Intestinal Microflora of Larval and Juvenile Stages in Japanese Flounder (*Paralichthys olivaceus*)

Varin TANASOMWANG and Kiyokuni MUROGA

Faculty of Applied Biological Science,
Hiroshima University, Higashi-Hiroshima 724, Japan

(Received October 21, 1987)

Investigations on the aerobic bacterial flora in the intestine of larval and juvenile stages in Japanese flounder (*Paralichthys olivaceus*) were carried out at two different fisheries stations. The bacterial flora of ambient water and feeds were also analyzed. The number of bacteria in the intestine of larvae measuring 10 mm in total length was 10^5 CFU/fish, remained nearly constant until 14 mm, and decreased to 10^4 CFU/fish in 15-19 mm. The decrease in bacterial numbers was hypothesized to be due to the change in feeds from live diets (rotifer and brine shrimp) to artificial diets. The bacterial numbers on ZoBell's 2216e agar were 10^4 , 10^5 , 10^6 , and 10^8 CFU/ml or g in water, live feeds, artificial feed and minced fish, respectively.

The composition of intestinal flora was characterized by two predominating groups of *Vibrio* and *Pseudomonas* followed by *Moraxella*, *Cytophaga* and *Alcaligenes*. Similar generic composition was recovered in water and live diets, while those of artificial diets comprised of *Acinetobacter* and Gram-positive bacteria in addition to *Moraxella*. *Vibrio alginolyticus* was frequently isolated from fish examined in one station where no apparent fish mortality occurred.

Seed production of marine fishes has been remarkably developed in Japan recently. Juveniles of various economically important species such as red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli*), and Japanese flounder (*Paralichthys olivaceus*) are mass produced mainly to stock feral population for fishing and partly to supply the seedlings for net cage culture. Fish production techniques, particularly for the above mentioned three species, are apparently well established. However, in actual fish production, difficulties are encountered in controlling diseases because high mortalities often occur during the larval and juvenile stages of fish.

Mass mortalities of juveniles associated with bacterial infections like *Pasteurella piscicida*, *Vibrio anguillarum*, and *Flexibacter maritimus* infections in sea breams and tiger puffer (*Takifugu rubripes*) have been documented (MUROGA *et al.*, 1977, 1987b; MUROGA and TATANI, 1982; WAKABAYASHI *et al.*, 1984, 1986). However, few investigations have elucidated the causes of mortalities in the larval stage of these farmed fishes. The mortalities of red and black sea bream larvae with abdominal swelling have been reported to

be caused by *V. alginolyticus* (IWATA *et al.*, 1975, 1978; KUSUDA *et al.*, 1986). Flounder larvae with opaque intestine, known as chōkan-hakudaku-shō in Japanese, has been ascribed to *Vibrio* sp. (MURATA, 1987).

Intestinal infections during the larval stage of various fishes remain unresolved. Thus, studies on the intestinal bacterial flora of both diseased and normal fish are essential to elucidate the causes and mechanisms of these infections. A study on the normal intestinal microflora of red and black sea bream has been reported (MUROGA *et al.*, 1987a). Investigation on the intestinal flora of healthy Japanese flounder is presented in this study.

Materials and Methods

Fish samples

Flounder were sampled from two different stations. The main one was a station of Japan Sea-Farming Association (JASFA) located in Hakata Island, Ehime Prefecture and the other was Hiroshima Prefectural Fisheries Experimental Station (HPFES) in Onoda, Hiroshima Prefecture.

