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ORIGINAL RESEARCH

Dynamics of *Vibrio* populations in the rearing water of tiger puffer (*Takifugu rubripes*)

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Abstract Members of *Vibrio* spp. are important risk factors in aquaculture, acting as opportunistic pathogens in marine fish. Therefore, we investigated, using quantitative polymerase chain reaction (qPCR) with *Vibrio*-specific primers, the behavior of *Vibrio* spp. during short-term rearing of tiger puffer (*Takifugu rubripes*). The results showed that the abundance of *Vibrio* spp. and total bacterial counts increased markedly from the first week after the fish were placed in the tank, and that these values decreased when the fish were removed. The abundance (copies/mL) of *Vibrio* spp. increased linearly with total bacterial counts, but their density (cells/mL) was estimated to be about two orders of magnitude lower than total bacterial counts. *Vibrio* spp. in the rearing water were less abundant than other bacterial groups, indicating that many other non-*Vibrio* microbial communities exhibit similar dynamics. The increase in total bacterial counts, including *Vibrio* spp., during the rearing period may reflect the spread of pufferfish gut bacteria in the rearing water via the fish feces, along with growth of the bacteria on organic matters derived from mucus, feces, and uneaten food. These results strongly suggest that effective control of vibriosis in fish farms requires surveillance of *Vibrio* spp., not only in rearing water but also in the fish gut and live diet. The qPCR method used in this study is suitable for surveillance of *Vibrio* spp. in aquaculture because it is highly accurate and results can be obtained in 5 – 6 h.

Keywords Tiger puffer . Rearing water . *Vibrio* spp. . qPCR . *Vibrio*-specific primers . TCBS medium

Introduction

The global aquaculture industry is increasing its production every year, reaching 120,071,500 tons (including aquatic plants) in 2019 (FAO 2021). In many marine fish farms, opportunistic infections occur frequently due to the stress of rearing fish at high densities. Vibriosis, a disease caused by the typical opportunistic pathogens *Vibrio* spp. and related bacteria, is an extremely important risk factor in marine fish farms. For example, in 2019, the annual aquaculture production value of pufferfish, including tiger puffer (*Takifugu rubripes*) and purple puffer (*Takifugu porphyreus*), in Japan was 8.98 billion yen, and the economic damage caused by vibriosis was estimated to be 182 million yen (2%) (https://www.maff.go.jp/). Genus *Vibrio* is a group of physiologically flexible marine bacteria that are ubiquitous in oceans and have been identified in most marine ecosystems, including fish farms (Mansergh and Zehr 2014). Mizuki et al. (2005) reported that *Listonella anguillarum* (synonymous with *Vibrio anguillarum*) is a transient bacterium in the gut microflora of the Japanese flounder (*Paralichthys olivaceus*) but is a permanently indigenous bacterium for Japanese flounder hatcheries, given that this bacterium always is detected in the rearing water and in live diets such as rotifers. In addition, Kim and Lee (2017) found that the *Vibrio* spp. density in rearing water correlates highly with bacterial number in fish products such as fish filets. Because the genus *Vibrio* contains pathogens (e.g.,

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V. parahaemolyticus and *V. vulnificus*) commonly found in humans and marine fish, fish and processed products contaminated with these pathogens are considered problematic from a food safety perspective. Therefore, surveillance in marine fish farms using the abundance of *Vibrio* spp. in fish guts, rearing water, and live feed is required as an indicator of possible infection.

Conventionally, a selective medium such as thiosulfate-citrate-bile salts-sucrose (TCBS) agar typically is used to detect and count *Vibrio* spp. However, given that this method requires at least 24–48 hours for cultivation, there is a risk that this growth interval may delay the timing of treatment and other procedures. In our previous report (Chen et al. 2022), we demonstrated that the use of *Vibrio*-specific primers for quantitative polymerase chain reaction (qPCR) permitted the accurate detection and quantification of *Vibrio* spp. in samples. Therefore, the present study was conducted using qPCR to characterize the dynamics of *Vibrio* spp. in the rearing water of tiger puffer.

