Int Aquat Res (2023) 15:361–368
https://doi.org/10.22034/IAR.2023.1993945.1491

SHORT COMMUNICATION

Diversity of gut microbiota in Japanese pufferfish and wrasses as determined by next-generation sequencing

Haruo Sugita . Yusuke Kishi . Kai Harumi . Takuya Masuda . Yasuyuki Sasaki . Eitaro Sawayama . Shiro Itoi

Received: 14 August 2023 / Accepted: 05 November 2023 / Published online: 12 November 2023

Abstract The gut microbiota of four tiger puffer (*Takifugu rubripes*), five grass puffer (*T. alboplumbeus*), one multicolorfin rainbowfish (*Parajulis poecilepterus*) and one bambooleaf wrasse (*Pseudolabrus sieboldin*) collected from coastal waters, along with four raised tiger puffer were analyzed using next-generation sequencing (NGS). As a result, Alphaproteobacteria (mean ± SEM, 16.6 ± 3.3%), Clostridia (13.9 ± 3.5%), Gammaproteobacteria (12.6 ± 2.8%), Epsilonproteobacteria (9.9 ± 6.6%), Bacilli (8.6 ± 3.4%), and Planctomycetia (6.4 ± 1.8%) had high relative abundance in more than 80% of the samples. The UPGMA dendrogram using the Bray-Curtis similarity index showed that the gut microbiota was similar among raised individuals of tiger puffer, whereas there are large individual differences among wild fishes, including tiger puffer, grass puffer and wrasses probably due to differences in their individual histories. Vibrionaceae were detected in 13 of 15 samples and the mean relative abundance of Vibrionaceae, including the genera *Aliivibrio*, *Enterovibrio*, *Photobacterium*, *Salinivibrio* and *Vibrio* was 3.981 ± 1.503%, which was estimated to be 3.5×10^5–4.9×10^8 cells/g. However, Vibrionaceae was not detected in two wild grass puffer samples, suggesting their absence or presence at densities too low to be detected by NGS. These results confirm that the density of Vibrionaceae in guts of coastal fishes varies widely. In addition, sequences of *Cetobacterium somerae*, a known dominant anaerobe of freshwater fish, and *Epulopiscium fishelsoni*, a giant bacterium larger than 600 µm × 80 µm, were detected, with mean relative abundances when present of 0.158 ± 0. 087% and 0.456 ± 0.176%, respectively.

Keywords Gut microbiota . Pufferfish . Wrasse . Vibrionaceae . Next-generation sequencing

Introduction

Studies on fish gut microbiota have typically been conducted by identifying strains isolated by agar plate culture, mainly based on phenotypic and morphological properties. Those studies have shown that the gut of marine fishes is occupied mainly by the family Vibrionaceae, including the genera *Vibrio*, *Listonella* and *Photobacterium* (Sugita et al. 1988a, 1989; Cahill 1990). However, since Woese (1987) proposed a new classification system based on 16S rRNA gene sequences, new culture-independent methods have been developed based on specific sequences of each taxon, such as clone library analysis, DGGE (denaturing gradient gel electrophoresis)/TGGE (temperature gradient gel electrophoresis) and FISH (fluorescence in situ hybridization) methods (Matsuki and Tanaka 2006). However, with the development of next-generation sequencing (NGS) in the mid-2000s, it became possible to analyze large numbers of DNA strands at once and NGS has been rapidly introduced into many areas of biology and medicine, including microbiota studies.

Bacteria of the family Vibrionaceae can be opportunistic pathogens and are extremely significant in risk management of fish aquaculture. For example, the economic damage caused by vibriosis was estimated to

Haruo Sugita (✉) . Yusuke Kishi . Kai Harumi . Takuya Masuda . Yasuyuki Sasaki . Eitaro Sawayama . Shiro Itoi
Department of Marine Science and Resources, Nihon University, Kameino 1866, Fujisawa, Kanagawa 252-0880, Japan
e-mail: harusugita4374@gmail.com

be 266 million yen, accounting for 3.6% of the total pufferfish production. Chen et al. (2022) measured the density of Vibrionaceae in the gut of coastal fish by real-time PCR using primers specific for Vibrionaceae and found that Vibrionaceae varied greatly from 1.1×10^5 to 9.9×10^{10} copies/g, but total numbers of bacteria remained relatively stable ($1.5 \times 10^9 – 2.2 \times 10^{11}$ cells/g). Sugita et al. (2005) measured viable counts and total number of bacteria in the gut of coastal fish and reported that the percentage of bacteria that could be cultured (culturability) ranged from 0.00003 to 80.9%. Considering that the majority of bacteria detected by the plate count method are Vibrionaceae, as noted, suggesting that the large fluctuations in the percentage of culturable bacteria were caused by great variations in Vibrionaceae density. In the present study, we used NGS to examine the gut microbiota of pufferfish and wrasse, primarily to confirm whether such large variation in Vibrionaceae actually occurs in marine fish.

