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Microbial community analysis of an eco-friendly recirculating aquaculture system for olive flounder (*Paralichthys olivaceus*) using complex microbial probiotics

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복합미생물 프로바이오틱을 이용한 환경친화적 넙치 순환여과양식시스템에서의 미생물군집 분석

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This study was conducted to evaluate effects of dietary microbial probiotics on the growth and disease resistance of olive flounder (*Paralichthys olivaceus*) in a recirculating aquaculture system (RAS), and the effects of the probiotic bioaugmentation on the microbial community structure and water quality. For the analysis, 80 juvenile fish (average weight, 25.7 ± 7.6 g; average length, 15.2 ± 1.7 cm) were fed a basal diet containing a commercial microbial product CES-AQ1 (CES; 1×10^9 CFU/kg diet) in an RAS for 8 weeks. Weight gain, the specific growth rate, feed efficiency, and protein efficiency ratio of the fish fed the CES diet in the RAS were 1.5~2.5 times higher than those of fish fed the basal diet alone, or the basal diet containing oxytetracycline (OTC), yeast plus bacterium, or *Bacillus subtilis* in a still water system. There was no significant difference in the pathogen challenge test between fish fed the OTC diet and fish fed the CES diet in the RAS, suggesting the CES-AQ1 probiotic used in the RAS as a potential replacement for antibiotics. The RAS biofilter maintained the highest microbial diversity and appeared to harbor microbial communities with ammonium oxidation, denitrification, and fish pathogen suppression functions. Ammonia, which is hazardous to fish, was significantly decreased to < 0.5 mg/L in 19 days, indicating the effectiveness of probiotic supplementation to maintain good water quality in RAS. These results suggest that the intestinal microbial communities of fish are stabilized by a probiotic-containing diet (CES) and that bioaugmentation with probiotics may be an eco-friendly and economical supplement for aquaculture of olive flounder, promoting both good water quality and fish health in an RAS.

Keywords: disease resistance, microbial community, microbial consortium, olive flounder, probiotics, recirculating aquaculture system (RAS)

Because conventional catches have reached their maximum capacity, the current demand for good seafood is high, and the

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consumption trend is rising, the aquaculture industry has become one of the fastest growing animal production sectors in the world (Tacon and Metian, 2015; FAO, 2016; Awad and Awaad, 2017). The high demand for high-quality seafood in a short time period is a challenge for the global aquaculture industry. Most large-scale aquaculture systems use excessive feed and antibiotics to increase fish yield and control disease outbreaks, which can significantly degrade water quality (Lin *et al.*, 2017). Therefore, on many such farms, more frequent water exchanges are necessary, and contaminated water is continuously released without proper treatment (Cho *et al*., 2013). Thus, intensive aquaculture systems can cause environmental problems and disease outbreaks (Ramirez and Romero, 2017).

In most East Asian countries, including Korea, Japan, and China, olive flounder (*Paralichthys olivaceus*) is one of the most important aquaculture species in terms of economy and food security (Cho *et al*., 2013). In Korea, olive flounder production in 2017 was 41,207 tons (KOSTAT, 2018), which is the highest production for all species produced by marine aquaculture. Large-scale aquaculture has increased the production rate in a short period of time by increasing the density of the fish and over feeding, resulting in a variety of diseases due to stress and poor water quality (FAO, 2016). Antibiotics, which are effective for disease control, have been used extensively in aquaculture to reduce mortality, despite their adverse effects on the fish. The use of antibiotics in aquaculture has been shown to promote the emergence of fish pathogens and bacteria carrying antibiotic resistance genes (Lin *et al.*, 2017). Furthermore, plasmids carrying antibiotic resistance genes can be transferred to bacteria of different genera that could threaten fish health (Jang *et al.*, 2018).

Excessive feeding in high-density culture systems quickly reduces water quality, requiring more frequent water exchanges and consuming large amounts of water (Chambel *et al*., 2015; Banerjee and Ray, 2017). Untreated aquaculture wastewater carrying feed, fish meal, feces, and antibiotic residues is released into the environment, and its impact on the ecosystem could be significant. Then, polluted water from the environment is likely to be reintroduced into most typical flow-through aquaculture systems, in a vicious cycle (Jang *et al*., 2018). As the demand for eco-friendly farming has increased, so has the use of probiotics to control pathogens as a sustainable alternative to antibiotics (Gatesoupe, 1999; Balcázar *et al.*, 2006). Previous studies showed that the use of probiotics increases survival rates and improves the immune system of fish (Goncalves *et al.*, 2017; Srisapoome and Areechon, 2017). Fish fed diets with probiotics showed better nutritional status and energy use than fish fed a control diet (Zhou *et al.*, 2009; Yamashita *et al*., 2017). In addition, the recirculating aquaculture system (RAS) is an alternative system that should enable sustainable global seafood production (Midilli *et al*., 2012). In recent years, a variety of aquatic species, including marine species, have been cultivated by using small and large RAS (Buřič *et al*., 2016).

