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EFFECT OF BACTERIAL LOAD IN FEEDS ON INTESTINAL MICROFLORA OF SEABREAM *(SPARUS AURATA)* LARVAE AND JUVENILES

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Abstract

Aerobic bacterial flora in the intestines of seabream *(Sparus aurata)* larvae and juveniles, in diets they were fed (rotifers, brine shrimp, artificial diet), and in their rearing water were analyzed. Fish fed live feeds had a higher bacterial count than the fish fed the artificial diet. In rotifers, the total bacteria count was 8.7 x 10^6 and *Pseudomonas* dominated the flora (60.2%). In larvae fed rotifers, the bacteria count was 9.8 x 10^2, with *Pseudomonas* (48.4%) and *Vibrio* (28.3%) dominating. In brine shrimp, the bacterial count was 1.7-3.5 x 10^7 cfu/g and *Vibrio* (73.7-81.3%) was more prevalent than *Pseudomonas* (10.2-15.5%). In larvae fed brine shrimp, the bacterial count was 5.3 x 10^4-1.8 x 10^5 and *Vibrio* (61.2-70.1%) dominated. The count for the artificial feed was 1.2 x 10^4 with *Pseudomonas* slightly dominating while for juveniles fed artificial feed the count was 2.3 x 10^4, with *Vibrio* slightly dominating. Bacterial microflora in the rearing water ranged from 1.3 x 10^2 to 3.2 x 10^3 cfu/ml. The study showed that the microflora of fish feeds quantitatively and qualitatively affect the intestinal microflora of seabream larvae and juveniles.

Introduction

Marine fish culture production has increased tremendously in Turkey. Warm water species, especially seabass *(Dicentrachus labrax)* and seabream *(Sparus aurata)*, are produced on the Mediterranean and Aegean coasts. The most critical period in the production of marine fish is the larvae stage (Muroga et al., 1990; Griez et al., 1997; Muroga, 2001; Olafsen, 2001). After resorption of the yolk-sac, marine fish larvae must be fed live feed. The rotifer

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(Brachionus plicatilis) and the brine shrimp *(Artemia* sp.) are common live food organisms used to feed marine fish larvae. Bacteria associated with mass cultivated rotifers and brine shrimp have a detrimental effect on the fish larvae and may contribute to poor reproducibility in terms of survival and growth (Perez-Benavente and Gatesoupe, 1988; Gatesoupe, 1989; Tanasomwang and Muroga, 1992; Skjermo and Vadstein, 1993). The bacterial load of live feeds can contain pathogenic bacteria that transfer to the larvae (Munro et al., 1993; Olafsen, 2001; Verner-Jeffreys et al., 2003). It has been observed that intestinal microflora of larvae are similar to the microflora of their live food. Verdonck et al. (1994) reported that the microflora of live food differs among hatcheries. Vibrionaceae and Pseudomonadaceae are dominant bacteria in the flora of live food with high bacterial loads (Skjermo and Vadstein, 1993; Vadstein et al., 1993).

When the larvae metamorphose into juveniles, live food is gradually replaced by pelleted dry food. It is generally thought that most technical problems have been solved in the larviculture of marine fish. However, survival rates from hatching until weaning are not always stable and mass mortality caused by bacterial infection is common (Muroga et al., 1987; Tanasomwang and Muroga, 1988; Nicolas et al., 1989; Verner-Jeffreys et al., 2003). Improved rearing techniques for seabream larvae require quantitative and qualitative determination of the bacterial flora that develop in seabream hatcheries. It is important to know the types, numbers, and sources of bacteria commonly associated with seabream eggs and larvae in the various stages of development to increase the survival rate. This information can also be used to target microbial contamination pathways, identify potential pathogens, and aid in identifying potential probiotic candidates (Griez et al., 1997; Muroga, 2001).

The aim of the present study was to qualitatively and quantitatively determine the intestinal aerobic microflora of seabream larvae and juveniles and the bacterial flora of the rearing water and feeds (rotifer, artemia, artificial diet) given to fish.

Materials and Methods

Fish. Newly-hatched seabream larvae *(Sparus aurata)* were obtained from the hatchery of the Ministry of Agriculture and Rural Affairs in the Beymelek Mariculture Center at Antalya, Turkey. They were reared in 18 m^3 cylindrical tanks. The water temperature was kept at 20±1°C with running water. At the outlet, the water was filtered through a 60 µm net to keep the rotifers in the larvae tanks. The water was not recirculated. The initial stocking density was 80 larvae/l. The fish were fed and sampled as shown in Table 1.

