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SHORT COMMUNICATION

Predominant gut microbiota in the early life stages of red seabream (*Pagrus major*) raised in indoor tanks

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Abstract This study was performed to assess, by molecular methods, the gut microbial community associated with early life stages of the reared red seabream (*Pagrus major*). This work sought to better understand the gut microbiota during seed production, which may help control disease in the near future. At the larval fish stage, Alphaproteobacteria, Flavobacteria, and Gammaproteobacteria (excluding Vibrionaceae) were predominant in fish guts. These bacteria also predominated in rotifers, the primary component of the larval fish diet. At the juvenile stage, Gammaproteobacteria, Alphaproteobacteria, and Flavobacteria (except for Flavobacteria in one of two juvenile libraries) were predominantly detected in fish guts. Again, these bacteria also predominated in *Artemia* nauplii, a main component of the juvenile fish diet. These results suggested that the gut microbiota of larval and juvenile red seabream is influenced primarily by the microbiota of their diets. On the other hand, members of the family Vibrionaceae were not detected in larval fish guts, the rearing seawater, or rotifers, whereas bacteria of this family represented 28.6–64.6% of the reads in two libraries of juvenile fish guts. These observations suggested that hygiene management and administration of probiotics at the early life stage of red seabream are effective in suppressing the onset of opportunistic infections.

Keywords Gut microbiota . Red seabream . Early life stage . Vibrionaceae . Clone library analysis

Introduction

Multiple literature reviews have suggested that the gut microbiota of fish plays an important role in nutrition, development, immunity, and resistance to invading pathogens (Gomez et al. 2008; Ray et al. 2012; Wang et al. 2018). At the same time, the normal gut microbiota of marine fish also includes opportunistic bacterial pathogens, particularly members of the family Vibrionaceae that have been shown to be causative agents of disease and mass mortality (Eddy and Jones 2002). Muroga et al. (1987) examined the intestinal microflora of reared red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegelii*) at the larval and juvenile stages using the agar plate method, and those authors found that bacteria of the genera *Vibrio* and *Pseudomonas* were the most dominant bacteria. Mizuki et al. (2006) examined the distribution of *Listonella anguillarum*, a representative opportunistic pathogen belonging to the family Vibrionaceae, in larval and juvenile Japanese flounder (*Paralichthys olivaceus*) raised in a seed production facility, and suggested that this bacterium was derived primarily from the fish's live diet, e.g., rotifers and *Artemia* nauplii. Together, these reports suggested that the gut microbiota of marine fish at early life stages is very important for understanding the opportunistic infections that occur in aquaculture facilities.

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On the other hand, we have reported that less than 1% of the total count of bacteria in the intestinal tract of many fish caught along the coast is culturable on agar plates (Sugita et al. 2005). This phenomenon may reflect the fact that the intestinal microbiota comprises unknown species that do not grow under general laboratory cultivation conditions (e.g., certain media compositions, levels of salinity, O_2 tension, and temperatures), and that cells may exist in a viable but non-culturable (VBNC) state. Tanaka et al. (2012), using clone library analysis, demonstrated that the intestinal microbiota of coastal fish comprised a diverse group of bacterial species belonging to the classes Actinobacteria, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria (excluding Vibrionaceae). These results strongly suggested that the intestinal microbiota of coastal fish comprises a highly diverse group of species, and as such, only a small portion of the intestinal microbiota can be cultivated under a given set of specific conditions.

The red seabream is an important marine cultured fish species in Japan, with a production of 62,400 t in 2020, accounting for 25.1% of the total cultured fish production (248,900 t) in Japan. However, although Muroga et al. (1987) reported the gut microbiota of larvae and juveniles of red seabream in a culture-dependent manner as described above, the intestinal microflora in this species has not yet (to our knowledge) been investigated in a culture-independent manner; such an approach would reveal the microbiota that actually predominate in the intestinal tract. The present study was performed to examine, using molecular techniques, the gut microbiota associated with early life stages of the raised red seabream, with the goal of better understanding the gut microbiota during seed production, which may help control disease in the near future.