Table 1. Fish sampling data taken at JASFA*¹ and HPFES*²

	Sampling date (1986)	Days after hatching	Total length (mm)	Water temperature (°C)	No. of fish examined
JASFA					
Pond 6	April 23	1	3.2	18.6	20×4 groups
	30	8	5.6	20.0	20×4
	May 7	15	9.1	19.0	20×4
	14	22	13.3	18.8	20×4
	21	29	14.7	18.9	20×4
	28	36	14.3	18.4	10×4
Pond 8	April 16	6	4.1	19.7	20×6
	23	13	7.6	19.6	20×4
	30	20	11.0	21.0	20×4
	May 7	27	13.7	19.0	20×6
	14	34	13.9	19.0	20×4
	21	41	15.8	18.8	10×4
	28	48	19.2	17.7	10×4
HPFES					
Tank 1	June 2	20	10.0	17.3	20×2
	11	29	12.2	18.8	20×2
Tank 2	June 2	20	9.0	17.3	20×2
	11	29	12.3	18.5	20×2

*¹ Japan Sea-Farming Association of Hakata Island*² Hiroshima Prefectural Fisheries Experimental Station

Fig. 1. Bacterial counts in the intestine of larval and juvenile flounder. ●—●: count on ZoBell, ○—○: count on BTB, JASFA: Japan Sea-Farming Association of Hakata Island, HPFES: Hiroshima Prefectural Fisheries Experimental Station, R: rotifer, BS: brine shrimp, AF: artificial feed, MF: minced fish, S-R: S-type rotifer, L-R: L-type rotifer.

In JASFA, fish were sampled once a week from two rearing ponds, pond 6 and 8, from April 16 to May 28, 1986. Details of fish sampling are given in Table 1. The fish were reared in well-

aerated circular concrete ponds containing 130 m³ water with temperature ranging from 17.7 to 21.0°C. Initial stocking rate was 9,000 fish/m³ in pond 6 and 11,200 fish/m³ in pond 8. Live

feeds consisting of rotifers (*Brachionus plicatilis*) and brine shrimp (*Artemia salina*), artificial feed and minced fish were supplied to the fish in the order shown in Fig. 1. The pelagic larvae metamorphosed into juveniles approximately 30 days after hatching or 13–14 mm in total length. The fish were raised for about 50 days until they reached 25 mm and then transported to various areas for secondary rearing in net cages. The survival rates in pond 6 and 8 were 69.7% and 68.0%, respectively.

In HPFES, fish were examined twice on July 2 and 11, 1986. The fish in tank 1 were fed S-type rotifers while L-type rotifers to tank 2 (FUKUSHO and OKAUCHI, 1982). The water temperature was maintained at about 18°C in both tanks.

Bacteriological examination

The intestinal microflora of larvae and juveniles were examined according to the isolation technique described by MUROGA *et al.* (1987a). Groups

of fish in 20 or 10 were immersed in 0.1% benzalkonium chloride for 1 min and washed thoroughly under tap water for 30 s to remove the surface bacteria. It was confirmed by a negative result in bacterial isolation from thus-treated flounder samples without visceral part that the surface bacteria were inactivated and/or removed. After washing, the samples were homogenized in 0.85% sterile saline in a sterile glass homogenizer. Ten-fold diluted samples at 0.1 ml were spread on ZoBell's 2216e and BTB teepol agar media. Water and feed samples were simultaneously examined for viable bacterial counts. After incubation at 25°C for 48 h, the colonies on the two media were counted and 4 or 5 representatives of predominant species on ZoBell agar were selected and stocked in ZoBell medium with 0.5% agar for characterization tests. When swarming bacteria appeared, colony counting and pure culture were made after 24 h incubation.

Table 2. Percentage bacterial flora of flounder at different body lengths

		Total length (mm)	Flora (%)									
			A	B	C	D	E	F	G	H	I	J
JASFA												
Pond 6		3.2						70.6				29.4
		5.6	42.0					44.3		2.9		10.8
		9.1	29.6	46.7		11.1		5.7				6.9
		13.3		17.5	51.2	24.3						7.0
		14.7	33.8			63.3						2.9
		14.3				38.7		54.9				6.4
Mean			17.6	10.7	8.5	22.9		29.3		0.5		10.5
Pond 8		4.1	59.3	11.8				19.0				9.9
		7.6	2.7	3.6		93.7						
		11.0	24.3			12.2		58.1				5.4
		13.4		10.6		68.3					18.7	2.4
		13.9	3.4			96.6						
		15.8			8.3	25.0		8.3		16.7		41.7
Mean			12.8	3.7	1.2	47.5		12.2	60.0	2.4	2.7	3.4
HPFES												
Tank 1		10.0					26.3	48.2				25.5
		12.2	24.4			1.0		60.1				14.5
Mean			12.2			0.5	13.1	54.2				20.0
Tank 2		9.0	1.9				96.9					1.2
		12.3		57.0				35.1				7.9
Mean			1.0	28.5			48.4	17.6				4.5