Materials and methods

Fish specimens

Four coastal fish species were collected by fishing in an unpolluted rocky area of Sagami Bay, Kanagawa, Japan. The animals analyzed included five grass puffer (*Takifugu alboplumbeus*; 28.7–47.8 g body weight), four tiger puffer (*T. rubripes*; 21.9–26.5 g), one multicolorfin rainbowfish (*Parajulis poecilepterus*; 69.6 g) and one bambooleaf wrasse (*Pseudolabrus sieboldi*; 46.8 g). In addition, four tiger puffer (7.4–9.3 g) were purchased from a seed production company (Marinetech Co., Aichi, Japan), kept in a 800-L tank with a recirculating water system at 20 ± 1°C and fed ad libitum on a commercial feed, EP-1 (48% protein, 12% fat, 2% fiber, 17% ash; Marubeni Nisshin Feed Co., Tokyo, Japan) before being subjected to the experiment. All fish specimens were euthanized by ice cooling immediately after collection, and treated as follows:

Gut contents were obtained aseptically by dissection and squeezing extrusion. Aliquots of each gut sample were stored at -80°C prior to use and then analyzed by NGS. Separately, bacterial cells in aliquots of each gut sample were fixed with a Lugol iodine solution (Pomroy 1984) and stained with 4′, 6-diamidino-2-phenylindole (DAPI); stained samples were used to determine total number of bacteria, using a BX50 fluorescence microscope (Olympus, Tokyo, Japan), as described by Porter and Feig (1980).

Next-generation sequencing

Gut contents were freeze-dried using a VD250R Freeze Dryer (TAITEC, Tokyo, Japan), physically ground using a Shake Master Neo (BMS, Tokyo, Japan), and DNA was extracted using the Mpure Bacterial DNA Extraction kit (MP Biomedicals, Irvine, CA, USA). The V3–V4 variable region of the 16S rRNA gene was amplified by PCR using the nested primers set including forward primer (5′–ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG–3′) and reverse primer (5′–GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC–3′) (Ong et al. 2018), and the amplicons were paired-end (PE) sequenced on the Illumina MiSeq platform (Illumina, CA, USA), as reported by Higo et al. (2019). Sequencing was conducted at Bioengineering Lab. Co., Ltd (Kanagawa, Japan). In total, 15 sequencing libraries were constructed and sequenced. The Illumina raw reads were demultiplexed in each sample based on their index sequences. Sequence read processing was performed using QIIME version 1.9.1 (Caporaso et al. 2010). After removing Illumina adaptor sequences, the reads were truncated at any site that received an average quality score <20 over a 40 bp sliding window, and the reads shorter than 40 bp were discarded using FASTX-Tool kit (http://hannonlab.cshl.edu/fastx_toolkit/). Then, PE reads were assembled according to their overlap sequence with a minimum overlap length of 10 bp, while reads that could not be assembled were discarded. The clean sequences were analyzed using the FLASH (Fast length adjustment of short reads) (Magoč and Salzberg 2011). Chimeric sequences were identified and removed using UCHIME (Edgar et al. 2011). MiSeq data were analyzed with QIIME 1.9.1 using the Greengenes 13.8 reference OTU database (97% similarity threshold). The OTUs proportions in the total sequence number were counted to obtain the relative abundance (%). These sequence data were deposited at the DDBJ Sequence Read Archive (DRA016849).

PAST 4.0.3 software (Hammer et al. 2001) was used to calculate the Bray-Curtis similarity index for pairwise comparisons between different libraries. The similarity matrix then was subjected to cluster anal-

ysis by UPGMA (unweighted pair-group average) to develop a dendrogram.

Results and discussion

Characteristics of the high-throughput sequences

Paired-end amplicon 16S rRNA gene sequencing (V3–V4) of bacterial DNA resulted in a total of 787,640 quality-controlled reads and 488,689 effective reads after trimming, processing, and removing chimera sequences, which resulted in an average of 32,580 ± 2,016 reads (mean ± SEM) per sample. This included removal of chimeric sequences and plant-derived sequences. Chloroplast sequences (13.2% of the raw reads) and mitochondrial sequences (2.1% of the raw reads) were removed, presuming that they were mainly derived from the diet. The total number of OTUs assigned across all samples was 9,581 at the 3% divergence level.

Total numbers of bacteria in gut contents of pufferfish and wrasse

Table 1 shows total numbers of bacteria in gut contents stained with DAPI, as follows: five specimens of wild grass puffer (WGP1–5), 3.5×10^8–6.3×10^9 cells/g; four specimens of wild tiger puffer (WTP1–4), 7.3×10^8–5.1×10^9 cells/g; four specimens of raised tiger puffer (RTP1–4), 4.9×10^8–6.3×10^9 cells/g; two specimens of wild wrasse (KYU and HOSH), 4.4×10^9–6.5×10^9 cells/g. The total number of bacteria, ranging from 3.5×10^8–6.5×10^9 cells/g was similar to the coastal fish including pufferfish and wrasse as described previously (Sugita et al. 2005; Chen et al. 2022). Therefore, there were no particularly unusual results.