Preparation of the feeds. Rotifers (S type) were cultured in 2.7 m^3 cylindrical tanks at 25°C and 20‰ salinity. They were initially fed *Isochrysis galbana* once a day, then bakers yeast twice a day for three days. The rotifers were enriched with commercial Protein Selco (INVE, Belgium) before being given to the larvae. Brine shrimp, *Artemia franciscana* (EG-grade, INVE Aquaculture NV, Belgium), cysts were decapsulated, incubated for 24 h at 28°C and 35‰ salinity in 1 m^3 cylindrical tanks. The nauplii were enriched with Super Selco (INVE, Belgium) for 24-36 h. Metanauplii were fed Super Selco during cultivation. The rotifers and *Artemia* were concentrated on a nylon net (60 µm) and carefully washed with sea water before given to larvae. Artificial feed (sizes 300-500 and 500-800; EPAC ALFA, INVE) contained 56% protein and 15% lipids.

Bacteria counts. Fish samples for bacteriological analysis were taken according to the method of Muroga et al. (1987). For each sample, fish larvae were disinfected externally by immersion in 0.1% (w/v) benzalkonium chloride prepared in 1.5% (w/v) saline for 1 min and rinsed three times with a sterile solution. Ten to fifteen larvae or 5-10 juveniles were transfered to 2 ml of a sterile saline solution and homogenized (IKA). Bacteria counts from the rearing water and diets were obtained by same method, except that diet samples were tested without disinfection. Rotifers and brine shrimp were washed with sterile sea water prior to homogenization. After homogenizing, the total volume was adjusted to 10 ml with saline and the homogenate was serially diluted ten-fold in

Table 1. Feeding regimes and sampling times for the seabream.

Sample	*Days after hatching*	*Larvae stage*	*Feed*
1	2-3	Larvae with yolk sac	None
2	15	Larvae	Rotifers
3	30	Larvae	Shrimp nauplii
4	45	Juveniles	Shrimp nauplii and metanauplii
5	90	Juveniles	Artificial feed

sterile saline. 0.1 ml of each dilution was spread on triplicate agar plates. Bacterial counts were determined on three types of agar media. Marine agar (Difco) was used to estimate the total number of aerobic bacteria, cholera medium (TCBS, Oxoid) to estimate the number of *Vibrio* spp., and CFC agar (Oxoid) to count *Pseudomonas* spp. (Skjermo and Vadstein, 1993; Qie et al., 1994). Plates were incubated at 25°C for 48 hours. Following incubation, plates containing 30-300 colonies were used to calculate bacterial population results.

Identification of bacteria. Representative colonies of the morphologies from plates with well isolated colonies were purified on TSA agar (Difco) supplemented with 2% sodium chloride and stored at 4°C. Routine tests for determining biochemical characteristics of the isolates were carried out as described in Cowan and Steel (1970) and Collins and Lyne (1976). Various tests in Bergery's Manual of Systematic Bacteriology (Holt et al., 1994) and Muroga et al. (1987) were used to identify the bacterial isolates. API 20 E test systems (Biomerieux, France) strips were also used.

Results

The bacterial counts differed among feeds, being highest in brine shrimp and lowest in the artificial diet (Table 2). The bacterial count and flora of the seabream are shown in Tables 3 and 4 and of the rearing water in Table 5. Intestinal bacterial counts increased until the fish were transferred to artificial feed, as did the count in the rearing water, and exceeded the counts in the water. When the larvae were fed rotifers (sample 2), *Pseudomonas* and *Vibrio* constituted the dominant microflora of the intestine; *Pseudomonas* was also dominant in the flora of the rotifers. When the larvae were fed brine shrimp (samples 3 and 4), *Vibrio* (including *V. alginolyticus*, *V. metschnikovii,* and *V. orientalis*) were the dominant intestinal bacteria. The intestinal flora of juveniles fed the artificial diet were also dominated by *Pseudomonas* and *Vibrio* (including *V. alginolyticus, V. metschnikovii,* and *V. orientalis*).