A: *Vibrio* spp. I, B: *Vibrio* spp. II, C: *Vibrio* spp. III, D: *V. alginolyticus*, E: *V. anguillarum*, F: *Pseudomonas*, G: *Moraxella*, H: *Cytophaga*, I: *Alcaligenes*, J: Others.

Bacterial identification

A total of 213 isolates, 153 from JASFA and 60 from HPFES, were tested for morphological and biochemical characteristics. The identification was based on the scheme described by MUROGA *et al.* (1987a) and Bergey's Manual of Systematic Bacteriology Vol. 1 (KRIEG and HOLT, 1984).

Results

Bacterial counts and flora of flounder

Bacterial counts of larval and juvenile flounder are shown in Fig. 1. In JASFA, few bacteria on ZoBell or BTB teepol agar were observed in one day old 3.2 mm unfed larvae. After the fish were fed rotifers, the bacteria increased in direct proportion to the size of the fish until they reached about 10 mm. The number of bacteria did not change at 10^5 CFU/fish from 10 to 14 mm, and

decreased thereafter. In HPFES, the bacterial counts obtained on ZoBell agar were almost the same as those at JASFA, but the numbers on BTB agar were lower.

The intestinal microflora of the fish revealed Gram-negative bacteria, predominantly *Vibrio* and *Pseudomonas* as indicated in Table 2. At JASFA, *Pseudomonas* was most dominant (70.6%) in 3.2 mm larvae (first sampling in pond 6), but after feeding, vibrios were most frequently detected throughout the larval and juvenile stages examined. Among the several species of *Vibrio* isolated, *V. alginolyticus* was the most prevalent (22.9% for pond 6 and 47.5% for pond 8) at JASFA. On the other hand, *V. anguillarum* (13.1% for tank 1 and 48.4% for tank 2) predominated at HPFES.

Bacterial counts and flora of rearing water and feeds

The bacterial counts of rearing water are given

Table 3. Baterial counts and flora in rearing water of flounder

	Bacterial count (CFU/ml)		Flora (%)							
	ZoBell	BTB	A	B	C	D	E	F	G	H
JASFA										
Pond 6	5.8×10^3	3.7×10^2				62.0	19.0			19.0
	1.1×10^4	2.9×10^3	29.0	0.9		63.6				6.5
	2.5×10^5	1.0×10^5	13.0			28.1	58.1			0.8
	9.5×10^4	5.8×10^4	61.1	3.2						35.7
	1.5×10^6	2.6×10^6	99.0	1.0						
Mean	5.0×10^4	1.0×10^4	38.0	6.0			40.0			16.0
	7.0×10^4	2.3×10^4	40.0	1.8		25.7	19.5			13.0
	2.7×10^4	5.0×10^3	11.6			11.2	77.2			
	4.1×10^4	1.8×10^4	26.9	2.4			63.4			7.3
	8.4×10^4	9.5×10^3	4.8	16.7		71.4				7.1
Pond 8	3.1×10^4	3.8×10^4	19.4	38.7				35.5		6.4
	8.3×10^4	3.2×10^4	37.3			15.6	34.9			12.2
	8.9×10^4	1.6×10^4	56.2	13.5						30.3
	1.5×10^5	1.5×10^4	2.6	0.7		87.6	5.2			3.9
	6.1×10^4	1.6×10^4	22.7	10.3		26.5	25.8	5.1		9.6
HPFES										
Tank 1	3.4×10^4	6.9×10^3	23.5		17.6	29.4				29.4
	1.5×10^4	3.9×10^3	72.0							28.0
Mean	2.3×10^4	5.2×10^3	47.8		8.8	14.7				28.7
Tank 2	1.4×10^4	3.2×10^3	6.6		27.9	47.8			7.4	10.3
	3.7×10^4	2.0×10^4	83.8			8.1				8.1
Mean	2.3×10^4	8.0×10^3	45.2		14.0	27.9			3.7	9.2

