.L., W.Y.B., K.H.M.; visualization, S.H., J.B.Y.; supervision, K.H.M.; funding acquisition, W.Y.B., K.H.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal studies were performed in compliance with the ethical guidelines for animal studies and approved by the Korea Maritime & Ocean University Institutional Animal Care and the Use Committee (KMOU-IACUC). Approval Code: KMOU IACUC 2021-02. Approval Date: 18 March 2021.

Informed Consent Statement: Not applicable.

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