Gut microbiota of pufferfish and wrasse

A total of 9,581 OTUs from gut contents of pufferfish and wrasse were utilized for the taxonomic analysis. The mean number of OTUs in gut microbiota of fish specimens was as follows: wild grass puffer, 1,230 ± 466 (mean ± SEM); wild tiger puffer, 1,260 ± 514; raised tiger puffer, 1,256 ± 45; and two wrasses, 362 ± 10.

The most abundant phyla detected in pufferfish and wrasse were Proteobacteria (41.3 ± 5.0%), Firmicutes (27.7 ± 6.8 %), Actinobacteria (10.6 ± 2.0%) and Planctomycetes (6.9 ± 2.0%). Proteobacteria and Firmicutes were the most prevalent phyla detected in all specimens.

The gut microbiota at the class level of raised tiger puffer, wild tiger puffer, wild grass puffer, and two wild wrasse species are shown in Fig. 1. Alphaproteobacteria (16.6 ± 3.3%), Clostridia (13.9 ± 3.5%), Gammaproteobacteria (12.6 ± 2.8%), Epsilonproteobacteria (9.9 ± 6.6%), Bacilli (8.6 ± 3.4%), and Planctomycetia (6.4 ± 1.8%) had high relative abundance in more than 80% of the samples. On the other hand, Tanaka et al. (2012) reported that Alphaproteobacteria, Gammaproteobacteria (excluding Vibrionaceae), Actinobacteria, Betaproteobacteria and Bacilli were dominant in the clone library analysis of the gut microbiota of six coastal fishes (excluding pufferfish). Of those studies, Alphaproteobacteria, Gammaproteobacteria, and Bacilli were commonly dominant, although the analytical methods used were different.The cluster analysis

Table 1 Total numbers of bacteria in the guts of pufferfish and wrasse

Fish specimen (symbol)	Total bacteria (cells/g)
Wild grass puffer No. 1 (WGP1)	6.3×10^9
Wild grass puffer No. 2 (WGP2)	3.5×10^8
Wild grass puffer No. 3 (WGP3)	1.4×10^9
Wild grass puffer No. 4 (WGP4)	5.8×10^8
Wild grass puffer No. 5 (WGP5)	7.9×10^8
Wild tiger puffer No. 1 (WTP1)	5.1×10^9
Wild tiger puffer No. 2 (WTP2)	1.1×10^9
Wild tiger puffer No. 3 (WTP3)	1.2×10^9
Wild tiger puffer No. 4 (WTP4)	7.3×10^8
Wild multicolorfin rainbowfish (KYU)	6.5×10^9
Wild bambooleaf wrasse (HOSH)	4.4×10^9
Raised tiger puffer No. 1 (RTP1)	4.9×10^8
Raised tiger puffer No. 2 (RTP2)	6.3×10^9
Raised tiger puffer No. 3 (RTP3)	4.1×10^9
Raised tiger puffer No. 4 (RTP4)	5.1×10^9

results are shown in Fig. 2. The results show that all four raised tiger puffer samples exhibited similar microbiota, consisting of Bacilli (26.9–34.9%), Gammaproteobacteria (12.3–31.2%), Clostridia (14.2–25.0%),