A: *Vibrio* spp. (I, II and III), B: *V. alginolyticus*, C: *V. anguillarum*, D: *Pseudomonas*, E: *Moraxella*, F: *Alcaligenes*, G: Gram-positive bacteria, H: Others.

Table 4. Mean bacterial counts and flora of feeds for flounder

Feed	Bacterial count (CFU/g)		Flora (%)								
	ZoBell	BTB	A	B	C	D	E	F	G	H	I
JASFA											
Rotifer	4.7×10^8	4.0×10^8	34.0	0.9		40.8	10.4	2.9			11.1
Brine shrimp	1.3×10^8	2.6×10^8	30.0	47.1							22.9
Artificial feed	2.4×10^5	3.0×10^2					17.3		65.0	15.6	2.1
Minced fish	4.3×10^6	5.0×10^4	46.5			53.5					
HPFES											
S-type rotifer	1.6×10^8	4.7×10^7	17.3		9.5	25.0	26.2			9.5	12.5
L-type rotifer	3.5×10^8	1.1×10^8	40.5		19.3	33.4					6.8

A: *Vibrio* spp. (I, II and III), B: *V. alginolyticus*, C: *V. anguillarum*, D: *Pseudomonas*, E: *Moraxella*, F: *Cytophaga*, G: *Acinetobacter*, H: Gram-positive bacteria, I: Others.

in Table 3. Lower bacterial count was observed in the first sampling in pond 6 as compared to other samplings. The mean viable counts on ZoBell agar were 10^4 CFU/ml in all the ponds and tanks, whereas the BTB counts at HPFES ($5.2\text{--}8.0 \times 10^8$ CFU/ml) were a little lower than those at JASFA ($1.6\text{--}2.3 \times 10^4$ CFU/ml). For the bacterial flora at JASFA, *Vibrio* (41.8 and 33.0% for pond 6 and 8, respectively) and *Pseudomonas* (25.7 and 26.5%) were observed to be dominant, followed by *Moraxella* (19.5 and 25.8%). Similarly, *Vibrio* and *Pseudomonas* were predominant at HPFES.

Table 4 shows the mean bacterial counts and flora of feeds given to the fish. Among the feeds examined, the live feeds, rotifer and brine shrimp at JASFA or S-type and L-type rotifer at HPFES, contained the highest bacterial populations (10^8 CFU/g). The microflora of rotifers at JASFA was comprised of *Pseudomonas* (40.8%), *Vibrio* (34.9%) and *Moraxella* (10.4%). Greater number of *Vibrio* (77.1%) was detected in brine shrimp. In contrast, *Acinetobacter* (65.0%) and Gram-positive bacteria (15.6%) in addition to *Moraxella* were isolated from artificial feed. The bacterial flora of minced fish likewise consisted of *Pseudomonas* and *Vibrio*. At HPFES, the microflora of S-type rotifers was composed of *Vibrio* (26.8%), *Moraxella* (26.2%) and *Pseudomonas* (25.0%), while that of the L-type rotifers was comprised mainly of *Vibrio* (59.8%) and *Pseudomonas* (33.4%).