In this study, an RAS was used to test the possible application of probiotics for olive flounder culture to achieve higher diet efficiency and maintain good water quality. The microbial community was analyzed to understand the effects of dietary probiotics on growth and disease resistance in olive flounder and to address roles of probiotic bioaugmentation in an eco-friendly RAS.

Materials and Methods

Consortium culture

A commercial microbial consortium culture obtained from Bayo, Inc. (Jinju, South Korea) (CES-AQ1; KCTC13483BP, Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea) was used for aquaculture. The microbial community structure of the consortium was analyzed by pyrosequencing of the 16S rRNA genes in the total DNA. Based on the NCBI BLAST analysis (National Center for Biotechnology Information, NIH) and a search of the EzbioCloud database (ChunLab, Inc.), the following bacteria were dominant in CES-AQ1: *Gluconacetobacter* (25.3%), *Frateuria* (24.7%), *Dyella* (12.7%), *Asaia* (11.1%), *Novosphingobium* (3.0%), *Acidisoma* (4.1%), *Acetobacter* (1.9%), *Massilia* (1.3%), and *Sphingomonas* (0.6%). The experimental culture was usually obtained as a fresh product in a bulk container (20 L) from Bayo, Inc.

Preparation of experimental diets

A commercial feed (Flatfish No. 7S; National Federation of Fisheries Cooperatives Feed, South Korea) was used as a basal

diet and to make the experimental diets. Basal diet without probiotic supplementation was used as the negative control (CON). Basal diet mixed with oxytetracycline (OTC), which was called the OTC diet (5 g/kg diet), was also included. Three other diets were formulated, each of which contained one the following three cultures at 1×10^9 CFU/kg of basal diet: *Bacillus subtilis* (BS, positive control; Lee *et al*., 2017), the commercial microbial product CES-AQ1 (CES), and a mixture of the yeast *Groenewaldozyma salmanticensis* and the bacterium *Gluconacetobacter liquefaciens* (PI; pure isolates), discovered in this study. The diets were prepared and stored as previously described (Kim *et al.*, 2016). To make diets with the same volume, 5 g of cellulose was added to all diets except for the OTC diet. After thoroughly mixing the dry ingredients with fish oil and 30% filtered tap water, experimental diets were pelleted with a laboratory pelleting machine using a 2-mm diameter module (Baokyong Commercial) without heating and air dried at room temperature.

Construction and operation of the recirculating aqua-culture system (RAS)

The RAS consisted of a cylindrical fish culture tank (∅ 1,500 mm × H 1,000 mm), a water supply tank (1,500 L), and a filtration system containing basalt medium inoculated with the microbial consortium (CES-AQ1; Fig. 1). The recirculating system was housed inside the aquarium room, which was maintained at 20~25°C. In the filtration system, a 20-cm thick layer of the basalt medium was used as substratum for the growth of the microbial biofilm carrying indigenous microorganisms as well as CES-AQ1. The medium was porous in nature, with a large surface area, which facilitated microbial growth. Circulation was continuous, and the flow rate was maintained at 26 L/min. The water temperature in the growth tank and filter bed were maintained at ~21 ± 2°C with an automatic temperature controller and heating system. Dissolved oxygen (DO; 7–9 mg/L) was maintained with an aerator. Residual fish feed was filtered out through the biofilter system and removed every 3 days. Water loss was monitored during operation, and 10% of the water in the growth tank was replaced with fresh water every week, and all the water was changed once 30 days after starting the experiment. The growth tank and water storage tank were inoculated daily with the CES-AQ1 microbial consortium (0.02% v/v).

Feeding trial and monitoring of the RAS

The feeding trial was carried out in an aquarium building at Korea Maritime and Ocean University (Busan, South Korea). Olive flounder were obtained from Uljin Aquaculture (South Korea). For the experiment, 80 juvenile olive flounder (initial average weight and length, 25.7 ± 7.6 g and 15.2 ± 1.7 cm, respectively) were introduced into the RAS tank (working volume, 500 L). All fish were fed the basal diet during a 1-week acclimation period. Prior to the start of the feeding trial, all fish were assessed and fasted for 24 h. Fish were fed the CES diet (CES-AQ1) once a day for 8 weeks at a rate of 2.0% of wet body weight per day. The total fish weight in the growth tank was measured after 4 weeks, and the feeding rate was adjusted accordingly.