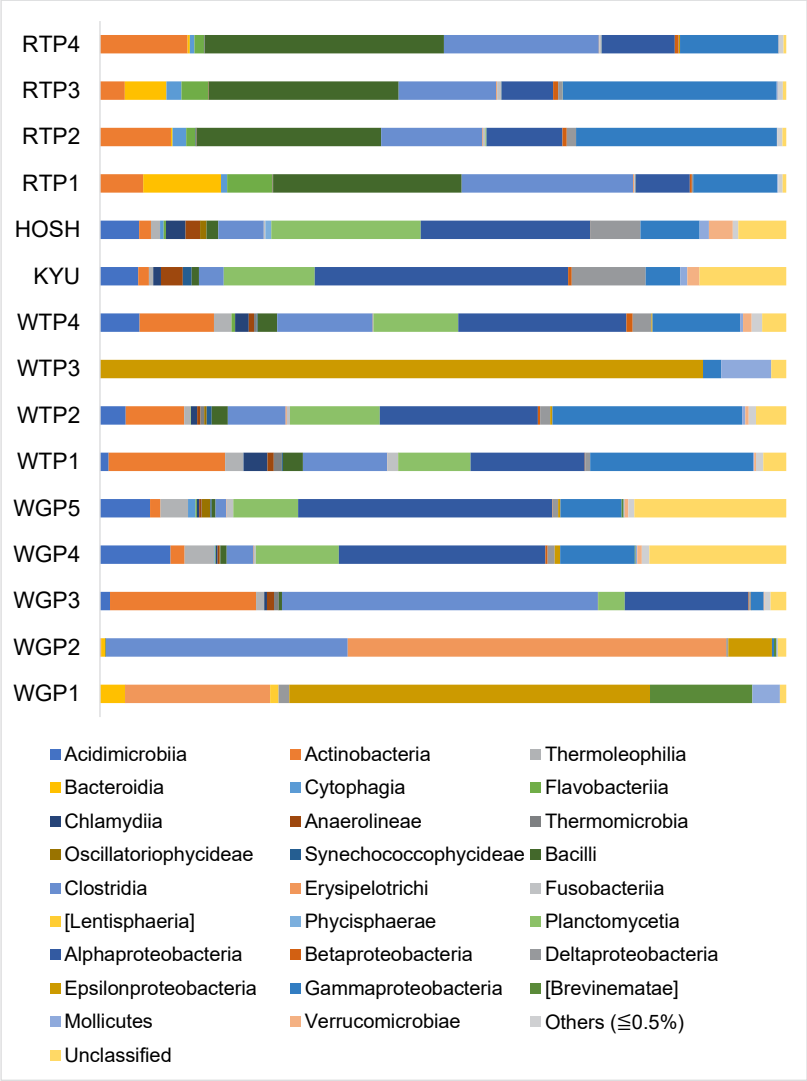

Fig. 1 Relative abundance (%) of the classes in the gut microbiota* of 15 specimens of pufferfish and two wrasse species. * Refer to the symbols in Table 1.

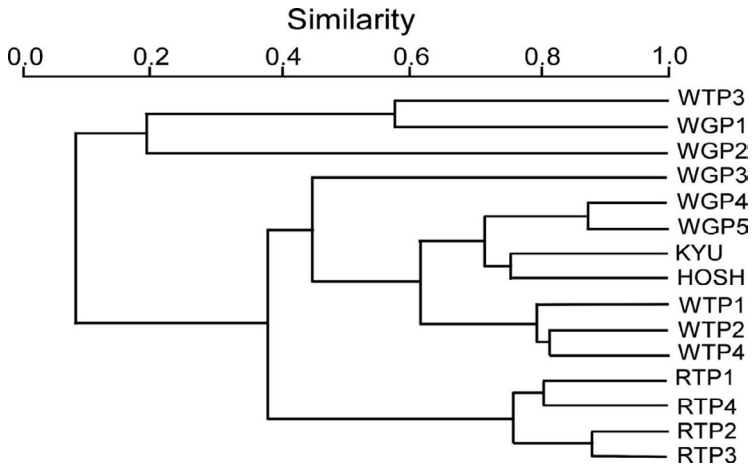

Fig. 2 A dendrogram showing the relationship among the gut microbiota* of 15 specimens of pufferfish and two wrasse species, based on the distribution of microbial classes using the Bray-Curtis similarity index and UPGMA analysis. * Refer to the symbols in Table 1.

Alphaproteobacteria (7.5–11.1%) and Actinobacteria (3.6–12.8%). The microbiota of two wild wrasse samples consisted of Alphaproteobacteria (24.7, 36.9%), Planctomycetia (13.2, 21.8%), Deltaproteobacteria (7.3, 10.8%), and Gammaproteobacteria (5.0, 8.6%), which were similar to each other. On the other hand, three of four wild tiger puffer samples were dominated by Alphaproteobacteria (16.7–24.5%), Gammaproteobacteria (12.7–27.6%), Planctomycetia (10.5–13.1%), Actinomycetes (8.5–17.0 %) and Clostridia (8.3–13.9%), forming the same cluster that were identical to each other but different from the cluster of raised tiger puffer. Of five wild grass puffer samples, two were characterized mainly by Alphaproteobacteria (30.1–37.0%), Planctomycetia (9.4–12.1%), Gammaproteobacteria (8.9–10.9%) and Acidimicrobiia (7.3–10.3%), which formed the same cluster, while the microbiota of the remaining three samples were quite different. The remaining wild tiger puffer (WTP3) and wild grass puffer (WGP1) samples were characterized by the genus *Arcobacter* within Epsilonproteobacteria accounting for 87.9% and 52.6%, respectively. The relative abundance of *Arcobacter* spp. in other four samples of wild grass puffer ranged from 0.003% and to 6.37%. In one sample (WTP2) of wild tiger puffer, it was 0.09%, and in four samples of raised tiger puffer, it ranged from 0.002% to 0.012%. This result suggests that *Arcobacter* spp., although present in low densities, are widely distributed in the gut of both tiger and grass puffers. In this study, *Arcobacter* spp. were found in high proportions in one sample each of wild tiger puffer (WTP3) and grass puffer (WGP1). This could indicate significant individual differences in the gut microbiota among wild pufferfish.