Materials and methods

Rearing fish

Juvenile tiger puffer were purchased from Marinetech Co. (Aichi, Japan) and raised in a 800-L fiber-reinforced plastic (FRP) tank with a recirculating water system at 19 ± 1 °C; these animals were provided with *ad libitum* access to a commercial feed (Nosan Corp., Yokohama, Japan). Five 57-L glass tanks equipped with recirculating water systems (flow rate: 8.3 L/min) were employed as experimental tanks in which the water temperature again was maintained at 19 ± 1 °C. For the first 7 days, no fish were housed in each tank, permitting stabilization of the water quality. On Day 7 of the experiment, 10 (for Tanks A and B), 3 (for Tanks C and D), and 0 (for Tank E; control) juvenile puffer (7.4–9.3 g) were transferred into the respective tanks. During the subsequent 28-day rearing period, the fish were fed each day with 2% of their body weight of a commercial feed. After all fish were removed from the tanks on Day 35, the tanks were allowed to run for another 14 days. During the experiment, no disease outbreaks or deaths of captive fish were observed in any of the tanks. Water samples were collected on study Day 0 (no fish housed); Day 7 (before introduction of pufferfish); Days 14, 21, 28, and 35 (during rearing); and Day 49 (two weeks after removal of the fish). The sample names are shown in Table 1. Each sample consisted of 600 mL of rearing water, which was collected from the surface water of each tank into a sterile glass bottle. For each sample, 500 mL of the rearing water were filtered through a 0.22-μm pore size Type-GS membrane filter (Merck Millipore, MA, USA). The membrane filter then was stored immediately at -80 °C prior to further analysis, while the filtrate was returned to the respective source tank.

Counting and isolation of viable bacteria

Aliquots of the collected water samples were subjected to serial dilution with sterile seawater, and 50 μL of each diluted sample was inoculated onto 1/20 peptone-yeast extract-beef extract-glucose (1/20 PYBG) agar medium (Sugita et al. 1989) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium (Eiken Chemical Co., Tokyo, Japan); the latter is a *Vibrio* spp.-selective medium. The 1/20 PYBG medium contains (per 1000 mL of 50% aged seawater): Trypticase peptone (Beckton Dickinson, Franklin Lakes, NJ, USA), 0.5 g; Phytone peptone (Beckton Dickinson), 0.25 g; Bacto-yeast extract (Beckton Dickinson), 0.1 g; Lab-lemco powder (Thermo Fisher Scientific, Waltham, MA, USA), 0.1 g; glucose, 0.1 g; and agar, 15 g; the resulting solution was adjusted to pH 7.5. Since the 1/20 PYBG agar medium does not contain any special selective agents, bacteria appearing on this medium were considered to be culturable heterotrophic bacteria. TCBS medium is often incubated at 35 –37°C to selectively isolate *V. cholerae* and *V. parahaemolyticus*, but this temperature is too high for marine vibrios, including fish pathogens, which are often incubated at 25°C or habitat temperature with these bacteria (Farmer III and Hickman-Brenner 1992). Therefore, in this study, the inoculated 1/20 PYBG and TCBS agars were incubated under aerobic conditions for 5 days at 20°C, close to the rearing temperature (19 ± 1°C). After incubation, the number of colonies on the agar medium containing the appropriately diluted samples was counted, and some 20–30 colonies were randomly selected from each sample and purified by repeated streaking and incubation on agar plates. All isolates were speciated based on the sequences of 16S rRNA genes as described below.

Table 1 Changes in the viable counts of bacteria as measured by plating to 1/20 PYBG and TCBS media, including abundance of *Vibrio* spp. and total bacterial counts (TBC) in the rearing water of Tanks A-E.