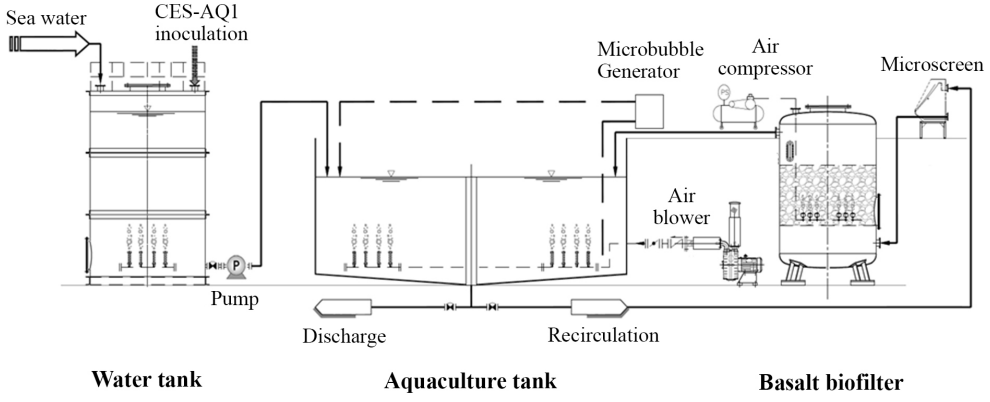

Fig. 1. Schematic of the recirculating aquaculture system (RAS) used in this study.

The environmental conditions in the RAS, including the temperature, pH, DO, and oxidation/reduction potential (ORP), were monitored throughout the experiment. Temperature and pH were measured with a pH and temperature sensor (YSI550) and DO was measured with a DO meter (YSI63; both from YSI, Inc.). ORP was measured with an ORP meter (Model Orion Star™ A211; ThermoFisher Scientific Korea). Chemical parameters, including COD, total nitrogen (T-N), ammonia (NH_3-N), Nitrate (NO_3^--N), and total phosphorus (T-P), were also measured daily using a comprehensive water quality analyzer (HS-3300), preprocessor (HS-R200), and corresponding measurement kits [HS-COD(Mn)-L, HS-TN-L, HS-NH_3(N)-L, HS-NO_3(N)-L, and HS-TP-L, respectively; all from Humas, Ltd.).

Fish sample collection and growth analysis

At the end of the feeding trial, the total number and weight of the fish in the RAS tank were measured to calculate weight gain (WG), feed efficiency (FE), the specific growth rate (SGR), protein efficiency ratio (PER), and survival rate. In addition, three fish were randomly selected, and their hepatosomatic index (HSI, %), viscerosomatic index (VSI, %), and condition factor (CF) were determined. These parameters were calculated by using the following equations: weight gain (WG, %) = (final weight - initial weight) × 100/initial weight; specific growth rate (SGR, %/day) = ($\log_e$ final weight - $\log_e$ initial weight) × 100/days; feed efficiency (FE, %) = (wet weight gain/dry feed intake) × 100; protein efficiency ratio (PER) = (wet weight gain/protein intake); survival rate (SR, %) = (total no. of fish - no. of dead fish)/total no. of fish × 100; hepatosomatic index (HSI, %) = liver weight × 100/body weight; and viscerosomatic index (VSI, %) = (viscera weight/body weight) × 100; condition factor (CF) = (fish weight/fish length^3) × 100.

Challenge test

At the end of the growth experiment in the RAS or still water tank, fish were redistributed in an aquarium for the challenge test. The aquarium tank (100 L; 685 × 485 × 415 mm) was equipped with an aerator and a thermostat and filled with 70 L of seawater, which was maintained at 19 ± 0.5°C under a 12-h light/dark period during the experiment. The fish pathogen *Edwardsiella tarda* (ATCC 15947), was obtained from the Department of Biotechnology, Pukyong National University (Busan, Republic of Korea). The bacterial strain originated from a diseased brook flounder. *E. tarda* was grown in tryptic soy broth (TSB; Merck KGaA) at 26°C for 24 h. Ten fish per aquarium were injected intraperitoneally with 0.1 ml (2×10^7 CFU/ml) of *E. tarda*. The mortality rate of the fish in each aquarium was recorded for 15 days.