Previous studies have reported that the fish gut microbiota varies greatly among species (Cahill 1990; Sugita et al. 1991; Sullam et al. 2012; Li et al. 2014; Yi et al. 2019; Yoshida et al. 2022), developmental stage (Yoshimizu and Kimura 1976; Sugita et al. 1988b; Yan et al. 2016; Kurosaki et al. 2021), trophic level (Sullam et al. 2012; Li et al. 2014; Egerton et al. 2018), season and water temperature (Sugita et al. 1989; Egeton et al. 2018), salinity (Yoshimizu and Kimura 1976; Hamid et al. 1978; Sugita et al.1982; Sullam et al. 2012; Dulski et al. 2020), daily fluctuation (Sugita et al.1990; Asfie et al. 2003), food type (Sullam et al. 2012; Ingerslev et al. 2014; Li et al. 2014; Miyake et al. 2015; Ringø et al. 2016; Niu et al. 2020; Yoshida et al. 2022), habit condition (Ramirez and Romero 2017a,b; Romero et al. 2022) and starvation (Xia et al. 2014). All raised tiger puffers used in this study were purchased from the same supplier at the same time and reared under the same conditions, including tank and formula feed. On the other hand, even though the wild samples were collected on the same day at the same location, it is highly likely that their conditions for growth in the wild, as described above, were different for each. Moreover, the wild fish collected were from natural habitats with a lot of fluctuations in the environmental factors. Star et al. (2013) found that the gut microbiota varies significantly in individual Atlantic cod (*Gadus morhua*) specimens caught at a single location, suggesting that a complex combination of factors influenced the species distribution of these gut microbiota. It is important to note that even among individuals of the same species collected from the same location on the same day, their individual histories can vary greatly. Therefore, it is not surprising that the gut microbiota of raised and wild fish differs to some extent, even in the same species. Nevertheless, these results suggest that gut microbiota was similar among raised individuals, whereas there are large individual differences among wild individuals.

Table 2 shows the relative abundance (%) of Vibrionaceae, *Cetobacterium/C. somerae* and *Epulopiscium/E. fishelsoni* in the gut microbiota of pufferfish and wrasse. The mean relative abundance of Vibrionaceae, including *Alivibrio*, *Enterovibrio*, *Photobacterium*, *Salinivibrio* and *Vibrio*, was 3.981 ± 1.503%, detected in 13 of 15 samples. The density estimated from the total number of bacteria (cells/g) and relative

Table 2 Relative abundance (%) of Vibrionaceae, *Cetobacterium* and *Epulopiscium* in the gut microbiota of pufferfish and wrasse

Taxon	Wild grass puffer (*n*=5)	Wild tiger puffer (*n*=4)	Raised tiger puffer (*n*=4)	Wild wrasses (*n*=2)
Vibrionaceae				
Aliivibrio sp.	0.000±0.000 (0)*	0.001±0.001 (25)	0.000±0.000 (0)	0.000±0.000 (0)
Enterovibrio spp.	0.001±0.001 (40)	4.280±4.271 (75)	0.001±0.001 (25)	0.000±0.000 (0)
Photobacterium angustum	0.000±0.000 (0)	0.001±0.001 (25)	0.014±0.005 (75)	0.000±0.000 (0)
Photobacterium rosenbergii	0.000±0.000 (0)	0.064±0.047 (75)	0.000±0.000 (0)	0.000±0.000 (0)
Photobacterium spp.	0.027±0.020 (60)	1.388±0.838 (75)	0.127±0.013 (100)	0.000±0.000 (0)
Salinivibrio costicola	0.000±0.000 (0)	0.490±0.252 (75)	0.032±0.009 (100)	0.000±0.000 (0)
Vibrio fortis	0.000±0.000 (0)	0.020±0.020 (25)	0.001±0.001 (25)	0.551±0.551 (50)
Vibrio rumoiensis	0.000±0.000 (0)	0.000±0.000 (0)	0.001±0.001 (25)	0.000±0.000 (0)
Vibrio shilonii	0.017±0.014 (40)	0.211±0.108 (75)	0.149±0.050 (100)	0.000±0.000 (0)
Unclassified *Vibrio* genus	3.050±1.864 (60)	2.685±0.627 (100)	0.0068±0.0021 (100)	0.099±0.097 (100)
Unclassified Vibrionaceae family	0.006±0.004 (40)	0.187±0.086 (75)	0.059±0.057 (50)	0.001±0.001 (50)
Total Vibrionaceae	3.102±1.895 (60)	9.328±4.214 (100)	0.453±0.067 (100)	0.651±0.455 (100)
Cetobacterium/C. somerae	0.044±0.027 (40)	0.431±0.303 (50)	0.068±0.027 (100)	0.000±0.000 (0)
Epulopiscium/E. fishelsoni	0.043±0.020 (100)	1.054±0.553 (75)	0.454±0.152 (100)	0.081±0.048 (100)

*Mean ± SEM of relative abundance (Occurrence, %).

abundance (%) was 3.5×10^5–4.9×10^8 cells/g. The reason why Vibrionaceace was not detected in the two samples, other than the complete absence of Vibrionaceae, is that sequences of this organism were not included in total reads of 30,689–37,346 decoded by NGS. Thus, there remains the possibility that it is present in small numbers. In any case, this result indicates that Vibrionaceae densities are highly variable in coastal fishes such as pufferfish. Chen et al. (2022), using the quantitative PCR (qPCR) technique, found that the abundance of *Vibrio* spp. in the gut of coastal fish was 1.1×10^5–9.9×10^{10} copies/g. These results revealed that the total number of bacteria in the gut of coastal fish is relatively constant (ranging from 1.5×10^9–2.2×10^{11} cells/g), while the abundance of Vibrionaceae varies greatly. These results strongly indicate that Vibrionaceae is not necessarily the dominant organism in the gut microbiota of marine fishes, although it remains unclear why Vibrionaceae varies greatly in number. However, it is important to keep in mind that Vibrionaceae presents the greatest risk factor in aquaculture, and hygiene must be constantly monitored.