Discussion

To date, many studies of the intestinal microflora of fishes have been carried out. A method for isolating and enumerating the aerobic intestinal bacteria in larvae and juvenile fish was devised by Muroga et al. (1987). The intestinal bacterial flora of several marine fishes have been investigated (Muroga et al., 1987; Nicolas et al., 1989; Tanasomwang and Muroga, 1988; Griez et al., 1997) and it was found that major intestinal flora are derived from live foods such as rotifers and brine shrimp.

According to Miyakawa and Muroga (1988) and Olsen et al. (2000), the total bacterial count in rotifers and brine shrimp is about 10^7-10^8 cfu/g. Nicolas et al. (1989) reported the bacterial load of rotifers as 7.1 x 10^6 to 3.9 x 10^7 cfu/ml. Our finding agrees with these earlier results.

Tanasomwang and Muroga (1988) found that the microflora of rotifer is comprised of

Table 2. Bacterial count (means±standard deviation) and flora of feeds.

Feed	*Bacterial count (cfu/g)*			*Flora (%)**				
	Total	*Vibrio*	*Pseudomonas*	A	B	C	D	E
Rotifers	$8.7\pm1.8 \times 10^6$	$1.6\pm0.3 \times 10^4$	$3.0\pm0.4 \times 10^5$	20.1	60.2	8.3	10.1	1.3
Nauplii	$1.7\pm0.3 \times 10^7$	$1.5\pm0.5 \times 10^6$	$2.5\pm0.5 \times 10^5$	73.7	15.5	-	-	11.0
Metanauplii	$3.5\pm1.2 \times 10^7$	$3.0\pm2.3 \times 10^6$	$3.2\pm1.8 \times 10^5$	81.3	10.2	-	-	8.5
Artificial diet	$1.2\pm0.9 \times 10^4$	$1.5\pm0.5 \times 10^2$	$2.5\pm0.9 \times 10^1$	20.4	40.3	-	-	39.3

* A = *Vibrio*, B = *Pseudomonas*, C = *Flavobacter*, D = *Alcaligenes*, E = other

Pseudomonas (40.8%), *Vibrio* (34.9%), and *Moraxella* (10.4%), while the dominant microflora of brine shrimp is *Vibrio* (77.1%). Muroga et al. (1987) detected that the most dominant bacterium in live feeds is *Pseudomonas* (48.3%), while *Vibrio* constitutes 11.4%, *Flavobacter* 10.2%, and *Moraxella* 8.1%. In the present study, the *Pseudomonas* spp. count was higher than the *Vibrio* spp. count in rotifers, while *Alcaligenes* and *Flavobacter* were also identified. The predominant bacteria of brine shrimp (both nauplii and metanauplii) was *Vibrio*, in agreement with Tanasomwang and Muroga (1988) and Olsen et al. (2000).

Intestinal flora change according to qualitative and quantitave properties of digested feed (Muroga et al., 1987; Perez-Benavente and Gatesoupe, 1988; Griez et al., 1997; Olafsen, 2001; Verner-Jeffreys et al., 2003). Although the bacteria that were dominant in the intestine were dominant in the feeds and rearing water in the current study, the incidence of each genus differed. When larvae were fed rotifers, *Pseudomonas* and *Vibrio* constituted the dominant intestinal flora whereas when they were fed brine shrimp, *Vibrio* dominated. The bacterial flora of larvae fed rotifers and brine shrimp were similar in genera to those of the live foods. It is thought that bacteria are transferred to larvae from the live food.

V. alginolyticus was the dominant species in intestinal flora of larvae fed brine shrimp (Tanasomwang and Muroga, 1988; Griez et al., 1997). It is not clear whether *V. alginolyticus* is a pathogen or a beneficial and protective bacterium for marine fish larvae (Griez et al., 1997). Kusuda et al. (1986) reported that *V. alginolyticus* caused mortalities in red and black seabream. Tanasomwang and Muroga (1988) reported high numbers of *V. alginolyticus* in the intestine of Japanese flounder larvae without the occurence of mortality. A positive correlation between larvae survival rates and an increasing number of *V. alginolyticus* was demonstrated by Gatesoupe (1990). In the current study, *V. alginolyticus* was isolated from the live foods, the artificial diet, and the intestines of healthy fish, however no infection or mortality was observed.

Table 3. Bacterial count of seabream larvae and juveniles.