Materials and methods

Fish and rearing condition

Fertilized eggs of the red seabream were placed in two 500-L fiber-reinforced plastic tanks (referred to as tanks A and B) in which the water temperature was maintained between 23.0 °C and 24.7 °C, and oxygen was adequately supplied by aeration. After hatching, the larvae were kept in the same tanks. At 18 days post-hatching (dph), the larvae were transferred to two 30-L polycarbonate tanks (referred to as tanks C and D). All tanks were filled with seawater, and half of the volume was replaced daily with fresh seawater (salinity, 33.6 to 34.5 psu) to maintain good water quality. Animals were fed daily at 2 to 26 dph with rotifers (*Brachionus plicatilis* sp. complex S-type) raised on an alga (*Nannochloropsis oculata*), followed by *Artemia* nauplii (at 26 to 38 dph) and NF2 feeds (Fuji Flour, Shizuoka, Japan; at 39 to 50 dph).

Larval specimens at both 17 and 13 dph (referred to as larvae A and B, respectively) and juvenile specimens at 38 and 40 dph (referred to as juveniles C and D, respectively) were collected from tanks A, B, C, and D, respectively, anesthetized with ice, and euthanized. Groups of 50 larvae (larvae A and B) were rinsed in sterile seawater filtered through a GS membrane filter (0.22-µm pore size, Millipore Sigma, Burlington, MA, USA) according to the method of Campbell and Buswell (1983) to remove the bacteria loosely attached to the external surfaces; the larvae then were homogenized with sterile glass mortars. The digestive tracts were removed aseptically from 25 juveniles from each of tanks C and D (juveniles C and D, respectively) under a stereomicroscope. Separately, rearing seawater (200 mL) from tanks A, B, C, and D (referred to as seawater A, B, C, and D, respectively) was collected and filtered through a GS membrane filter. The filter then was cut into small pieces and processed for DNA extraction. The rotifers for both larvae A and B (referred to as rotifers), and *Artemia* nauplii for juveniles C and D (referred to as *Artemia* C and D, respectively) also were rinsed with 100 mL of sterile seawater on sterile nets (80-µm opening) and homogenized with sterile glass mortars. The feeds were processed in the same manner.

Aliquots of the prepared specimens and samples were stained with 4′,6-diamidino-2-phenylindole, and the total counts of bacteria (cells/g or mL) were determined using an epifluorescence microscope (BX-50, Olympus, Tokyo, Japan; Porter and Feig 1980).

16S rDNA clone library analysis

Genomic DNA was extracted from the microbial cells in specimens and samples, and purified using the

Table 1 Total counts of bacteria in the seabream, seawater, and diets

Tank	Collection data (dah)	Specimen/sample	Total counts of bacteria
A	17	Larvae A	2.2×10^9 cells/g
		Seawater A	6.7×10^6 cells/ml
B	13	Larvae B	6.9×10^8 cells/g
		Seawater B	6.1×10^6 cells/ml
C	38	Juveniles C	1.3×10^9 cells/g
		Seawater C	1.1×10^7 cells/ml
D	40	Juveniles D	1.7×10^9 cells/g
		Seawater D	1.1×10^7 cells/ml
Diets		Rotifers	2.5×10^{10} cells/g
		Artemia nauplii C	4.0×10^8 cells/g
		Artemia nauplii D	6.2×10^8 cells/g
		Feeds	3.0×10^{10} cells/g

FastDNA SPIN Kit for Soil (Funakoshi, Tokyo, Japan) according to the manufacturer's instructions. The purified DNA then was stored at -20 °C until used in the experiments.