Discussion

The fact that *Pseudomonas* predominated both in the intestine of newly hatched larvae and in the rearing water indicates that the bacterial flora of non-feeding larvae might reflect the flora of the environment. After feeding, the intestinal microflora of larval and juvenile flounder was influenced both quantitatively and qualitatively by the microflora of foods ingested. The bacterial flora of the larvae fed rotifers exhibited similar generic pattern as that of the rotifers. Later, when the larvae were fed brine shrimp as supplemental diet, *V. alginolyticus*, which is the most dominant bacterium (47.1%) among the brine shrimp flora, was most isolated in the intestine. Maximum bacterial counts were observed when the fish attained approximately 10–14 mm in total length. Thereafter, a distinct decrease in the number of bacteria was observed in the fish fed artificial feed and/or minced fish. Low bacterial population in artificial diets and minced fish as compared to the live diets affected the number of intestinal microflora of the fish. At the same time, a decrease in the incidence of *Vibrio* accompanied by an increase of *Pseudomonas* and *Moraxella* was observed. On the contrary, the predominant bacteria of artificial diet, *Acinetobacter* and Gram-positive bacteria, were not found in the intestine suggesting that these bacteria might have failed to colonize in the intestine. These findings are in agreement with those of CAMPBELL and BUSWELL (1983) who suggested that the microflora of young Dover sole reflected the bacterial

flora of its ambient water. In their study, in older fish, the intestinal microflora arising from the diets were established in accordance with the development of special ecological conditions in the gut.

The maximum bacterial count was 10^5 CFU/fish in 10–14 mm flounder. This value is comparatively higher than that of 10 mm red sea bream (7×10^4 CFU/fish) reported by MUROGA *et al.* (1987a). The predominant occurrence of *Vibrio* and *Pseudomonas* in the intestine of flounder is in agreement with the results obtained in other marine fishes (LISTON, 1957; AISO *et al.*, 1968; MACFARLANE *et al.*, 1986; MACDONALD and AUSTIN, 1986). In addition to these dominant genera, *Moraxella*, *Cytophaga* and *Alcaligenes* were also observed in the flounder as in Dover sole (CAMPBELL and BUSWELL, 1983) and sea breams (MUROGA *et al.*, 1987a).

In an earlier investigation, the mortality of flounder fed S-type rotifers was often higher than those fed L-type rotifers (MASUMURA and FUSHIMI, 1985). However, no apparent difference in bacterial number between S- and L-type rotifers was observed in the present study. Also in the case of this experiment no difference in mortality between these two tanks was recorded by MASUMURA and FUSHIMI (1987). In order to clarify this problem, more detailed investigations on both bacterial and nutritional aspects have to be conducted.

Vibrio alginolyticus was remarkably the most dominant species (35.2%) in the intestine of flounder given brine shrimp at JASFA. Although *V. alginolyticus* has been associated with fish mortalities in red and black sea breams (IWATA *et al.*, 1975, 1978; KUSUDA *et al.*, 1986), this bacterium may not be pathogenic to flounder as evidenced by low mortalities obtained in spite of the high incidence of the bacterium. Using the same sample, the colony count on BTB agar was generally lower than that on ZoBell agar, which may be explained by the selectivity of the former medium. However, the reverse results were occasionally obtained in the present study in that *V. alginolyticus* was predominantly isolated. This suggests that the swarming character of the bacterium might have inhibited the growth of other bacteria on ZoBell agar.

The present study shows the intestinal micro-

flora of only a few groups of larval and juvenile flounder in healthy condition. The similar occurrence of the dominant bacteria observed in the present study needs to be investigated in different groups of flounder representing a wider geographical distribution. In addition, the role of these normal flora or predominant bacteria on nutritional process and/or disease occurrence in larval and juvenile flounder needs to be examined.

Acknowledgements

This work was supported by the Ministry of Education, Science and Culture of Japan grant No. 61440018. The authors wish to thank Mr. Shigeo Imamura, head of Hakata Island Station of Japan Sea-Farming Association, and Mr. Kazuhiko Masumura of Hiroshima Prefectural Fisheries Experimental Station for their facilitating the supply of flounder.