DNA extraction from fish guts, water, and biofilter media, and microbial community analysis by pyrosequencing

After termination of the experiment, the guts of three randomly selected fish from the RAS tank were extracted under aseptic conditions and stored at -80°C until extraction, and samples of the water and biofilter media were collected and stored at -20°C. Genomic DNA was extracted from three replicates of each sample using the MO BIO PowerSoil® DNA Isolation Kit (Mo Bio Laboratories). DNA isolation and purification were performed according to the manufacturer's protocol. The DNA from the three replicates of each sample was pooled, and the pooled DNA was used for the microbial community analysis. All DNA samples were stored at -20°C. For microbial community analysis based on pyrosequencing, the bacterial 16S rRNA gene was first amplified from the isolated genomic DNA. Then, library construction, sequencing, and sequencing analyses were performed using a 454 GS FLX Junior Sequencing System (Roche), according to the manufacturer's protocols. Statistical analyses of the microbial communities were performed with Mothur, using a 3% difference cut-off value (Schloss *et al*., 2009). To compare the microbial communities, α-diversity and β-diversity indices were analyzed using BioPLUG (ChunLab).

Results and Discussion

Physical and chemical analysis of the water quality in the recirculating aquaculture system

Water quality was monitored by measuring physical and chemical parameters (Fig. 2). The temperature in the aquaculture room was maintained at 19°C. However, the temperature decreased to 15°C for several days during a temperature control failure in the winter. The pH was maintained at 6.6~7.7, but it could have been affected by the regular addition of feed and the CES-AQ1 consortium. Dissolved oxygen gradually decreased

for the first 30 days, from 9.31 mg/L to 8.5 mg/L, and then further decreased to 7.4 mg/L at the end of the experiment. The ORP was maintained between 136 mV and 183 mV. The highest ORP was observed in 31 days, after the total water change occurred and all chemical pollutants from fish discharge and leftover feed were removed.

The lowest COD (1.05 mg/L) was observed at the beginning of the experiment, while the highest COD (4.13 mg/L) was observed at 19 days (Fig. 2). COD levels were maintained at 3~4 mg/L throughout most of the experimental period. The highest concentration of T-N (2.97 mg/L) was observed in 16 days and was maintained at 1~3 mg/L. The concentrations of NO_3^--N, NH_3-N, and T-P were 1~3 mg/L during the first 13 days of the experiment, and then significantly decreased to less than 0.5 mg/L at 19 days, during which all other water quality parameters were stably maintained. In fact, the average ratios of COD/T-N, NH_3/T-N, and NO_3^-/T-N on days 19~64 were 2.26, 0.09, and 0.67, respectively. A previous report (Fernandes *et al*., 2017) showed that ammonia and nitrite were maintained at concentrations below 0.20 mg/L, while nitrate levels ranged between 40 and 44 mg/L under constant feed loading (1 kg feed/m^3 of make-up water). The average ratio of COD:N during the last 4 weeks of the RAS experiment was 2.54:1, which is less than the previous reported optimal value (5:1) for efficient nitrogen removal (Pungrasmi *et al*., 2013). Therefore, nitrogen removal through denitrification in the RAS could be more effective at a COD:N of 5:1.

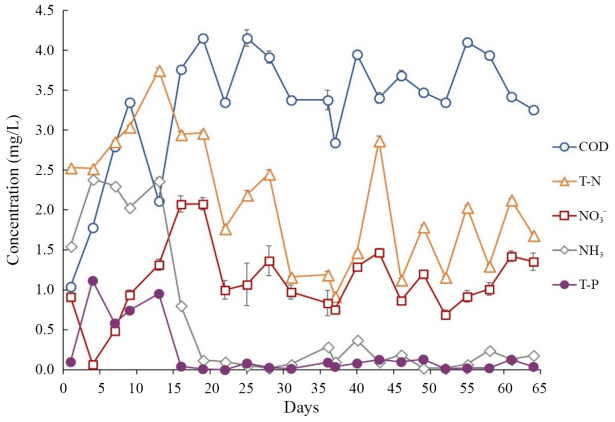

Fig. 2. Chemical analysis of water quality in terms of chemical oxygen demand (COD), total nitrogen (T-N), ammonia (NH_3-N), Nitrate (NO_3^--N), and total phosphorus (T-P) in the RAS.