Tank	Sample name	Day	1/20 PYBG	TCBS	*Vibrio* spp.	TBC
			(log CFU/mL)	(log CFU/mL)	(log copies/mL)	(log cells/mL)
A	A0	0	3.95	<1.30	3.01	5.87
	A7	7	5.14	2.40	3.22	5.78
	A14	14	5.34	4.93	7.84	8.20
	A21	21	4.93	4.87	7.72	7.99
	A28	28	4.82	4.58	6.82	7.23
	A35	35	5.39	4.76	7.09	8.06
	A49	49	4.17	1.95	1.86	5.68
B	B0	0	3.55	<1.30	1.57	5.90
	B7	7	5.11	3.02	3.01	6.05
	B14	14	5.32	4.81	7.89	8.13
	B21	21	5.47	5.13	7.34	8.14
	B28	28	5.06	4.34	6.43	7.40
	B35	35	6.48	2.90	6.88	8.05
	B49	49	4.11	3.00	2.17	5.70
C	C0	0	4.10	1.60	3.26	5.86
	C7	7	5.81	4.01	4.18	5.94
	C14	14	4.53	4.27	7.42	8.05
	C21	21	4.28	4.18	6.66	7.12
	C28	28	4.33	3.49	6.87	7.13
	C35	35	4.41	4.31	5.33	7.14
	C49	49	4.57	2.69	0.97	4.88
D	D0	0	4.30	<1.30	2.66	5.95
	D7	7	3.88	3.78	1.39	5.85
	D14	14	4.88	4.38	7.72	8.05
	D21	21	4.98	4.67	6.61	7.15
	D28	28	4.37	3.91	6.42	7.34
	D35	35	4.28	4.36	4.33	7.20
	D49	49	4.19	1.30	0.92	5.79
E	E0	0	4.08	<1.30	2.06	5.97
	E7	7	5.45	<1.30	3.48	5.94
	E14	14	2.70	<1.30	3.52	6.00
	E21	21	3.58	<1.30	4.23	5.71
	E28	28	3.51	<1.30	1.19	5.73
	E35	35	3.41	<1.30	1.78	5.69
	E49	49	3.31	<1.30	1.60	5.16

Direct counting

Aliquots of water samples were stained with 4′,6-diamidino-2-phenylindole, and the total bacterial count (TBC; cells/mL) was determined using an BX-50 epifluorescence microscope (Olympus, Tokyo, Japan) according to the method of Porter and Feig (1980).

Construction and analysis of 16S rDNA and *Vibrio* libraries

Both 16S rDNA libraries and *Vibrio* libraries were constructed according to the methods of Sugita et al. (2005), with minor modifications. DNA of the microbial cells collected on the membrane filter was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, CA, USA) according to a manufacturer's instruction. The sequences of 16S rDNA were amplified by polymerase chain reaction (PCR) using the universal primers 20F (5'-AGAGTTTGATCCTGGCTCAG-3') and r2L (5'-CATCGTTTACGGCGTGGAC-3'; Hiraishi 1992); the sequences of *Vibrio*-specific DNA were amplified by PCR using the *Vibrio*-specific primers VIB-F (5'-CTACTTGGAGGTTGTGGCCT-3') and VIB-R (5'-GCTGGCAAACAAGGATAAG-3', Chen

et al. 2022). The resulting amplicons were cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions, yielding both 16S rDNA libraries and *Vibrio* libraries.

Identification of bacterial isolates and clones

DNA sequences of clone inserts and bacterial isolates were amplified by PCR and analyzed according to the method of Hiraishi (1992) with a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, MA, USA). The sequences were resolved on a Model 3130*xl* automated DNA sequencer (Applied Biosystems). The final sequences (approximately 780 bp for 16S rDNA; 290 bp for *Vibrio*-specific DNA) were determined from overlapping sequence data using the AutoAssembler ver. 2.1 computer program (Applied Biosystems). Chimeric sequences were identified and removed using Bellerophon software (Huber et al. 2004). Bacterial isolates and clones were identified using EZBioCloud (Yoon et al. 2017) based on the isolates' and clones' 16S rDNA sequences. Representative sequences from this study have been deposited into the DDBJ/GenBank/EMBL databases under Accession Numbers LC685585 to LC685592.