The partial DNA fragments of the bacterial 16S ribosomal RNA genes (16S rDNA) were amplified by polymerase chain reaction (PCR) using oligonucleotides 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525r (5′-AAAGGAGGTGATCCAGCC-3′; Hiraishi 1992) as the primer set. The resulting PCR products were subcloned into the TA site of the pGEM T-Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Each library was constructed from 50 clones. Both strands of the inserted DNAs were sequenced using an ABI PRISM 3100 Genetic Analyzer and a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster, CA, USA) with the 27f and 685r (5′-TCTACGCATTTCACCGCTAC-3′) primers. The final sequence (approximately 690 bp) was determined from overlapping sequence data using the AutoAssembler ver. 2.1 computer program (Applied Biosystems). Chimeric sequences were identified and removed using USEARCH ver. 6.1.544_i86 software (Edgar et al. 2011). The clones were identified using EZBioCloud (Yoon et al. 2017) on the basis of their 16S rDNA sequences. Representative sequences from this study have been deposited into the DDBJ/GenBank/EMBL databases under accession numbers LC581476 to LC581489.

PAST 4.0.3 software (Hammer et al. 2001) was used to calculate the Morisita index of community similarity for pairwise comparisons between different libraries. The similarity matrix then was subjected to cluster analysis by UPGMA (unweighted pair-group average) to develop a dendrogram.

Results

Microbiota of the seabream, rearing water, diets, and feeds

The total counts of bacteria in red seabream were relatively stable throughout the rearing period (6.9×10^8 – 2.2×10^9 cells/g), and the density was similar to that seen for marine fishes (Sugita et al. 2005) (Table 1). Total counts in the seawater, rotifers, *Artemia* nauplii, and feeds were 6.1×10^6 – 1.1×10^7 cells/mL, 2.5×10^{10} cells/g, 4.0×10^8 – 6.2×10^8 cells/g, and 3.0×10^{10} cells/g, respectively.

A total of 600 clones in 12 constructed libraries were sequenced to determine the corresponding bacterial identities. Thirty-three chimeric sequences were detected and excluded from further analysis. Among the remaining 567 sequences, 269 (47.4%) were identified to the species level (>97% similarity to the nearest type strain), and 103 (18.2%) were at least 90% similar to the nearest type strain, and therefore

Table 2 Composition of bacterial classes in clone libraries constructed from the red seabream, seawater and diets

Class	Number of clones in the library of:											
	Larvae A	Larvae B	Juveniles C	Juveniles D	Seawater A	Seawater B	Seawater C	Seawater D	Rotifers	*Artemia* C	*Artemia* D	Feeds	Total
Actinobacteria	2	2	4	0	0	0	8	0	3	0	0	7	26
Alphaproteobacteria	17	17	4	4	32	32	19	26	12	29	20	0	212
Bacilli	3	3	0	0	0	0	0	0	0	0	0	21	27
Bacteroidia	0	2	0	0	0	0	0	0	0	0	0	0	2
Betaproteobacteria	2	0	0	0	0	0	0	5	0	0	0	1	8
Chroobacteria	2	6	0	0	10	10	0	0	0	1	0	1	30
Cytophagia	0	1	0	0	0	1	0	6	0	0	0	0	8
Deltaproteobacteria	0	2	0	0	0	0	0	0	0	0	0	0	2
Flavobacteria	12	5	14	1	1	0	15	2	27	9	6	1	93
Gammaproteobacteria	10	7	23	42	0	0	1	2	4	8	16	19	132
Oligoflexia	1	1	1	1	0	4	0	0	0	1	4	0	13
Peribacteria	0	0	2	0	0	0	0	0	0	0	0	0	2
Planctomycetia	0	0	0	0	0	0	1	0	0	0	0	0	1
Saprospiria	0	0	1	0	0	0	0	2	0	0	1	0	4
Sphingobacteriia	0	0	0	0	0	0	0	0	0	1	0	0	1
Verrucomicrobiae	0	3	0	0	3	0	0	0	0	0	0	0	6
Total	49	49	49	48	46	47	44	43	46	49	47	50	567

were classified into the nearest genus. There were 195 sequences (34.4%) with <90% similarity that were identified to the class level.

Of the 16 classes detected, Alphaproteobacteria, Gammaproteobacteria, and Flavobacteria accounted for 37.4%, 23.3%, and 16.4% of all clones, respectively. The predominant sequences that were identified to the species level were *Photobacterium damselae* subsp. *piscicida* (accession no. X78105; 24 clones), *Marivita cryptomonadis* (EU512919; 19 clones), *Donghicola eburneus* (DQ667965; 16 clones), *Vibrio japonicus* (LC143378; 13 clones), *Marivita litorea* (EU512918; 12 clones), and *Tritonibacter mobilis* (AB255401; 10 clones).