Growth performance of olive flounder fed the experimental diets

The growth performance and FE of olive flounder fed different experimental diets were shown in Table 1. At the end of the 8-week feeding trial, WG, the SGR, FE, and PER of fish fed the OTC and PI diets (in the still water system) were higher than

Table 1. Growth performance of juvenile olive flounder fed experimental diets and grown in a recirculating aquaculture system (RAS) or still water tanks for 8 weeks

Growth parameters	CON*	OTC*	PI*	CES** (RAS)
WG (%)[a]	62.5 ± 8.11	80.3 ± 2.32	81.6 ± 8.09	140
SGR (%/day)[b]	0.87 ± 0.09	1.05 ± 0.02	1.06 ± 0.06	1.56
FE (%)[c]	42.3 ± 5.30	51.9 ± 0.25	52.8 ± 3.42	130.9
PER[d]	0.77 ± 0.10	0.98 ± 0.01	0.97 ± 0.06	2.40
SR (%)[e]	100 ± 0.00	100 ± 0.00	97.5 ± 3.54	100
HSI (%)[f]	1.55 ± 0.09	1.61 ± 0.18	1.54 ± 0.16	0.99
VSI (%)[g]	3.04 ± 0.32	3.05 ± 0.19	2.98 ± 0.37	1.88
CF[h]	0.95 ± 0.07	0.93 ± 0.09	0.95 ± 0.08	0.83

* Cultured in still water tanks (70 L) in triplicate
** Cultured in a RAS (500 L) without replicate
[a] Weight gain (WG, %) = (final weight - initial weight) × 100/initial weight
[b] Specific growth rate (SGR, %/day) = ($\log_e$ final wt. - $\log_e$ initial wt.) × 100/days
[c] Feed efficiency (FE, %) = (wet weight gain/dry feed intake) × 100
[d] Protein efficiency ratio (PER) = (wet weight gain/protein intake)
[e] Survival rate (SR, %) = (total no. of fish - no. of dead fish)/total no. of fish × 100
[f] Hepatosomatic index (HSI, %) = liver weight × 100/body weight.
[g] Viscerosomatic index (VSI, %) = (viscera weight/body weight) × 100
[h] Condition factor (CF) = (fish weight/fish length^3) × 100

those of fish fed the CON diet (in the still water system). All these parameters were much higher in fish fed the CES diet (in the RAS) than in fish fed the CON, OTC, and PI diets. In fact, the WG, SGR, FE, and PER of the fish in the RAS were 140%, 1.56%, 130.9%, and 2.4, respectively, which were 1.5~2.5 times higher than the corresponding values for fish fed the other diets. Improved growth through dietary supplementation with *B. subtilis* has been reported for other aquatic biota, including starry flounder (*Platichthys stellatus*) (Park *et al.,* 2016), sea cucumber (*Apostichopus japonicus*) (Zhao *et al*., 2012), and Japanese eel (*Anguilla japonica*) (Lee *et al*., 2017). Moreover, there were no significant differences in growth performance between fish fed the CES and OTC diets. This study demonstrated that the dietary administration of CES exerted a beneficial effect on growth performance that was equivalent to that of *B. subtilis* or antibiotics.

Challenge test

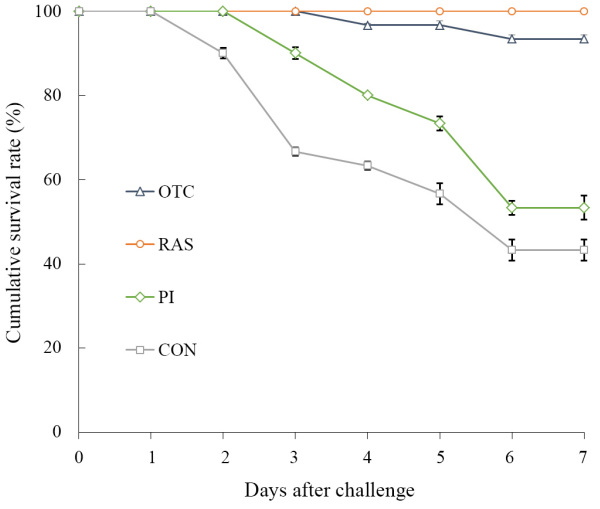

Fig. 3. Cumulative survival of juvenile olive flounder after challenge with *Edwardsiella tarda* for 7 days. The fish were fed experimental diets and grown in either still water tanks (CON, OTC, and PI) or in a RAS and fed the CES diet (RAS).

Edwardsiella tarda, a Gram-negative bacterium of the Enterobacteriaceae, is the causative agent of edwardsiellosis that leads to extensive losses in olive flounder (Kwon *et al*., 2007). The cumulative survival rate in olive flounder challenged with *E. tarda* for 7 days is shown in Fig. 3. During the challenge test, the first death occurred on the first day post-injection. The survival rate of the fish fed the OTC diet and challenged with *E. tarda* was significantly higher than the survival rate of fish fed the CON, BS, and CES diets. In contrast, there was no significant difference among fish fed the PI and OTC diets for up to 7 days (Fig. 3).