Real-time PCR

Abundance (copies/mL) of *Vibrio* spp. in the rearing water of tiger puffer was estimated by qPCR with the *Vibrio*-specific primer set, VIB-F and VIB-R, according to the method of Chen et al. (2022). qPCR analysis was performed in triplicate with QuantiFast SYBR Green PCR kit (Qiagen, Dusseldorf, Germany). A pGEM plasmid containing a 310-bp length of 16S rDNA from *Vibrio proteolyticus* strain No. 442 was used as the standard for the calibration curve.

Statistical analysis

The Euclidean distance defines the similarity between two samples, and the distance can be represented as the difference between analytical values of the samples (Zhang et al. 2011). Therefore, the Euclidean distance was calculated from the logarithmic values of each item, and a cluster analysis by UPGMA (unweighted pair-group average) was performed using PAST 4.03 software (Hammer et al. 2001) to produce a dendrogram.

Results and discussion

Bacterial densities in the rearing water

Detection level of qPCR assay was approximately 30 copies per reaction. There was a good correlation between Ct (cycle threshold) values and concentrations of standard DNA (copies) prepared from *V. proteoluticus*, yielding a linear plot with a regression coefficient (r^2) of 0.985-0.989. Since 50 µL of each diluted sample was inoculated into TCBS medium for incubation, the detection limit for this medium was 2×10^1 CFU/mL.

Table 1 shows the viable counts (colony forming units, CFU/mL) on 1/20 PYBG and TCBS agars, the abundance (copies/mL) of *Vibrio* spp., and the total bacterial counts (cells/mL) in rearing water samples during the experiment. In Tanks A–D (containing 10 or 3 pufferfish), the abundance of *Vibrio* spp. and total bacterial counts, along with the viable counts of bacteria as assessed by plating on 1/20 PYBG and TCBS agars, were low on Days 0 and 7, before the addition of fish, whereas on Days 14 – 35, the densities of all bacterial parameters increased by one to four orders of magnitude. These parameters also decreased two weeks after the fish were removed from the tanks, approaching baseline (Day-0) levels. On the other hand, in Tank E, which did not house any fish during the experimental period, there was no apparent change in any of the parameters, unlike the other tanks, which housed puffer fish.

Fig. 1 shows the relationship between the abundance (copies/mL) of *Vibrio* spp. estimated by qPCR and the total bacterial count (cells/mL) in seawater from Tanks A – E. The abundance of *Vibrio* spp. present increased linearly with the total bacterial count. Since the 16S rRNA gene copy number of *Vibrio* spp. is

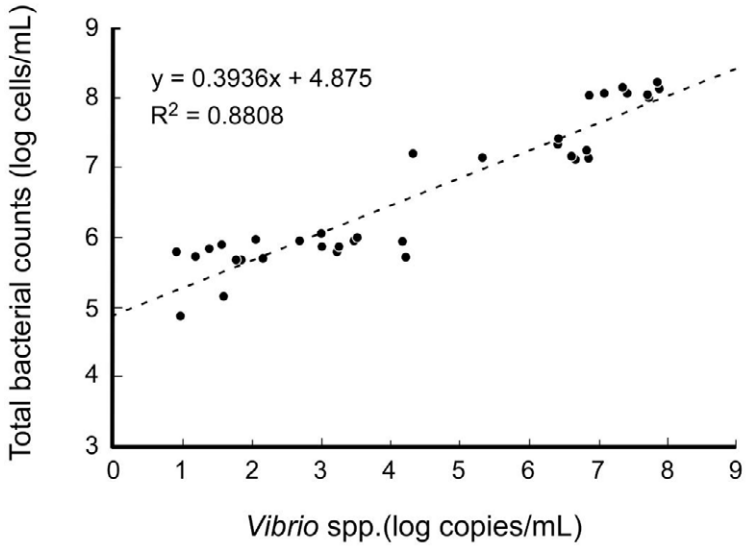

Fig. 1 Abundance of *Vibrio* spp. determined by *Vibrio*-specific qPCR method in 35 samples are plotted against the total bacterial count. The equation and r^2 values of the regression line are indicated.