The composition of the bacterial classes in the 12 libraries is shown in Table 2. The libraries of larvae A and B consisted primarily of Alphaproteobacteria, Flavobacteria, and Gammaproteobacteria (excluding Vibrionaceae). Clones related to Gammaproteobacteria (including Vibrionaceae) dominated the libraries of juveniles C and D. Libraries of seawater A, B, C, and D consisted primarily of Alphaproteobacteria. All diets, including rotifers, *Artemia* nauplii, and feeds, contained Flavobacteria and Gammaproteobacteria, while only rotifers and *Artemia* nauplii contained Alphaproteobacteria. The feed libraries consisted of Bacilli and Gammaproteobacteria. Morisita indices of similarity were calculated for the class distributions based on 16S rRNA gene sequence analysis. High similarities were observed for comparisons of libraries between larvae A and B (0.903), larvae A and seawater A (0.666), larvae B and seawater B (0.760), larvae A and rotifers (0.777), larvae B and rotifers (0.548), juveniles C and D (0.777), juveniles D and *Artemia* nauplii D (0.617), and juveniles D and feeds (0.597), as shown in Fig. 1. However, except for the comparisons of seawater A and B (0.752) and larvae A and B (0.500), a high similarity in the species distribution was rarely observed among the remaining libraries.

Members of Vibrionaceae in the seabream, rearing water, diets, and feeds

Table 3 shows the composition of species belonging to the family Vibrionaceae in the 12 libraries. Overall, 10 species of Vibrionaceae (32 clones) and 1 species of *Photobacterium* (23 clones) were detected. Of these, only two species, *Vibrio parahaemolyticus* and *Photobacterium damselae* subsp. *piscicida,* were known fish pathogens (Mohamad et al. 2019); sequences corresponding to these species were detected in the libraries of juveniles D, seawater D, and *Artemia* nauplii D. Interestingly, no Vibrionaceae were detected in the libraries

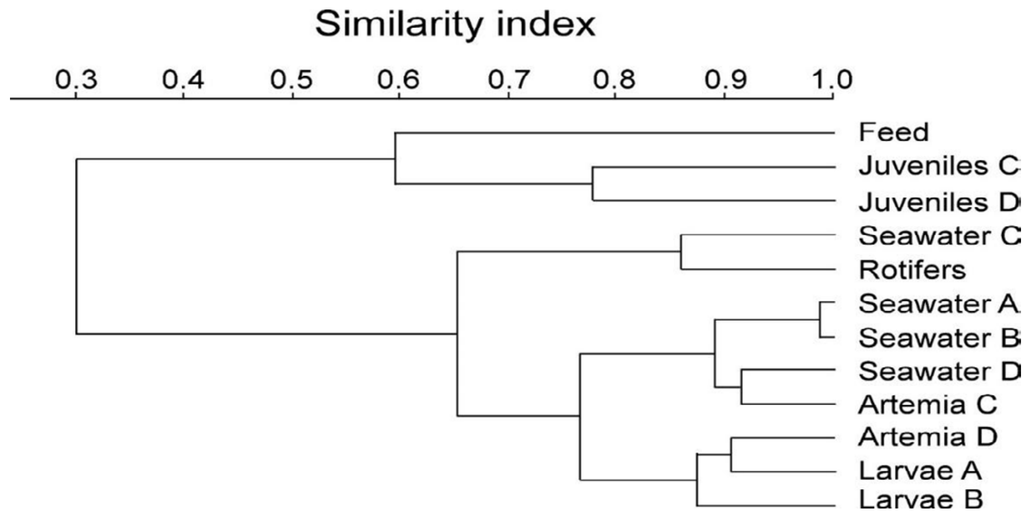

Fig. 1 A dendrogram showing the relationship among 12 libraries based on the distribution of clones belonging to the classes using Morisita's index and UPGMA analysis