References

- AISO, K., U. SIMIDU and K. HASUO (1968): Microflora in the digestive tract of inshore fish in Japan. *J. gen. Microbiol.*, **52**, 361–364.
- CAMPBELL, A. C. and J. A. BUSWELL (1983): The intestinal microflora of farmed Dover sole (*Solea solea*) at different stages of fish development. *J. Appl. Bacteriol.*, **55**, 215–223.
- FUKUSHO, K. and M. OKAUCHI (1982): Strain and size of the rotifer, *Brachionus plicatilis*, being cultured in Southeast Asian countries. *Bull. Natl. Res. Inst. Aquacult.*, **3**, 107–109.
- IWATA, K., O. ISHIBASHI, T. KITAO and T. AOKI (1975): On the cause of the mortality in farmed red seabream larvae. *Rep. Miyazaki Pref. Fish. Res. Stn.* (Showa 50), pp. 267–273.
- IWATA, K., Y. YANOYAMA and O. ISHIBASHI (1978): Studies on factors related to mortality of young red seabream (*Pagrus major*) in the artificial seed production. *Fish Pathol.*, **13**, 97–102.
- KRIEG, N. R. and J. G. HOLT (1984): *Bergey's Manual of Systematic Bacteriology*, Vol. 1, Williams and Wilkins, Baltimore, USA, 964 pp.
- KUSUDA, R., J. YOKOYAMA and K. KAWAI (1986): Bacteriological study on the cause of mass mortalities in cultured black sea bream fry. *Bull. Japan. Soc. Sci. Fish.*, **52**, 1745–1751.
- LISTON, J. (1957): The occurrence and distribution of bacteria type on flatfish. *J. gen. Microbiol.*, **16**, 205–216.
- MACDONALD, N. L., J. R. STARK and B. AUSTIN (1986):

- Bacterial microflora in the gastro-intestinal tract of Dover sole (*Solea solea* L.), with emphasis on the possible role of bacteria in the nutrition of the host. *FEMS Microbiol. Lett.*, **35**, 107–111.
- MACFARLANE, R. D., J. J. McLAUGHLIN and G. L. BULLOCK (1986): Quantitative and qualitative studies of gut flora in striped bass from estuarine and coastal marine environments. *J. Wildlife Dis.*, **22**, 344–348.
- MASUMURA, K. and T. FUSHIMI (1985): Studies on the mechanism of mass mortality in larval flounder. S-59 Gyobyō-Taisaku Gijutsu-Kaihatsu Kenkyū-Seika Hōkokusho, 16 pp.
- MASUMURA, K. and T. FUSHIMI (1987): Studies on the mechanism of mass mortality in larval flounder. S-61 Gyobyō-Taisaku Gijutsu-Kaihatsu Kenkyū-Seika Hōkokusho, 15 pp.
- MURATA, O. (1987): An infectious disease of larval flounder showing opaque intestine. *Fish Pathol.*, **22**, 59–61.
- MUROGA, K., M. HIGASHI and H. KEITOKU (1987a): The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegelii*) at larval and juvenile stages. *Aquaculture*, **65**, 79–88.
- MUROGA, K., T. SUGIYAMA and N. UEKI (1977): Pasteurellosis in cultured black seabream (*Mylio macrocephalus*). *J. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **16**, 17–21.
- MUROGA, K., V. TANASOMWANG and K. MOMOYAMA (1987b): *Vibrio anguillarum* infection in tiger puffer (*Takifugu rubripes*) fingerlings. *Fish Pathol.*, **22**, 29–30.
- MUROGA, K. and M. TATANI (1982): Isolation of *Vibrio anguillarum* from juvenile red seabream (*Pagrus major*). *Fish Pathol.*, **16**, 211–214.
- WAKABAYASHI, H., M. HIKIDA and K. MASUMURA (1984): *Flexibacter* infection in cultured marine fish in Japan. *Helgoländer. Meeresunters.*, **37**, 587–593.
- WAKABAYASHI, H., M. HIKIDA and K. MASUMURA (1986): *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. *Int. J. Syst. Bacteriol.*, **36**, 396–398.