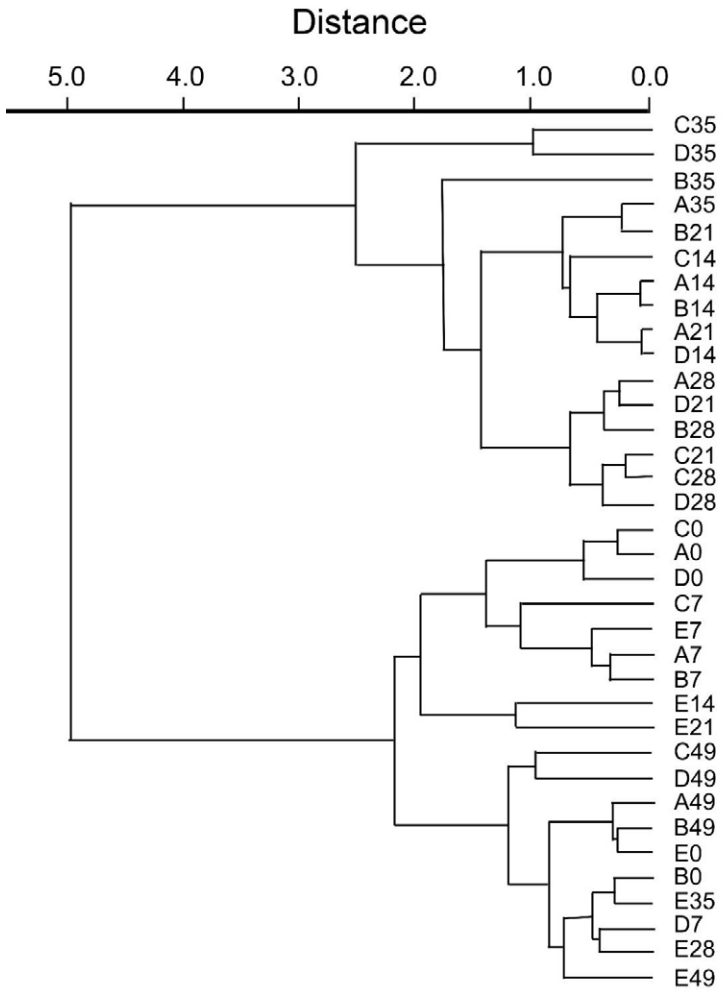

Fig. 2 A dendrogram showing the relationships among 35 water samples based on Euclidean distance of three bacterial parameters using UPGMA (unweighted pair group method with arithmetic mean) analysis. Log-transformed data for values in rearing water of viable counts (cells/mL) of bacteria (obtained by plating to 1/20 PYBG agars), abundance (copies/mL) of *Vibrio* spp., and total bacterial counts (cells/mL).

Table 2 Class composition of the clones in 16S rDNA-libraries and among the bacterial isolates obtained by plating rearing water samples on 1/20 PYBG medium.

Class	No. of clones in libraries of:				No. of isolates from:		
	B35	C35	E0	E35	B35	C35	E35
Actinomycetia	0	1	0	6	0	0	0
Alphaproteobacteria	4	24	39	33	0	2	10
Bacilli	0	0	0	3	0	0	0
Cytophagia	0	8	0	0	0	1	1
Flavobacteriia	0	1	3	0	0	2	1
Gammaproteobacteria (genus *Vibrio*)	1	0	0	0	0	7	0
Other Gammaproteobacteria	47	13	8	7	20	7	8
Holophagae	0	0	0	0	0	1	0
Peribacteria	0	2	1	2	0	0	0
Phycisphaerae	0	0	1	0	0	0	0
Saccharimonas	0	1	0	0	0	0	0
Saprospiria	0	2	0	0	0	0	0
Thermoanaerobaculia	0	0	0	1	0	0	0
Total	52	52	52	52	20	20	20

6-15 copies/cell (Ribosomal RNA Data Base (*rrn*DB) version 5.7; https://rrndb.umms.med.umich.edu/), it is assumed that the abundance (copies/mL) of *Vibrio* spp. is one order of magnitude higher than the density (cells/mL) of *Vibrio* spp. In other words, the density (cells/mL) of *Vibrio* spp. is about two orders of magnitude lower than that of total bacterial count, even in the rearing water on Days 14 to 28 in Tanks A– D, where the abundance of *Vibrio* spp. is high.