Aly *et al.* (2008) reported that dietary administration of a mixture of probiotics showed higher protection against different pathogens than a control diet. This suggests that the PI diet could be a possible replacement for antibiotics.

Pyrosequencing analysis of the microbial community structures in the fish gut, water, and biofilter in the RAS

The α-diversity indices (ACE, Chao1, NPShannon, and Shannon) showed the highest diversity in the biofilter and the lowest diversity in the gut of fish fed the BS diet and grown in the still water system (Table 2). The overall microbial α-diversity indices of the samples from the RAS were several times higher than those of samples from the still water systems, indicating that the growth environment of the fish could significantly affect the microbial diversity in the RAS as well as the fish gut.

The most dominant phyla in the gut of the fish in the RAS were Proteobacteria (77.3%), Bacteroidetes (9.6%), Firmicutes (4.2%), and Verrucomicrobia (2.7%; Fig. 4A). Proteobacteria, Bacteroidetes, and Verrucomicrobia were also the dominant phyla in the water and biofilter. Planctomycetes (4.6%) was uniquely dominant in the water. Firmicutes (4.2%) and Streptophyta (2.1%) were highly dominant in the fish gut.

Relatively higher diversity in the community structures at

Table 2. Diversity indices of the microbial communities in samples from the water and biofilter of the RAS and the guts of fish grown in the RAS based on pyrosequencing analysis[a]

Sample name	Target reads	OTUs	ACE	Chao1	NPShannon	Shannon	Simpson
Gut	63,631	631	646	644	3.1	3.1	0.21
Water	62,887	1,678	1,716	1,687	3.7	3.7	0.14
Biofilter	51,172	2,166	2,218	2,177	5.8	5.8	0.009

[a] Indices were obtained from Mothur (Schloss *et al*., 2009) using normalized reads from each sample.

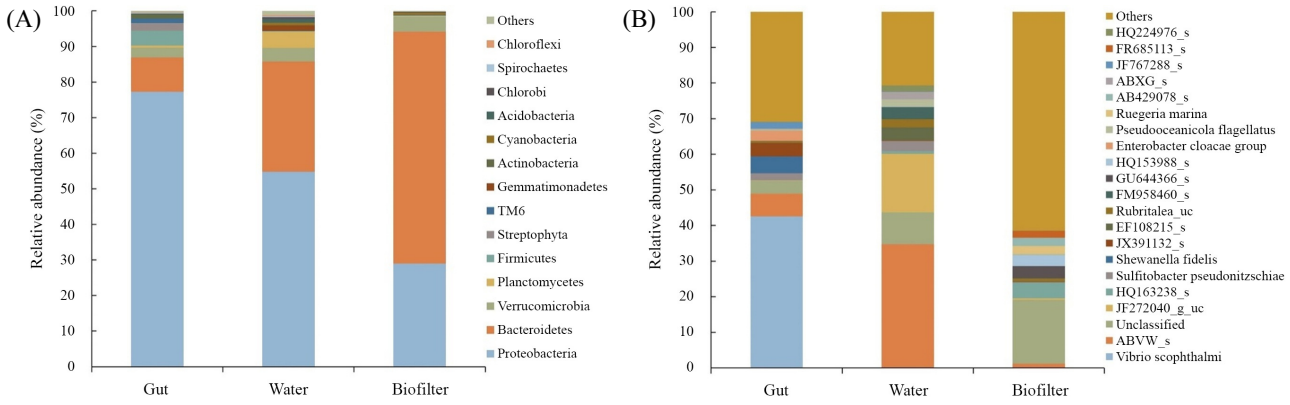

Fig. 4. Microbial community structures in the gut of the fish, water, and biofilter in the RAS at the (A) phylum and (B) species levels.