Interestingly, sequences of *Cetobacterium*/*C. somerae* were detected in 8 of 15 samples with mean relative abundances of 0.158 ± 0.087%. This bacterium, initially tentatively named "*Bacteroides* type A", is known to be the dominant anaerobe in the gut of freshwater fish such as Nile tilapia *Oreochromis niloticus*, ayu *Plecogrossus altivelis*, carp *Cyprinus carpio* and goldfish *Carassius auretus* (Sakata et al. 1980; Cahill 1990; Sugita et al. 1991; Tsuchiya et al. 2008). Sugita et al. (1982) showed that *C. somerae* grew well in the medium adjusted to 0–2% NaCl, and growth was also observed, albeit weakly, in 3% NaCl. Therefore, Nile tilapia, a euryhaline fish, were acclimated to 100% artificial seawater in a stepwise manner to determine changes in density of *C. somerae*. In the stomach and fore-intestine of Nile tilapia, the density of *C. somera*e decreased by one to two orders of magnitude, while in the post-intestine, it was almost the same order as in freshwater. In seawater, it is common for marine fish to drink large amounts of seawater, selectively excrete NaCl from ionocytes, and excrete small amounts of urine to compensate for osmotic dehydration (Wedemeyer 1996). Therefore, it can be assumed that the salinity of the hind-intestine was lower than that of the stomach and fore-intestine, which had less effect on the growth of *C. somerae*, thus keeping the density of *C. somerae* higher. This may mean that *C. somerae* is somewhat more likely to be present in the intestinal tracts of marine fish if it can overcome conditions other than salt tolerance, such as competition with other bacteria. In addition, it has been strongly suggested that *C. somerae* can efficiently produce vitamin B_{12} in the gut of freshwater fish, supplying the host with vitamin B_{12} (Sugita et al. 1991; Tsuchiya et al. 2008), and the same phenomenon may be occurring in marine fish to some extent. Nevertheless, it is necessary to isolate *C. somerae* from the gut of coastal fish and compare its properties with those of *C. somerae* from the gut of freshwater fish.

Another giant bacterium, *Epulopiscium fishelsoni*, measures more than 600 µm by 80 µm and is found in the intestinal tract of the brown surgeon fish (*Acanthurus nigrofuscus*: family Acanthuridae) in the Red Sea (Angert et al. 1993). Moreover, Miyake et al. (2015) reported that members of the phylum Firmicutes, especially of the genus *Epulopiscium*, were dominant in the gut microbiota of seven surgeon fishes from the Red Sea. In the present study, sequences of this bacterium or related organisms were detected in 14 of 15 gut samples of Japanese pufferfish and wrasses, with a relative abundance of 0.456 ± 0.176%. This fact suggests that this bacterium is widely distributed, albeit at low density, in the gut of fish along the coast of Japan. Since this bacterium cannot be cultured under normal culture conditions to date, in order to confirm its actual existence in the gut of coastal fish, it is necessary to detect this bacterium from the guts of coastal fish using the FISH method with a probe specific to this bacterium (Angert et al. 1993).

In this study, the sequences of bacteria such as *Cetobacterium somerae* and *Epulopiscium fishelsoni* were unexpectedly detected in coastal pufferfish and wrasses. The detection of these bacteria in coastal fishes is considered a further extension of the usefulness of NGS in the study of fish gut microbiota. In the future, it will be necessary to isolate these bacteria from marine fishes and study their properties to further our knowledge of intestinal bacteria. Moreover, using NGS to decipher the dynamics of the gut microbiota in farmed aquatic animals will play an essential role in improving animal health and aquaculture productivity (Diwan et al. 2022, 2023).

Acknowledgements This study was supported in part by a 2018 research grant from The Tokyo Suisan Foundation (*formerly* The Towa Foundation for Food Science and Research).

Authors' contributions HS, conceptualization, formal analysis, funding acquisition, supervision, Writing – original draft, Writing – review & editing; YK, investigation; KH, TM, and YS, resources; ES, data curation, Writing – review & editing; SI, Writing – review & editing. All authors reviewed the manuscript.

