キンギョの腸内細菌相に及ぼす餌料の影響

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Changes in the Fecal Microflora of Goldfish *Carassius auratus*, Associated with Diets

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Goldfish *Carassius auratus* was reared, fed on pelleted diets, tubifex worms and/or dried daphnia, and successively examined for microfloras of fecal pellets using six agar media. *Aeromonas hydrophia* and *Bacteroides* type A were predominant with bacterial densities ranging from 10^4 to 10^9 cells g^{-1} in almost all specimens of fish fed on eitheir the pelleted diets and tubifex worms, or the pelleted diets and dried daphnia, showing little diet dependency. Other bacterial components varied with the individuals, and it was suggested that a part of minor bacterial components were derived from the diets.

It was shown from the above results that the intestinal microflora of goldfish was not easily influenced by the diets used usually.

It is known that *Aeromonas hydrophila* and *Bacteroides* type A are ubiquitous to the intestinal tracts of freshwater fishes while other bacterial components vary with fish species.[1-4] However, the intestinal microflora of fish is not necessarily invariable and found to be influenced by various factors including the developmental stage[5-7] and structure of gastro-intestines of fish,[8,9] water temperature,*2 location[10] and salinity of water.[11] In addition to these factors, the diets seem to be important because it was reported that there were close relationships between the physiological conditions of fish and their diets.[12,13] Therefore, the present study was undertaken to evaluate the effect of the diets on the intestinal microflora of goldfish *Carassius auratus*.

Materials and Methods

Diets

Pelleted diets (Nissin Flour Milling), alive tubifex worms (oligochaeta) and dried daphnia (Sanryu Boueki) were used as experimental diets for goldfish.

Fish

Wakin-goldfish (85.4 to 142.6 g) was purchased from a commercial supplier, reared for more than 3 months and fed on the pelleted diets prior to the experiment. Fish was maintained for 20 to 22 days in glass aquaria equipped with recirculating water system at water temperatures ranging from 23 to 25°C in the experimental period.

Two individuals, Nos. 1 and 2, were fed on the pelleted diets from day 0 to 3 and from day 20 to 22, while the tubifex worms from day 4 to 19 during the 22-day experimental period.

Other two individuals, Nos. 3 and 4, were also fed on the pelleted diets from day 0 to 2 and from day 17 to 20 while the dried daphnia was given to them from day 3 to 16.

Bacteriological Sampling

The hanging fecal pellet of the fish was successively removed,[14] and liquified in a nine-fold volume of diluent of phosphate buffer (pH 7.6) containing 0.05% L-cysteine hydrochloride and 0.1% agar as described by Mitsuoka *et al.*[15] The sample was then diluted serially, and plated onto 6 different media including Trypticase soy blood agar[TS] (BBL), Phenylethyl alcohol blood agar[PEA] (BBL), MacConkey agar (Eiken), EG blood agar[EG] (Nissui), FM-CW blood agar [FM-CW] (Eiken) and *Bacteroides* type A-selective agar[AS]. The first three madia were incubated aerobically, and the last three media anaerobically, both at 25°C for 6 to 7 days. Anaerobiosis was established by the jar technique.[2,15] The pelleted diets, tubifex worms and dried daphnia were also homogenized and processed similarly.

Identification

After incubation, bacterial colonies were counted, and about 20 colonies were isolated at random

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*2 H. Sugita *et al.*: Abstr. Spr. Meet. Japan. Soc. Sci. Fish., Tokyo, 1987, p. 216.

from each plate. The isolated bacteria were identified according to the previous paper.[2)]

On suitably diluted samples, colonies of each bacterial group were counted and expressed as number per gram material.

The maximum count of each bacterial group through six media was expressed as "viable count." Total viable count (TVC) was obtained by summation of viable counts of all bacterial groups.

Results

Bacteria Isolated

A total of 2,200 strains of aerobic and facultatively anaerobic bacteria were isolated aerobically from the fecal pellets of goldfish and its diets. These were composed of *Vibrionaceae* (1,530 strains), *Pseudomonas* (342), *Enterobacteriaceae* (128), *Bacillus* (41), *Flavobacterium* (40), coryneforms (31), *Acinetobacter* (16), *Streptococcus* (11), *Micrococcus* (10) and others (51) which lost viability during subculture. The 1,530 strains of *Vibrionaceae* were divided into *Aeromonas hydrophila* (1,481 strains), *A. punctata* (42) and *Plesiomonas shigelloides* (7).