the species level was observed in the biofilter (Fig. 4B), and unclassified (18.0%), HQ163238_s (*Emcibacter* sp., 4.4%), GU644366_s (Flavobacteriaceae, 3.4%), HQ153988_s (Nannocystaceae, 3.0%), and *Ruegeria marina* (2.3%) were the dominant species. *Nitrosomonas* sp. (2.0%), an ammonia oxidizer group, was also a relatively dominant genus in the biofilter. However, this genus was not observed in the water or fish gut. The detected *Nitrosomonas* sp. included *Nitrosomonas aestuarii* (1.1%), *Nitrosomonas*_uc (0.76%), *Nitrosomonas cryotolerans* (0.01%), *Nitrosomonas marina* (0.01%), and *Nitrosomonas europaea* (0.01%). Moreover, denitrifiers, such as *Nisaea denitrificans* (0.004%) and *Paracoccus denitrificans* (0.004%), were also observed in the biofilter. Therefore, the ammonium in the RAS appeared to be oxidized by these genera and removed through nitrification and denitrification. *Nitrosomonas aestuarii* and *Nitrosomonas marina* have also been shown to be dominant in the flood/drain biofilters used to treat aquaculture wastewater (Gregory *et al*., 2012). The denitrifiers *Nisaea denitrificans* and *Paracoccus denitrificans* were also identified in the fluidized sand biofilters used to remove nitrogen aquaculture effluent in a previous study (Tsukuda *et al*., 2015). In this study, Planctomycetaceae (1.63%) was also one of the dominant families in the biofilter, indicating potential ammonium removal through anaerobic ammonium oxidation. They are mostly found in the free oxygen (O_2)-depleted area of the biofilter, where they directly oxidize ammonia into nitrogen gas (Mulder *et al*., 1995; Rurangwa and Verdegem, 2015). Most members of the Nannocystaceae are considered to be halotolerant and halophilic microbes capable of degrading complex macromolecules and lysing other microbes (Garcia and Müller, 2014). ABVW_s (Flavobacteriaceae, 34.7%), JF272040_g_uc (16.4%), and *Sulfitobacter pseudonitzschiae* (2.8%) were the dominant species in the water. Flavobacteriaceae is a family that includes many utilizers of macromolecules, such as polysaccharides and proteins (McBride, 2014). *Vibrio scophthalmi* (42.5%), *Shewanella fidelis* (4.7%), and *Enterobacter cloacae* group (2.9%) were dominant in the fish gut. *V. scophthalmi* is one of the most abundant marine aerobic or facultative anaerobic species present in the intestinal tract of turbot (*Scophthalmus maximus*) (Cerda-Cuellar *et al*., 1997, 2004). *V. scophthalmi* was originally isolated from the intestines of juvenile turbot as a non-pathogen (Cerda-Cuellar *et al*., 1997). However, this species was recently shown to be pathogenic in olive flounder (Qiao *et al*., 2012). *Enterobacter cloacae* is a member of the normal gut flora of humans and is not a major human pathogen but has is considered to be an important cause of nosocomial infections and may be a pathogen for some fish (Keller *et al*., 1998; Sekar *et al*., 2008; Mezzatesta *et al*., 2012). Fortunately, this species was not found in the water or biofilter system, indicating that this potential fish pathogen cannot survive in the system, except in the fish gut at a relatively low population density. In a previous study (Rud *et al*., 2017), a higher abundance of the potential pathogens was also observed in the water when compared to its abundance in the biofilms in both an RAS and semi-closed containment system (S-CCS). These results may indicate that the biofilm in the RAS could prevent the growth of pathogens.

A Unifrac UPGMA clustering analysis of the microbial community structures in fish fed the various diets, as well as the fish, water, and biofilter in the RAS is shown in Fig. 5. All

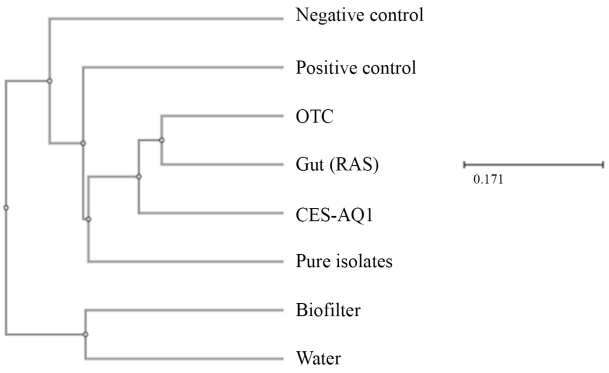

Fig. 5. Unifrac UPGMA clustering analysis of the microbial community structures in the guts of fish fed various experiment diets (OTC, Pure isolates, and CES-AQ1) and the guts of fish, as well as the water and biofilter, in the RAS (RAS). The "negative control" is equivalent to fish fed the CON diet (in a still water tank). The "positive control" fish were fed the BS diet; "CES-AQ1" fish were fed the CES diet; "Pure isolates" fish were fed the PI diet; and "Gut (RAS)" fish were fed CES diet and reared in the RAS. "Biofilter" is a sample from the RAS biofilter, and "Water" is the water from the RAS growth tank.