Table 3 Composition of species belonging to the family Vibrionaceae in the 12 clone libraries

Related taxon (accession no.; identity, %)	Number of clones in the library of:											
	Larvae A	Larvae B	Juveniles C	Juveniles D	Seawater A	Seawater B	Seawater C	Seawater D	Rotifiers	*Artemia* C	*Artemia* D	Feeds
Photobacterium damselae ssp. *piscicida* (X78105; 98.8-100%)	0	0	0	23	0	0	0	1	0	0	0	0
Vibrio azureus (BATL01000140 ; 99.5%)	0	0	0	0	0	0	0	0	0	0	1	0
Vibrio brasiliensis (AEVS01000097; 99.2%)	0	0	1	0	0	0	0	0	0	0	0	0
Vibrio fortis (AJ514916; 98.4%)	0	0	0	0	0	0	0	0	0	0	1	0
Vibrio galatheae (JXXV01000023; 99.0–99.7%)	0	0	3	0	0	0	0	0	0	0	0	0
Vibrio japonicus (LC143378; 97.3–99.3%)	0	0	6	7	0	0	0	0	0	0	0	0
Vibrio kanaloae (AJ316193; 99.1%)	0	0	0	0	0	0	0	0	0	0	0	1
Vibrio owensii (JPRD01000038; 99.2–99.4%)	0	0	0	1	0	0	0	0	0	0	1	0
Vibrio parahaemolyticus (BBQD01000032; 99.1– 99.8%)	0	0	0	0	0	0	0	0	0	0	3	0
Vibrio tasmaniensis (AJ514912; 99.3%)	0	0	0	0	0	0	0	0	0	0	0	1
Vibrio xuii (AJ316181; 99.5%)	0	0	1	0	0	0	0	0	0	0	0	0
Vibrio spp.	0	0	3	0	0	0	0	0	0	0	1	0
Total	0	0	14	31	0	0	0	1	0	0	7	2

of larvae A and B, seawater A and B, or rotifers. In contrast, *V. brasiliensis, V. galatheae, V. japonicus,* and *V. xuii* were detected in the gut of juveniles C, while members of the Vibrionaceae family were not detected in the libraries of seawater C and *Artemia* nauplii C. Moreover, *V. japonicus* and *V. owensii* were detected in the gut of juveniles D, whereas *V. parahaemolyticus, V. azureus, V. fortis*, *V. owensii, V. kanaloae, and V. tasmaniensis* were detected in the library of *Artemia* nauplii D and feeds. Thus, species of the genus *Vibrio* were detected in the guts of red seabream during the juvenile stage, but no species of the genus *Vibrio* was found to be shared between the rearing seawater and diets, with the exception of *Vibrio owensii*.

Additionally, one clone of *Lactococcus garvieae* subsp. *garvieae,* which belongs to the class Bacilli, was identified in the library of feeds; this species also is classified as a pathogen.

Discussion

Gut microbiota of the red seabream at early life stages

In a previous report (Sugita et al. 1988), we examined the gut microbiota of goldfish (*Carassius auratus*) at each growth stage by a culture-dependent method and classified the bacterial species into three types based on their dynamics: (i) a transient type that appears infrequently and for a relatively short period of time, (ii) a permanently indigenous type that appears abundantly at all stages of fish development, and (iii) an adult type that appears for a period of time after hatching and then becomes established. In contrast, it is generally accepted that the gut microbiota of marine fish at early life stages is established by incorporating members of the genera *Vibrio* and *Pseudomonas* from their diets and rearing seawater, as was determined by a culture-dependent method (Muroga et al. 1987). Sera et al. (1974) found that a specific member of the genus *Vibrio* with abilities to resist gastric juice and bile acids colonized the intestinal tract of marine fish. However, Tanaka et al. (2012) reported that the genus *Vibrio* is not always predominant in the intestinal tract of wild fish caught along the coast of Japan; rather, the intestinal tract of such fish is dominated by members of the classes Actinobacteria, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria (excluding Vibrionaceae). Similar results were observed in the guts of many marine fish analyzed by next-generation sequencing (Yi et al. 2019). As mentioned earlier, an explanation for these contradictory phenomena may be that the predominant gut microbiota of marine fish comprises a highly diverse bacterial class, while only a small portion of the gut microbiota, such as the family Vibrionaceae, can be cultivated under laboratory conditions.