On the other hand, a total of 2,106 strains of anaerobic bacteria were isolated anaerobically. They were composed of 1,381 strains of obligate anaerobes and 725 strains of facultative anaerobes. The obligate anaerobes were *Bacteroides* type A (1,273 strains), *Clostridium* (63), other *Bacteroidaceae* (42) and Gram-positive cocci (3) while the facultative anaerobes were composed of Gram-negative rods (702), Gram-positive rods (17), Gram-positive cocci (4) and *Bacillus* (2).

Microflora of Diets

Generic composition of the pelleted diets, tubifex worms and dried daphnia as diet of goldfish, it shown in Table 1. In the pelleted diets, only genus *Bacillus* was detected with viable count of 1.3×10^3 cells g^{-1}. In the tubifex worms, *Enterobacteriaceae*, *Clostridium*, *Bacteroidaceae* (other than *Bacteroides* type A), *A. hydrophila*, coryneforms, *Pseudomonas* and *Streptococcus* were detected with viable counts ranging from

Table 1. Generic composition of the bacteria in the pelleted diets, tubifex worms and dried daphnia

Component	Pelleted diets	Tubifex worms	Dried daphnia
A. hydrophila	nd*1	6.26*2	nd
Enterobacteriaceae	nd	7.28	nd
Pseudomonas	nd	6.00	5.79
Flavobacterium	nd	nd	6.39
Micrococcus	nd	nd	6.09
Streptococcus	nd	5.78	nd
Coryneforms	nd	6.08	6.27
Bacillus	3.11	nd	nd
Bacteroidaceae	nd	7.12	nd
Clostridium	nd	7.17	nd
TVC	3.11	7.71	6.79

*1 Not detected.
*2 Log number of viable count per gram.

Table 2. Generic composition of the intestinal bacteria from the goldfish No. 1 fed on the pelleted diets or tubifex worms

Component	Day*1								
	0	3	4	5	6	12	19	20	22
A. hydrohila	8.83*2	7.80	8.92	8.18	9.02	8.20	9.02	8.58	9.98
A. punctata	nd*3	nd	nd	6.90	nd	7.32	nd	nd	nd
P. shigelloides	nd	nd	nd	nd	nd	nd	nd	nd	7.87
Enterobacteriaceae	nd	nd	nd	5.30	8.45	7.41	7.02	nd	7.72
Pseudomonas	7.56	nd	7.24	nd	nd	7.32	7.58	8.57	8.68
Acinetobacter	nd	nd	nd	4.60	nd	nd	nd	nd	7.82
Flavobacterium	nd	6.52	nd	nd	nd	nd	nd	nd	nd
Coryneforms	nd	6.30	nd	nd	nd	nd	nd	nd	7.82
Bacillus	nd	nd	nd	nd	nd	nd	nd	nd	7.52
Bacteroides type A	8.23	7.22	8.04	8.79	8.19	8.45	8.59	7.93	8.76
Clostridium	nd	5.94	6.90	nd	nd	nd	7.28	7.20	nd
Gram-positive cocci	nd	nd	nd	nd	nd	nd	nd	7.02	nd
TVC	8.95	7.93	9.02	8.89	9.37	8.73	9.21	8.97	9.36

*1 Goldfish No. 1 was fed on the pelleted diets from day 0 to 3 and from day 20 to 22 while tubifex worms were fed from day 4 to 19.
*2 Log number of viable count per gram.
*3 Not detected.

