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Short Communication

Culturable microflora of *Artemia franciscana* reared under laboratory conditions

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Abstract: *Artemia* is widely used as an initial food for larval and juvenile fish in aquaculture facilities around the world. However, several lines of research have strongly suggested that *Artemia* larvae may carry opportunistic pathogens such as *Listonella anguillarum*, thereby serving as a source of infection of fish. In the present study, we investigated the dynamics of the culturable microflora of *Artemia* reared under laboratory conditions, with the goal of understanding the risk of opportunistic infection mediated by this animal. After hatching decapsulated cysts of *A. franciscana*, the larvae were reared for an additional 27 days to examine, using the culture-dependent method, the culturable microflora of the rearing water and of washed *Artemia*. The results showed that Vibrionaceae, Flavobacteriaceae, Pseudoalteromonadaceae, Alteromonadaceae and Rhodobacteriaceae accounted for 8.3-35.8% of the rearing water isolates. In contrast, Vibrionaceae dominated in *Artemia* isolates, accounting for 79.2% of the flora. However, Vibrionaceae were not detected in either decapsulated or undecapsulated cysts, or in the algal concentrates used as feed, suggesting that Vibrionaceae is not indigenous to *Artemia* cysts and instead is derived primarily from natural seawater. These results strongly suggest that hatching and rearing live diets such as *Artemia* under sanitary conditions may reduce the risk of opportunistic infection.

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Introduction

Artemia is one of the most important foods for marine fish in aquaculture facilities worldwide, as its cysts can be incubated in seawater or salt water for one to two days to produce nauplii of appropriate size. In addition, *Artemia* can be enriched with special nutrients such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for the use of *Artemia* in the diets of captive animals that require these substances (Lavens and Sorgeloos, 1996). However, Kurosaki et al. (2021), in an examination of the gut microbiota of red seabream, *Pagrus major* during early development, found that *Vibrio* spp. in larval guts are derived primarily from rotifers *Brachionus plicatilis* sp. complex S-type and *Artemia* sp. nauplii used as the diet. Previously, Mizuki et al. (2006), in an investigation of the dynamics of the fish pathogen, *Listonella anguillarum* in a hatchery of Japanese flounder, *Paralichthys olivaceus*, suggested that *L. anguillarum* was being transmitted from rotifers, *B. plicatilis* and *Artemia* nauplii. Similar results have been reported by many researchers (Grisez et al., 1977; Tanasomwang and Muroga, 1988; López-Torres et al., 2001; Eddy and Jones, 2002). However, to our knowledge, there are no reports on the dynamics of the microflora of *Artemia* during the long-term rearing of this animal.

In marine fish farming, *Vibrio* spp. often is detected in the fish gut, and the rearing environment contains a number of opportunistic pathogens; the presence of these pathogens is an extremely important risk factor in the aquaculture industry. Therefore, in this study, we investigated the dynamics of the culturable microflora of *Artemia* reared for relatively long periods of time, specifically by employing a culture-dependent approach in combination with 16S rRNA gene (16S rDNA) sequencing.

Materials and Methods

Experiments were performed with *A. franciscana*

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cysts obtained from the Great Salt Lake, Utah, USA, as supplied by OSI Marine Lab (Snowville, UT, USA). *Artemia* cysts were decapsulated as described by Lavens and Sorgeloos (1996). For hatching and rearing *Artemia*, a glass medium bottle with a capacity of 1,200 mL was used; two glass tubes with cotton filters attached were inserted into the lid of the bottle, and the air was delivered from an air pump into one of the tubes. The tip of another glass tube was placed above the water surface, permitting exhaust of spent air into the atmosphere. After the apparatus without an air pump was autoclaved at 121°C for 15 min and allowed to cool to room temperature, 1,000 mL of unsterilized natural seawater and 0.01 g of decapsulated cysts were placed in the bottle; the culture then was incubated at 25°C with aeration at a flow rate of 2.9 mL/sec and exposed to constant incandescent light. The natural seawater collected on Enoshima Island, Fujisawa, Kanagawa was used. *Artemia* larvae were fed an algal concentrate (Chlorella Industry Co., Tokyo, Japan) once every two days for an additional 27 days.

Surface bacteria loosely attached to the *Artemia* body were removed according to the method of Austin and Allen (1982). Specifically, sterile seawater was added to ten washed *Artemia* larvae to make a total volume 100-500 μL depending on *Artemia* size, which then was homogenized to generate a washed sample. *Artemia* homogenates and rearing water sampled on days 0, 1, 7, 14, 21, and 28 were diluted serially with sterile seawater and inoculated onto 1/20 PYBG agar medium, which contains the following (per 1000 mL of 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 is adjusted to pH 7.5 (Sugita et al., 2005). The inoculated agar plates were incubated at 25°C for 7 days under aerobic conditions. Additionally, homogenates of both decapsulated and undecapsulated cysts, as well as natural seawater and an algal concentrate, were processed similarly. After incubation, the viable counts (colony-forming units (CFUs) per mL or nauplius) were determined, and 19-20 colonies per sample were chosen and purified by streaking to a fresh solid medium.