At the larval stage of red seabream, Alphaproteobacteria, Flavobacteria, and Gammaproteobacteria (excluding Vibrionaceae) were predominant in fish guts and in rotifers, while Alphaproteobacteria and Chroobacteria were predominant in seawater. At the juvenile stage, Gammaproteobacteria, Alphaproteobacteria, and Flavobacteria were predominantly detected in fish guts and in *Artemia* nauplii, with the exception of juveniles D, in which Flavobacteria did not predominate. Meanwhile, Alphaproteobacteria and Flavobacteria were predominantly detected in the rearing seawater, with the exception of seawater D, in which Flavobacteria did not predominate. The strong similarities indicated by the Morisita index suggested that the gut microbiota of larval specimens is similar to that of seawater and rotifers, while the microbiota of one of the two juvenile specimens (juvenile D) was similar to that of *Artemia* nauplii and feeds (Table 2 and Fig. 1). Fish larvae ingest bacteria by drinking and thus are primed with antigens before active feeding commences. This pattern may result in the formation of an indigenous larval microflora (Olafsen 2001). Likewise, larvae and juveniles feed on live diets that have many attached bacteria, some of which also are members of the gut microbiota. Similar trends have been observed in the early stage of Atlantic halibut (*Hippoglossus hippoglossus*; Verner-Jeffreys et al. 2003), Atlantic salmon (*Salmo salar*; Navarrete et al. 2009), cod (*Gadus morhua*; Bakke et al. 2015), Nile tilapia (*Oreochromis niloticus*; Giatsis et al. 2015), and yellowtail kingfish (*Seriola lalandi*; Walburn et al. 2019). However, it is thought that the intestinal microflora of fish at the early life stage changes with the development of the host's digestive system, subsequently approaching the intestinal microflora of adult fish (Sugita et al. 1988).

Vibrionaceae in intestinal tract of the early life stage of red seabream

Members of the family Vibrionaceae were not detected in fish guts, rearing seawater, or rotifers at the larval stage, whereas representation of this family increased to 28.6–64.6% in two libraries of fish guts at the juvenile stage (Table 3). However, the species composition of the family Vibrionaceae in the gut microbiota of juvenile red seabream was not similar to that of their live diets or rearing seawater (Table 3). In addition, in one of the two tanks (tank D), a small number of Vibrionaceae was found in the libraries of seawater, *Artemia* nauplii, and feeds; however, with the exception of *V. owensii* and *P. damselae* subsp. *piscicida*, no species within the same family was found to be shared among the guts, seawater, and diets. This result suggests that during the early life stages, the predominant bacteria, including the *Vibrio* species in the guts of red seabream, are significantly replaced.

The family Vibrionaceae was not detected in the library of larval guts, but reached 28.6–64.6% in the library of juvenile guts. Mizuki et al. (2006) investigated the behavior of *L. anguillarum* in a seed

production facility of Japanese flounder and suggested that this bacterium is transmitted primarily from rotifers and *Artemia* nauplii to flounder. Similarly, in the present study, the Vibrionaceae was detected in rearing water, *Artemia* nauplii, and feeds. These results suggest that opportunistic infections of red seabream may be effectively controlled by hygiene management of seawater and diets, along with the administration of probiotics and the microalga *Nannochloropsis oculata* at the fish's juvenile stage (Sharifa and Eguchi 2011). Further studies along this line are currently in progress.

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Authors' contributions MKur collected materials and carried out the molecular genetic analysis. MKun participated in sequence alignment and bacterial identification. NA designed the aquaculture system and reared red seabream. SI discussed the results and commented on the manuscript. HS designed the experiment and wrote the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Conflicts of interest The authors declare that they have no conflict of interest.

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