Fig. 2 provides a dendrogram showing the relationship among 35 samples based on the Euclidean distance derived using UPGMA analysis. The samples on Days 14–35 from Tanks A–D formed a single cluster, showing distinct differences from the samples obtained from the tanks on Days 0, 7, and 45, and from all seven water samples obtained from Tank E, indicating that bacterial parameters in each tank were relatively similar to each other during the puffer rearing period.

Microbiota in the rearing water determined by 16S DNA libraries and by plating to 1/20 PYBG agars

Table 2 shows the class composition of the clones in the 16S rDNA libraries and among the bacterial isolates obtained by plating water samples to 1/20 PYBG agars. In the 16S rDNA libraries of Samples E0 and E35, Alphaproteobacteria was the predominant class, accounting for 63–75% of the total clones, and Gammaproteobacteria excluding *Vibrio* spp. accounted for 14–15%. In contrast, Gammaproteobacteria excluding *Vibrio* spp. was the predominant class, accounting for 90%, in Sample B35; Alphaproteobacteria accounted for 46% and Gammaproteobacteria excluding *Vibrio* spp. accounted for 25% in Sample C35. In addition, one clone of *Vibrio* sp. was detected but only in the B35 water sample.

Bacterial flora of Sample B35, isolated by plating to 1/20 PYBG medium, was composed only of Gammaproteobacteria excluding *Vibrio* spp. In Sample C35, *Vibrio* spp. accounted for 35% and Gammaproteobacteria excluding *Vibrio* spp. accounted for 35%. In Sample E35, Alphaproteobacteria accounted for 50% and Gammaproteobacteria excluding *Vibrio* spp. accounted for 40%. The fact that viable counts on TCBS agars in Samples B35, C25, and E35 were three to five orders of magnitude lower than the total bacterial count indicated that the percentages of *Vibrio* spp. in the microbiota of these samples were so low that *Vibrio* spp. would not have been detected by the non-selective 16S rDNA library or plating to 1/20 PYBG medium (Table 1).

Vibrio spp. in the rearing water, as determined by *Vibrio*-specific PCR and plating to TCBS medium

Table 3 shows species composition of the clones in *Vibrio* libraries constructed with *Vibrio*-specific PCR and among the bacterial isolates obtained by plating B35, C35, E0, and E35 water samples to TCBS agars. In

Table 3 Species composition of clones in *Vibrio*-libraries and among the bacterial isolates obtained by plating rearing water samples on TCBS medium"

Closest species (accesion no.; identity, %)	No. of clones in libraries of:				Isolates from samples of:	
	B35	C35	E0	E35	B35	C35
Shewanella schlegeliana (AB081760; 100)	0	0	0	0	0	1
Vibrio aestuarianus subsp. *francensis* (AJ845017; 99.0)	0	0	0	1	0	0
Vibrio alfacsensis (JF316656; 99.1-100)	0	6	1	0	8	9
Vibrio crassostreae (CCJW01000022; 99.2-100)	0	0	0	0	9	6
Vibrio crosai (JQ434120; 99.1-100)	0	15	1	7	0	0
Vibrio ezurae (BATM01000062; 97.7-100)	5	0	0	0	0	0
Vibrio pomeroyi (AJ491290; 100)	0	0	0	0	1	0
Vibrio renipiscarius (JTKH01000028; 100)	1	0	0	0	0	0
Vibrio rotiferianus (AJ316187; 98.2-100)	15	5	2	1	6	9
Vibrio scophthalmi (AFWE01000105; 100)	0	0	0	0	2	1
Vibrio tasmaniensis (AJ514912; 98.2-100)	34	26	9	23	4	3
Vibrio vulnificus (AMQV01000037; 99.1-100)	0	2	38	12	0	0
Total	55	52	13	32	30	29