Table 3. Generic composition of the intestinal bacteria from the goldfish No. 2 fed on the pelleted diets or tubifex worms

Component	Day*1								
	0	3	4	5	6	12	19	20	22
A. hydrohila	8.76*2	8.91	8.84	8.10	7.93	8.68	7.51	7.15	8.01
A. punctata	nd*3	nd	nd	6.89	nd	8.30	nd	6.34	7.20
P. shigelloides	7.53	nd	nd	nd	nd	nd	nd	nd	nd
Enterobacteriaceae	7.96	nd	nd	7.39	7.97	7.64	7.41	nd	7.03
Pseudomonas	nd	7.06	7.01	nd	nd	7.96	6.51	6.91	7.43
Acinetobacter	nd	nd	nd	nd	nd	7.96	6.91	nd	nd
Flavobacterium	7.41	nd	nd	nd	nd	nd	nd	nd	nd
Bacteroides type A	7.88	7.52	7.77	5.09	7.78	9.11	8.89	6.93	8.19
Bacteroidaceae	6.60	nd	nd	nd	nd	nd	nd	nd	nd
Clostridium	nd	nd	6.46	nd	nd	nd	8.48	nd	nd
TVC	8.91	8.94	8.90	8.21	8.41	9.39	9.06	7.55	8.48

*1,*2,*3 Refer to the footnotes in Table 2.

Table 4. Generic composition of the intestinal bacteria from the goldfish No. 3 fed on the pelleted diets or dried daphnia

Component	Day*1							
	0	2	4	7	10	16	18	20
A. hydrohila	9.03*2	8.66	8.33	8.21	4.89	6.69	8.42	9.11
A. punctata	7.86	7.41	5.88	nd	nd	nd	nd	8.06
P. shigelloides	nd*3	nd	nd	nd	6.27	nd	nd	nd
Enterobacteriaceae	7.56	nd	7.08	7.36	6.09	4.19	nd	nd
Pseudomonas	8.45	nd	7.08	8.44	6.57	6.39	8.31	8.69
Flavobacterium	nd	nd	nd	nd	4.98	nd	nd	nd
Micrococcus	nd	nd	nd	nd	5.79	nd	7.34	nd
Streptococcus	nd	nd	nd	nd	6.49	nd	7.34	nd
Coryneforms	nd	nd	nd	nd	5.59	nd	nd	nd
Bacteroides type A	8.84	8.36	8.49	8.07	7.13	5.19	7.33	8.58
Bacteroidaceae	7.56	5.74	nd	nd	nd	nd	nd	nd
Clostridium	7.61	5.74	nd	nd	nd	nd	nd	nd
TVC	9.34	8.85	8.74	8.76	7.39	6.88	8.69	9.36

*1 Goldfish No. 3 was fed on the pelleted diets from day 0 to 2 and from day 17 to 20 while dried daphnia was fed from day 3 to 16.
*2 Log number of viable count per gram.
*3 Not detected.

Table 5. Generic composition of the intestinal bacteria from the goldfish No. 4 fed on the pelleted diets or dried daphnia

Component	Day*1							
	0	2	4	7	10	16	18	20
A. hydrohila	8.59*2	7.56	8.27	7.20	7.24	8.03	8.55	7.84
A. punctata	7.80	6.78	nd	6.04	nd	nd	nd	nd
P. shigelloides	nd*7	nd	nd	6.34	nd	nd	nd	nd
Enterobacteriaceae	nd	nd	7.04	nd	nd	8.05	nd	nd
Pseudomonas	nd	6.14	7.34	6.64	7.04	7.54	7.65	7.77
Micrococcus	nd	nd	nd	nd	nd	nd	nd	6.69
Bacteroides type A	9.38	7.32	7.64	7.49	7.19	7.78	7.97	8.68
Bacteroidaceae	8.71	nd	nd	nd	6.59	nd	nd	nd
Clostridium	7.83	5.68	nd	nd	nd	nd	nd	nd
TVC	9.54	7.82	8.42	7.74	7.68	8.50	8.69	8.79

*1,*2,*3 Refer to the footnotes in Table 4.

6.0×10^5 to 1.9×10^7 cells g^{-1}, and with a TVC of 5.1×10^7 cells g^{-1}. In the dried daphnia, *Flavobacterium*, coryneforms, *Micrococcus* and *Pseudomonas* occurred with viable counts ranging from 6.2×10^5 to 2.5×10^6 cells g^{-1}, with a TVC of 6.2×10^6 cells g^{-1}.

Fecal Flora of Goldfish

Tables 2 and 3 show the intestinal microflora of the goldfish (individual Nos. 1 and 2) fed on the pelleted diets and tubifex worms.