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

References

Angert ER, Clements KD, Pace NR (1993) The largest bacterium. Nature 362:239–241.https://doi.org/10.1038/362239a0

Asfie M, Yoshijima T, Sugita H (2003) Characterization of the goldfish fecal microflora by the fluorescent in situ hybridization method. Fish Sci 69:21-26. https://doi.org/ 10.1046/j.1444-2906.2003.00583.x

Cahill MM (1990) Bacterial flora of fishes: a review. Microb Ecol 19:21-41. https://doi.org/10.1007/BF02015051

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303

Chen CH, Sagara K, Minamishima R, Yoshino Y, Yamaguchi D, Itoi S, Sugita H (2022) Abundance of *Vibrio* populations in the gut of Japanese coastal fishes. Int Aquat Res 14:41–50. https://doi.org/10.22034/IAR.2022.1942260.1201

Diwan AD, Harke SN, Gopalkrishna, Panche AN (2022) Aquaculture industry prospective from gut microbiome of fish and shellfish: An overview. J Anim Physiol Anim Nutr 106:441-469. https://doi.org/10.1111/jpn.13619

Diwan AD, Harke SN, Panche AN (2023) Host-microbiome interaction in fish and shellfish: An overview. Fish Shellfish Immunol Rep 4: 100091. https://doi.org/10.1016/j.fsirep.2023.100091

Dulski T, Kujawa R, Godzieba M, Ciesielski S (2020) Effect of salinity on the gut microbiome of pike fry (*Esox lucius*). Appl Sci 10:2506. https://doi.org/10.3390/app10072506

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinform 27:2194-200. https://doi.org/10.1093/bioinformatics/btr381

Egerton S, Culloty S, Whooley J, Stanton C, Ross P (2018) The gut microbiota of marine fish. Front Microbiol 9:873. https://doi.org/10.3389/fmicb.2018.00873

Ingerslev HC, Jørgensen LVG, Strube ML, Larsen N, Dalsgaard I, Boye M, Madsen L (2014) The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. Aquaculture 424-425:24-34. http://dx.doi.org/10.1016/j.aquaculture.2013.12.032

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Palaeontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9

Hamid A, Sakata T, Kakimoto D (1978) Microflora in the alimentary tract of gray mullet-II A comparison of the mullet intestinal microflora in fresh and seawater. Bull Jpn Soc Sci Fish 44:53-57. https://doi.org/10.2331/suisan.44.53

Higo M, Tatewaki Y, Gunji K, Kaseda A, Isobe K (2019) Cover cropping can be a stronger determinant than host crop identity for arbuscular mycorrhizal fungal communities colonizing maize and soybean. PeerJ 7:e6403. https://doi.org/10.7717/peerj.6403

Kurosaki M, Kunimoto M, Akiyama N, Itoi S, Sugita H (2021) Predominant gut microbiota in the early life stages of red seabream (*Pagrus major*) raised in indoor tanks. Int Aquat Res 13:219–226. https://doi.org/10.22034/IAR.2021.1925333.1143

Li J, Ni J, Li J, Wang C, Li X, Wu S, Zhang T, Yu Y, Yan Q (2014) Comparative study on gastrointestinal microbiota of eight fish species with different feeding habits. J Appl Microbiol 117:1750-1760. https://doi.org/10.1111/jam.12663

Magoč T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinform 27:2957-2963. https://doi.org/10.1093/ bioinformatics/btr507

Matsuki T, Tanaka R (2006) Molecular methods in the analysis of human intestinal flora. J Intest Microbiol 20:25-33. https://doi.org/10.11209/jim.20.25

Miyake S, Ngugi DK, Stingl U (2015) Diet strongly influences the gut microbiota of surgeonfishes. Mol Ecol 24:656–672. doi:10.1111/mec.13050

Niu KM, Lee BJ, Kothari D, Lee WD, S Hur SW, Lim SG, Kim KW, K Kim KD, Kim NN, Kim SK (2020) Dietary effect of low fish meal aquafeed on gut microbiota in olive flounder (*Paralichthys olivaceus*) at different growth stages. MicrobiologyOpen 9:e992. https://doi.org/10.1002/mbo3.992

Ong IM, Gonzalez JG, McIlwain SJ, Sawin EA, Schoen AJ, Adluru N, Alexander AL, Yu J-PJ (2018) Gut microbiome populations are associated with structure-specific changes in white matter architecture. Transl Psychiatry 8:6. https://doi.org/10.1038/s41398-017-0022-5

Pomroy AJ (1984) Direct counting of bacteria preserved with Lugol iodine solution. Appl Environ Microbiol 47:1191–1192. https://doi.org/10.1128/aem.47.5.1191-1192.1984

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948. https://doi.org/ 10.4319/lo.1980.25.5.0943

Ramirez C, Romero J (2017a) Fine flounder (*Paralichthys adspersus*) microbiome showed important differences between wild and reared specimens. Front Microbiol 8:271. https://doi.org/10.3389/fmicb.2017.00271

Ramírez C, Romero J (2017b) The microbiome of *Seriola lalandi* of wild and aquaculture origin reveals differences in composition and potential function. Front Microbiol 8:1844. https://doi.org/10.3389/fmicb.2017.01844

Ringø E, Zhou Z, Vecino JLG, Wadsworth S, Romero J, Krogdahl A, Olsen RE, Dimitroglou A, Foey A, Davies S, Owen M, Lauzon HL, Martinsen LL (2016) Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? Aquac Nutr 22:219-282. https://doi.org/10.1111/anu.12346