Vibrio libraries of B35, C35, E0, and E35, clones belonging to *Vibrio tasmaniensis* (AJ514912) and *Vibrio rotiferianus* (AJ316187) accounted for 2.3 – 61.8% of clones in all libraries. The B35 library consisted of *V. tasmaniensis* and *V. rotiferianus*, while the C35 library consisted of *V. tasmaniensis* and *Vibrio crosai* (JQ434120). In contrast, the E0 and E35 libraries consisted of *V. vulnificus* (AMQV01000037), *V. tasmaniensis*, and *V. crosai*. Although these four *Vibrio* libraries differed in terms of the represented species and the proportions thereof, these four libraries consisted exclusively of *Vibrio* species, confirming that the *Vibrio*-specific primers have high specificity and are suitable for quantifying *Vibrio* species in seawater along with fish gut (Chen et al. 2022).

Furthermore, the bacterial flora isolated from Samples B35 and C35 by plating to TCBS medium were characterized as *Vibrio alfacsensis* (JF316656), *Vibrio crassostreae* (CCJW01000022), and *V. rotiferianus* (AJ316187), of which *V. alfacsensis* and *V. rotiferianus* also were detected in the *Vibrio* libraries. However, one isolate of *Shewanella schlegeliana* (AB081760), which belongs to the Shewanellaceae of Gammaproteobacteria, was detected in Sample C35. Since the percentages of *Vibrio* spp. in the water samples in this study were extremely low, as shown in Table 2, it would have been essentially impossible to detect *Vibrio* spp. in the 16S rDNA libraries or among cells obtained by plating to 1/20 PYBG medium. However, the detection sensitivity of *Vibrio*-specific PCR and plating to TCBS medium for *Vibrio* spp. was found to be very high, given that these methods detected low densities of *Vibrio* spp. (Table 3). Nonetheless, plating to TCBS agar medium did not work well for the Day-0 water samples from Tanks A, B, and D and for all samples from Tank E, as the densities of *Vibrio* spp. in these samples were below the detection limit ($<2\times10^1$ CFU/mL).

Evaluation of qPCR with *Vibrio*-specific primers

We next considered the quantification of *Vibrio* spp. by qPCR with *Vibrio*-specific primers and plating to TCBS medium. Given that the former method involves a filtration step, *Vibrio* spp. can be concentrated by filtering large volumes of water samples, even if the density of these bacteria is so low that these organisms cannot be detected by plating to TCBS medium; thus, qPCR seems to possess superior detection ability. In addition, the density of *Vibrio* spp. can be estimated by the plate count method using TCBS medium at 24–48h (35–37°C incubation) or 96–120h (20–25°C incubation), whereas by the qPCR method at 5–6h. However, the plate counting method using TCBS medium is simpler and less expensive than qPCR and should not be discarded in non-urgent cases. It would be wise to use both methods according to the conditions required.

Dynamics of *Vibrio* spp. in the rearing water of tiger puffer

In the environment without fish, such as Day 0 rearing water in Tanks A-D and the rearing water in Tank E,

Vibrio spp. are very minor in the water because of the low organic matters and the absence of animals excreting fecal matters containing *Vibrio* spp. In such environment, Alphaproteobacteria, Gammaproteobacteria (excluding *Vibrio* spp.), and Flavobacteriia are predominant (Lee and Eom 2016; Kurosaki et al. 2021). When fish are housed in tanks and fed food, the bacteria that grow in their intestines are released into the water avia their feces, and the mucus and uneaten food of the fish are added to the rearing water, so that these organic substances become nutrients for the heterotrophic bacteria in the rearing water. *Vibrio* spp., along with other bacteria, increases by utilizing these organic materials and increases to about 1% of the total bacterial population. When the fish are removed again, the bacteria along with *Vibrio* spp. decrease again to their original level due to the reduced supply of nutrients. The dynamics of *Vibrio* spp. suggests that they are not necessarily the predominant bacteria in natural seawater as well as in the fish gut (Chen et al. 2022). The observed phenomenon, in which the number of heterotrophic bacteria increases when fish are reared and decreases when fish are removed, previously has been reported with carp (*Cyprinus carpio*) and Japanese flounder (*Paralichthys olivaceus*) (Sugita et al. 1985, 1988).