microbial communities from the guts of fish fed the five different diets and the community in the gut of the fish in the RAS grouped together. However, the microbial communities from the water and biofilter of the RAS were grouped separately. This indicates that the two different microbial communities developed separately under the different environmental conditions in the fish gut and tank/biofilter in the RAS. In a previous report (Rud *et al*., 2017), the microbial community structures in water samples and biofilms were also clearly different. The more abundant phyla in the MBBR-biofilm in the RAS were Proteobacteria (Rhizobiales, Myxococcales, and Marinicellales; in order), Bacteroidetes (Cytophagales), and Planctomycetes, while Proteobacteria (Rhodobacteraceae) and Bacteroidetes (*Polaribacter* sp.) were more abundant in the water (Rud *et al*., 2017). Likewise, Rhizobiales (0.65%), Myxococcales (4.76%), and Planctomycetes (4.55%) were dominant in the biofilter, while *Polaribacter* sp. (2.27%) was dominant in water.

Conclusion

In this study, we evaluated the effects of dietary probiotics on growth and disease resistance in olive flounder (*Paralichthys olivaceus*) as well as the effect of probiotic bioaugmentation on water quality in an eco-friendly RAS during olive flounder aquaculture. WG, the SGR, FE, and the PER of fish fed the CES diet in the RAS were much higher than those of fish fed the CON, PI, and OTC diets in still water systems. Moreover, the 7-day challenge test showed there was no significant difference between fish fed with diets containing OTC, PI (in still water systems), or CES (in the RAS) in terms of cumulative survival rate, indicating that PI and CES could be potential antibiotic replacements for disease control in olive flounder. Moreover, an 8-week feeding trial showed that WG, SGR, FE, and PER were 1.5~2.5 times higher in the RAS than in fish fed probiotic treatment diets in the still water system. Probiotic treatment appeared to control the pathogen population within fish cultured in the RAS as well as in the still water system. The biofilter system of the RAS maintained higher microbial diversity, and harbored microbial communities with potential ammonium oxidation, denitrification, and fish pathogen suppression functions. In fact, the early toxic level of ammonia (2.4 mg/L) was reduced to less than 0.5 mg/L after 19 days of operation, and this level was maintained thereafter, indicating the effectiveness of probiotic bioaugmentation for maintaining an eco-friendly system during olive flounder aquaculture. We concluded that the addition of probiotic organisms (CES-AQ1) to the flounder fish diet and bioaugmentation of probiotic organisms to the RAS allow eco-friendly, economical aquaculture of olive flounder by maintaining good fish health and water quality.

적 요

본 연구는 순환여과양식시스템(RAS)에 있어서 복합프로바이오틱스의 적용이 넙치의 성장과 병저항성에 미치는 영향과 이 프로바이오틱스를 RAS에 생물증강처리 시 미생물군집구조 및 수질에 미치는 영향을 평가하고자 실시하였다. RAS 내에서 80미의 넙치치어(25.7 ± 7.6 g; 15.2 ± 1.7 cm)에 프로바이오틱스 CES-AQ1를 첨가하여 사료를 제조하여(CES 사료; 1 x 10^9 CFU/kg) 8주일 동안 급이하였다. 이 경우 넙치의 증체율, 비성장속도, 사료효율, 및 단백질 전환효율은 비유수식 양식시스템에 있어서 CON, PI 및 OTC 사료를 처리한 경우에 비해 1.5~2.5배 정도 높게 나타났다. 1주일간 병원균 저항성 시험에 있어서 비유수식에서 항생제함유 사료(OTC)를 급이한 경우와 RAS에서 CES 사료를 처리한 경우간에는 별 차이가 나타나지 않았다. 따라서 이 CES 프로바이오틱스를 RAS에서

넙치를 양식하는데 있어서 항생제 대용으로 활용할 수 있을 것으로 판단되었다. RAS의 생물여과막에서는 가장 높은 미생물다양성이 나타났으며 암모니아의 산화 및 탈질능을 가진 미생물이 관찰되었고, 병원미생물의 성장억제도 관찰되었다. 더구나 RAS 운전 19일 경과 시 암모니아가 0.5 mg/L이하의 농도로 감소하여 양호한 RAS 수질의 유지에 있어서 프로바이오틱스 처리가 효과가 있음이 밝혀졌다. 사료에 프로바이오틱스(CES-AQ1)를 첨가하여 넙치 장내 미생물이 안정화되고 또한 이 프로바이오틱스를 RAS 양식수에도 처리하여 RAS를 운전할 경우 건강한 넙치의 양식과 양호한 수질을 유지할 수 있어서 경제적이고 환경친화적인 넙치양식이 가능할 것으로 판단되었다.

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