Romero J, Díaz O, Miranda CD, Rojas R (2022) Red cusk-eel (*Genypterus chilensis*) gut microbiota description of wild and aquaculture specimens. Microorganisms 10:105. https://doi.org/10.3390/microorganisms10010105

Sakata T, Sugita H, Mitsuoka T, Kakimoto D, Kadota H (1980) Isolation and distribution of obligate anaerobic bacteria from the intestines of freshwater fish. Bull Jpn Soc Sci Fish 46:1249–1255. https://doi.org/10.2331/suisan.46.511

Star B, Haverkamp THA, Jentoft S, Jakobsen KS (2013) Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. BMC Microbiol 13:248. https://doi.org/10.1186/1471-2180-13-248

Sugita H, Ishida Y, Deguchi Y, Kadota H (1982) Bacterial flora in the gastrointestine of *Tilapia nilotica* adapted in seawater. Bull Jpn Soc Sci Fish 48:987–991. https://doi.org/10.2331/suisan.48.987

Sugita H, Iwata J, Miyajima C, Kubo T, Noguchi T, Hashimoto K, Deguchi Y (1989) Changes in microflora of the puffer fish *Fugu niphobles*, at different water temperatures. Mar Biol 101:299–304. https://doi.org/10.1007/BF00428125

Sugita H, Kurosaki M, Okamura T, Yamamoto S, Tsuchiya C (2005) The culturability of intestinal bacteria of Japanese coastal fish. Fish Sci 71:956–958. https://doi.org/10.1111/j.1444-2906.2005.01050.x

Sugita H, Miyajima C, Deguchi Y (1991) The vitamin B_{12}-producing ability of the intestinal microflora of freshwater fish. Aquaculture 92:267–276. https://doi.org/10.1016/0044-8486(91)90028-6

Sugita H, Miyajima C, Iwata J, Arai S, Kubo T, Igarashi S, Deguchi Y (1988a): Intestinal microflora of Japanese coastal fishes. Nippon Suisan Gakkaishi 54:875–882. https://doi.org/10.2331/suisan.54.875

Sugita H, Miyajima C, Kobiki Y, Deguchi Y (1990) The daily fluctuation and inter-individual variation of the faecal flora of carp, *Cyprinus carpio* L. J Fish Biol 36:103-105. https://doi.org/10.1111/j.1095-8649.1990.tb03525.x

Sugita H, Tsunohara M, Ohkoshi T, Deguchi Y (1988b) The establishment of an intestinal microflora in developing goldfish (*Carassius auratus*) of culture ponds. Microbial Ecol 15:333-344. https://doi.org /10.1007/bf02012646

Sullam KE, Essinger SD, Lozupone CA, O'connor MP, Rosen GL, Knight R, Kilham SS, Russell JA (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol Ecol 21:3363-3378. https://doi.org/10.1111/j.1365294X.2012.05552.x

Tanaka Y, Chen CH, Kozaki T, Komiya Y, Itoi S, Sugita H (2012) Bacterial diversity of the intestinal tracts of the Japanese coastal fish as determined by the clone library method. Aquac Sci 60:333–340. https://doi.org/10.11233/aquaculturesci. 60.333

Tsuchiya C, Sakata T, Sugita H (2008) Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett Appl Microbiol 46:43–48. https://doi.org/10.1111/j.1472-765X.2007.02258.x

Wedemeyer GA (1996) Physiology of fish in intensive culture systems. Chapman and Hall. New York

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271. https://doi.org/10.1128/mr.51.2.221-271.1987

Xia JH, Lin G, Fu GH, Wan ZY, Lee M, Wang L, Liu XJ, Yue GH (2014) The intestinal microbiome of fish under starvation. BMC Genomics 15:266. https://doi.org/10.1186/1471-2164-15-266

Yan Q, Li J, Yu Y, Wang J, He Z, Nostrand JDV, Kempher ML, Wu L, Wang Y, Liao L, Li X, Wu S, Ni J, Wang C, Zhou J (2016) Environmental filtering decreases with fish development for the assembly of gut microbiota. Environ Microbiol 18:4739–4754. doi:10.1111/1462-2920.13

Yi Y, Liang L, Wang Z, Ai P, You X, Bian C, Shi Q, Dong B (2019) A comparative metagenomics study on gastrointestinal microbiota in amphibious mudskippers and other vertebrate animals. Animals 9:660. https://doi.org/10.3390/ani9090660

Yoshida M, Tanabe T, Akiyoshi H, Kawamukai M (2022) Gut microbiota analysis of Blenniidae fishes including an algae-eating fish and clear boundary formation among isolated *Vibrio* strains. Sci Rep 12:4642. https://doi.org/10.1038/s41598-022-08511-7

Yoshimizu M, Kimura T (1976) Study on the intestinal microflora of salmonids. Fish Pathol 10:243-259. https://doi.org/10.3147/jsfp.10.243

Publisher's Note