Furthermore, Mougin et al. (2021) found that biofilms on the concrete walls of tanks harbor Vibrionaceae, especially *Vibrio harveyi*, which are released into the water. In other words, since biofilms are potential reservoirs of pathogenic bacteria, these concrete walls as well as the rearing water should be monitored regularly; if necessary, these surfaces and biofilms also should be disinfected with chlorine and ozone after the water is drained (Sugita et al. 1992).

In other work, Kurosaki et al. (2021) investigated the intestinal microbiota of red seabream (*Pagrus major*) at early life stages and found that *Vibrio* spp. were not detected in the larval stage, whereas *Vibrio* spp. accounted for 29-65% of gut microbiota in the juvenile stage. This result showed that the gut microbiota of red seabream varies greatly depending on the developmental stage. Similar trends have been observed in the early stages 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), and yellowtail kingfish (*Seriola lalandi*; Walburn et al. 2019). Moreover, Ringø et al. (1995) and Asfie et al. (2003) reported that the intestinal microbiota of salmonid fish and goldfish (*Carassius auratus*) displays day-to-day fluctuations and individual variation, respectively. Parris et al. (2019) examined the intestinal microbiota of maroon clownfish (*Premnas biaculeatus*) at five time points per day over a 2-day interval; the resulting data indicated that feeding rapidly alters the microbiome composition of the clownfish gut. Together, these reports suggest that the microbiota and *Vibrio* density in the fish gut vary depending on internal and external factors of the fishes. These facts indicate that the behaviors of gut microbiota must be kept in mind while testing for abundance of *Vibrio* spp. in the fish gut, environments, and live diets; this surveillance is essential as a risk management measure to prevent opportunistic infections.

However, since the 16S rRNA gene in one bacterial cell varies greatly among bacterial species, it is difficult to convert from abundance (copies/mL) to cell density (cells/mL) when assessed by the qPCR method. Since the *Vibrio* spp. in the actual samples from fish farms are composed of a variety of species (Table 3), the copy number of the 16S rRNA gene varies from species to species and cannot be expressed simply, as a single number. However, it is also true that in fish farms, abundance (copies/mL) does not give a clear picture of *Vibrio* density. According to the Ribosomal RNA Data Base (*rrn*DB) version 5.7 (https://rrndb.umms.med.umich.edu/), the mean copy number of 16S rRNA gene in *Vibrio* spp. was 10.4 (sd, 1.9), ranging from 6 to 15 copies/cell. Therefore, in fish farms, it is considered realistic to divide the qPCR-measured abundance (copies/mL) by 6-15 (copies/cell) to estimate the actual density of *Vibrio* spp. (cells/mL) as a matter of convenience, but this approach necessarily will introduce some error in the calculated density.

Conclusion

Vibrio spp. are a major causative agent of opportunistic infections in marine fishes and are an important risk factor in marine fish farm management. Therefore, the surveillance of *Vibrio* spp. in the fish gut, rearing water, and live diets is necessary for protecting against vibriosis in farmed fish. In the present study, juvenile tiger puffer (*Takifugu rubripes*) were housed in experimental tanks for a short period of time, and the total bacteria, viable counts (as obtained by plating to agar media), and abundance of *Vibrio* spp. in the rearing water were determined over time. The results showed that qPCR using *Vibrio*-specific primers is suitable

for the detection and quantification of *Vibrio* spp. in the gut and environmental water of farmed fish.

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Authors' contributions KS performed molecular genetic analysis; RM, and YT collected material and conducted bacteriological experiment. 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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