Sample	*Intestinal bacterial count (CFU/fish)*		
	Total	*Vibrio*	*Pseudomonas*
1	2.0±0.4 x 10	-	-
2	9.8±1.8 x 10^2	0.6±0.2 x 10^2	2.8±0.7 x 10^2
3	5.3±1.9 x 10^4	1.5±1.1 x 10^3	3.5±1.2 x 10^2
4	1.8±0.9 x 10^5	2.1±0.8 x 10^4	1.2±0.5 x 10^3
5	2.3±1.2 x 10^4	1.7±0.7 x 10^2	0.8±0.2 x 10^2

Table 4. Bacterial flora (%) of seabream larvae and juveniles.

Sample	*Bacterial flora (%)**								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
1	-	-	-	-	-	-	-	-	-
2	28.3	48.4	7.1	9.3	2.1	-	1.9	-	2.9
3	61.2	20.3	3.3	-	1.2	4.2	3.2	1.5	5.1
4	70.1	12.7	-	-	1.8	1.2	5.8	-	8.4
5	30.2	25.8	-	-	3.5	8.1	7.2	-	25.2

* A = *Vibrio*, B = *Pseudomonas*, C = *Flavobacter*, D = *Acinetobacter*, E = *Alcaligenes*, F = *Moraxella*, G = *Enterobacter*, H = *Alteromonas*, I = other

During the first 30 days, seabream larvae lack gastric secretion and enzyme development. After the development of the digestion system in the juveniles, the *Vibrio* spp. counts in the intestines increased. In juvenile and adult marine fish, intestinal microflora is dominated by *Vibrio* (Muroga et al., 1987; Griez et al., 1997). In the present study, intestinal *Pseudomonas* was high in the larvae stage and *Vibrio* was high during the juvenile stage. After the start of brine shrimp feeding, *Vibrio* counts in intestines increased, similar to the results of other researchers. *V. orientalis*, *V. metschnikovii*, *V. alginolyticus,* and *Pseudomonas* 2 were isolated from both brine shrimp and fish intestines. Straub and Dixon (1993) found that *V. alginolyticus*, *V. metschnikovii*, *V. fluvialis*, *V mimicus,* and others were present in adult *Artemia* from hypersaline ponds in California. It is thought that these bacteria, especially *V. alginolyticus* and *V. metschnikovii*, were transferred to the larvae during feeding. It has already been indicated by some researchers that intestinal microflora of larvae initiate in the diet and become established by selection through specific ecological

Table 5. Bacterial count and flora of rearing water.

Sampling	Bacterial count (CFU/ml)			Flora (%)*						
	Total	Vibrio	Pseudomonas	A	B	C	D	E	F	G
1	2.3±0.5 x 10									
2	1.3±0.8 x 10^2	3.0±0.5 x 10	0.9±0.2 x 10^2	-	-	28.9	-	-	-	71.1
3	2.8±0.7 x 10^3	0.9±0.3 x 10^2	1.5±1.0 x 10^2	32.1	13.3	11.3	-	8.4	3.5	31.4
4	3.2±1.1 x 10^3	2.2±1.2 x 10^3	1.1±0.7 x 10^2	28.4	17.8	5.1	-	8.3	-	40.4
5	1.4±0.7 x 10^3	1.4±0.6 x 10^2	0.3±0.1 x 10^2	23.1	31.2	-	10.3	14.8	-	20.6

* A = *Vibrio*, B = *Pseudomonas*, C = *Flavobacter*, D = *Alcaligenes*, E = *Moraxella*, F = *Alteromonas*, G = other

conditions in the gut. The bacterial load of the larvae fed live food was high on day 45 (sample 4). This increase in bacterial load might have been caused by ingestion of brine shrimp since the bacterial load of larvae fed artificial food was low in contrast. Compared to larvae fed brine shrimp, there was a decreased incidence of *Vibrio* and increase of *Pseudomonas* in the intestine of juveniles fed artificial feed. Tanasomwang and Muroga (1988) observed a decrease in bacterial load of intestines when feed was changed from live to dry. The decrease in bacterial count in juveniles fed artificial food confirmed other studies.

In conclusion, the present results indicate that the intestinal microflora of healthy seabream larvae and juveniles were influenced quantitatively and qualitatively by the microflora of the ingested feeds. Measures for reducing bacterial loads and selectively manipulating the microflora in live foods produced in the hatchery and in the rearing water during the initial feeding period of fish larvae should be studied. Information regarding microflora in the larvaculture system will help to recognize and correct situations that can lead to the onset of bacterial disease.

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