16S rDNA of each isolate was amplified and sequenced according to Hiraishi (1992), with some modifications. Briefly, 16S rDNA was amplified by polymerase chain reaction (PCR) using a primer set consisting of 20F (5'-AGA GTT TGA TCC TGG CTC AG-3') and r2L (5'-CATCGTTTACGGCGTGGAC-3'), and the resulting fragments were partially sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit with a Model ABI 3130*xl* automated sequencer (Applied Biosystems, Foster City, CA, USA). The final sequence (approximately 690 bp) was determined from overlapping sequence data using the AutoAssembler ver. 2.1 computer program (Applied Biosystems). The isolates 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 (Accession Numbers: LC13330-LC13337).

Results and Discussions

A total of 299 bacteria were isolated, and all were classified into 22 families based on 16S rDNA sequences. Vibrionaceae (140 isolates), Pseudoalteromonas (34), Flavobacteriaceae (26), Moraxellaceae (16), Alteromonadaceae (15), Stappiaceae (12), and Rhodobacteriaceae (12) were the major families of isolates obtained in this study. Notably, no bacteria were detected in the decapsulated cysts. Table 1 shows the composition of 16 families in bacterial isolates from the undecaspulated cysts, from an algal concentrate, and from natural seawater, which exhibited viable counts of 1.2×10^6 CFU/g, 6.5×10^5 CFU/mL, and 6.6×10^3 CFU/mL, respectively. Moraxellaceae and Planococcaceae accounted for 25.0-60.0% of the isolates from undecapsulated cysts; Microbacteriaceae, Bacilli, and Moraxellaceae accounted for 20.0-30.0% of the isolates from the algal concentrate. However, bacteria belonging to Vibrionaceae were not isolated from either of those

Table 1. Composition of bacterial families in isolates derived from the undecapsulated cysts, an algal concentrate, and natural seawater.

Closest family	Undecapsulated cysts	Algal concentrate	Natural seawater
Bacillaceae	1	6	0
Cellulomonadaceae	0	1	0
Erythrobacteraceae	0	0	1
Flavobacteriaceae	0	0	1
Gordoniaceae	0	1	0
Halomonadaceae	2	0	1
Intrasporangiaceae	0	1	0
Maricaulaceae	0	0	1
Microbacteriaceae	0	6	0
Microbulbiferaceae	0	0	7
Moraxellaceae	12	4	0
Nocardiaceae	0	1	0
Planococcaceae	5	0	0
Rhodobacteraceae	0	0	1
Salinisphaeraceae	0	0	1
Stappiaceae	0	0	4
Vibrionaceae	0	0	2
Viable counts (CFU/g, mL)	1.2×10^6	6.5×10^5	6.6×10^3

samples. Nine families were isolated from the natural seawater, with Microbulbiferaceae and Stappiaceae accounting for 21.1-36.8% and Vibrionaceae accounting for 10.5% of those isolates.

Ten families were isolated from the *Artemia* rearing water, with viable counts of 5.0×10^4-2.6×10^7 CFU/mL (Table 2). Vibrionaceae, Flavobacteriaceae, Pseudoalteromonadaceae, Alteromonadaceae, and Rhodobacteriaceae accounted for 8.3-35.8% of the rearing water isolates. Bacteria isolated from washed *Artemia* ranged in viable counts from 1.4×10^4-1.1×10^7 CFU/nauplius and consisted of six families that Vibrionaceae and Pseudoalteromonadaceae were the top-two-most-dominant bacteria, accounting for 79.2 and 14.2%, respectively. Viable counts of bacteria in rearing water and washed *Artemia* during the rearing period varied by as much as three orders of magnitude, and the families of bacteria detected varied widely, indicating that the densities of the dominant culturable bacteria in each sample also varied greatly.

Table 3 shows the species composition of Vibrionaceae in isolates from the natural seawater, rearing water, and washed *A. franciscana.* *Photobacterium* consisted of one species, and *Vibrio* of seven species (Table 3). Among the *Vibrio*, *V. parahaemolyticus* (BBQD01000032) and *V. tubiashii* (CP009354) are known to act as pathogenic bacteria in marine animals. *V. azureus* (BATL01000140), *V. hyugaensis* (LC004912), and *V. neocaledonicus* (JQ934828) were detected in 50.0-66.7% of the rearing water samples and 83.3-100% of the washed *Artemia* samples. These results suggested that bacteria of the genus *Vibrio* have a high affinity for *Artemia* because these bacteria are easily taken up into the gut of *Artemia* or adhere to these animals' body surfaces. *Vibrio* spp. are predominantly found in the gut and on the body surface of copepods, and *V. cholerae* and *V. parahaemolyticus* adhere to copepod shells, using this matrix as a nutrient source and as a cryoprotective environment (Kaneko and Colwell, 1975; Sochard et al., 1979; Shimodori et al., 1989). However, the differences among the *Vibrio* species in their frequencies and percentage of the bacteria may reflect the compatibility of each bacterial species with *Artemia* and the rearing environment. The large fluctuations in *Vibrio* representation in

Table 2. Composition of bacterial families in isolates derived from rearing water and washed *Artemia*.

Closest family	No. of isolates from rearing water						No. of isolates from washed *Artemia*					
	Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 0	Day 1	Day 7	Day 14	Day 21	Day 28
Alteromonadaceae	0	1	0	9	0	2	0	0	0	3	0	0
Bacillaceae	0	0	0	0	0	1	0	0	0	0	0	0
Cyclobacteriaceae	0	0	0	0	0	2	0	0	0	0	0	0
Enterobacteriaceae	0	0	0	1	0	0	0	0	0	0	0	0
Erythrobacteraceae	0	0	0	1	0	1	0	0	0	0	0	0
Flavobacteriaceae	0	0	13	0	4	8	0	0	0	0	0	0
Oceanospirillaceae	0	0	0	0	0	0	3	0	0	0	0	0
Pseudoalteromonadaceae	6	10	1	0	0	0	10	0	0	5	2	0
Rhodobacteraceae	0	0	1	3	2	4	0	0	0	1	0	0
Stappiaceae	0	0	0	5	0	2	0	0	0	1	0	0
Vibrionaceae	14	9	5	1	14	0	7	20	20	10	18	20
Viable counts (CFU/mL, nauplius)	1.8×10^{6}	2.6×10^{7}	1.2×10^{6}	4.3×10^{5}	5.0×10^{4}	1.3×10^{6}	1.4×10^{4}	7.5×10^{5}	4.9×10^{5}	2.4×10^{5}	1.4×10^{6}	1.1×10^{7}

rearing water and among *Artemia*-associated bacteria during the *Artemia* rearing period may be related to the effects of *Artemia* shell molting and other factors; further research will be needed to clarify these patterns.

Since no bacteria were detected in decapsulated cysts, the *Vibrio* spp. detected in *Artemia* and its rearing water presumably existed at low densities in natural seawater and/or in the algal concentrate, subsequently multiplying and becoming dominant in the *Artemia*-rearing environment.

We hypothesize, by extension, that many opportunistic pathogens, including *L. anguillarum*, are not cyst-derived, but instead derive from the live diets and/or the seawater environments in which cysts are hatched and reared for a period of time (Mizuki et al., 2006). López-Torres et al. (2001) reported that *Artemia* larvae are vectors of *Vibrio* spp. in aquaculture activities. However, the present work suggests that these *Vibrio* spp.. Instead, result from the manipulation of *Artemia* hatchery tanks and are not derived from the cysts themselves. In other words, *Vibrio* spp. are ubiquitous in aquaculture facilities, but keeping *Artemia* in an environment free of opportunistic pathogens may be an effective way to prevent opportunistic infections in the early developmental stages of marine fish being fed *Artemia*. These previous results, viewed in the context of the present study, lead us to recommend that *Artemia* cysts be hatched in seawater and containers that both have been disinfected, and be reared on foods and food additives that are confirmed to be free of opportunistic pathogens. We propose that similar studies also should be performed on rotifers to facilitate further effective control of opportunistic infections in seed production for aquaculture.

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Table 3. Composition of bacterial species belonging to Vibrionaceae among isolates derived from natural seawater, rearing water, and washed *Artemia*.

Closest species (accession no.; identity, %)	Natural seawater	No. of isolates from rearing water						No. of isolates from washed *Artemia*					
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 0	Day 1	Day 7	Day 14	Day 21	Day 28
Photobacterium salinisoli (KP054474; 97.7)	0	0	0	0	0	0	0	0	1	0	0	0	0
Vibrio azureus (BATL01000140; 98.8-100)	1	4	4	0	1	5	0	2	9	7	7	15	12
Vibrio hyugaensis (LC004912; 98.8-99.9)	0	1	2	0	0	8	0	2	9	9	0	2	6
Vibrio japonicus (LC143378; 99.4-99.6)	0	0	0	0	0	0	0	2	1	0	0	0	0
Vibrio natriegens (ATWU01000093; 100)	0	1	0	0	0	0	0	0	0	0	0	0	0
Vibrio neocaledonicus (JQ934828; 98.6-100)	0	7	3	5	0	1	0	1	0	4	3	1	1
Vibrio parahaemolyticus (BBQD01000032; 98.4-99.3)	0	1	0	0	0	0	0	0	0	0	0	0	1
Vibrio tubiashii (CP009354; 99.2)	1	0	0	0	0	0	0	0	0	0	0	0	0

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