In the fecal pellets of fish individual No. 1, a total of 12 bacterial components were detected with viable counts ranging from 2.0×10^5 to 1.1×10^9 cells g^{-1}, and TVCs from 8.5×10^7 to 2.3×10^9 cells g^{-1} during 22 days of the experiment (Table 2). *A. hydrophila* and *Bacteroides* type A were predominant in all specimens while other components occurred with low frequencies.

In the fecal pellets of individual No. 2, a total of 10 bacterial components were isolated with viable counts ranging from 1.2×10^5 to 1.2×10^9 cells g^{-1}, and TVCs from 3.5×10^7 to 2.5×10^9 cells g^{-1} during 22 days (Table 3). Similar to the microfloras of individual No. 1, *A. hydrophila* and *Bacteroides* type A occurred predominantly, except for one specimen on day 5 in which *Bacteroides* type A was a minor component.

On the other hand, Tables 4 and 5 show the intestinal microflora of the goldfish (individual Nos. 3 and 4) fed on the pelleted diets and dried daphnia.

In the fecal pellets of fish individual No. 3, a total of 12 bacterial components were detected with viable counts ranging from 1.5×10^4 to 1.3×10^9 cells g^{-1}, and TVCs from 7.6×10^6 to 2.3×10^9 cells g^{-1} during the 22-day period of the experiment (Table 4). *A. hydrophila*, *Pseudomonas* and *Bacteroides* type A were highly counted although they decreased or disappered on day 2, 10 or 16. Other components appeared occasionally.

In the fecal pellets of individual No. 4, a total of 9 bacterial components occurred with viable counts ranging from 4.8×10^5 to 2.4×10^9 cells g^{-1}, and TVCs from 4.8×10^7 to 3.5×10^9 cells g^{-1} during 20 days (Table 5). *A. hydrophila*, *Bacteroides* type A and *Pseudomonas* also predominated in at least seven out of the eight specimens as observed in the fecal specimens of individual No. 3. Other components were detected with low frequencies of occurrence.

Discussion

In the previous paper,[16] we reported that each individual of goldfish had a characteristic fecal flora and that there was a great day-to-day variation of the fecal flora. These results show that the effect of exogenous and endogenous factors on the intestinal microflora of fish should be analyzed by successive sampling from the same individual. In this situation, the present study was undertaken to determine how the diets influence the intestinal microflora of goldfish using the hanging fecal pellet method.[14]

In the fish individuals fed on the pelleted diets and tubifex worms during a total of 22 days, *Aeromonas hydrophila* and *Bacteroides* type A occurred predominantly in all the specimens at densities ranging from 10^8 to 10^9 cells g^{-1}, except for one specimen. The decreased viable counts of *A. hydrophila* and *Bacteroides* type A soon increased to the normal level during the feeding of the tubifex worms. This result indicates that the dominant microflora of goldfish intestines could not be significantly influenced by these diets. Of the bacterial components detected in the tubifex worms, *A. hydrophila*, *Enterobacteriaceae*, *Pseudomonas* and *Clostridium* appeared during the feeding period of the worm whereas *Streptococcus*, coryneforms and *Bacteroidaceae* could not be isolated from the fecal pellets of fish in this period. These results may suggest that *A. hydrophila*, *Enterobacteriaceae*, *Pseudomonas* and *Clostridium* in the fecal pellets originated partly from the worms.

Similarly, *A. hydrophila* and *Bacteroides* type A dominated in almost all fecal specimens of the fish fed on the pelleted diets and dried daphnia for a total of 20 days, although with some exceptions. In the fish individual No. 3, *A. hydrophila* and *Bacteroides* type A, along with TVCs, decreased 8 to 14 days after the feeding of dried daphnia and again increased after the feeding of the pelleted diets. However, the similar tendency of variation was not recognized in another fish (No. 4). This result may show that the response of intestinal microflora to the dried daphnia varied with the individual of goldfish. However, the variation of *Bacteroides* type A with densities ranging from 10^4 to 10^9 cells g^{-1} was also observed in the goldfish fed on only the pelleted diets.[16] These results, together with those of the fish fed on the pelleted diets and tubifex worms, may in-

dicate the dominant microflora consisting of *A. hydrophila* and *Bacteroides* type A, does not significantly respond to the change of diets. The bacterial components including *Pseudomonas*, *Flavobacterium*, *Micrococcus* and coryneforms detected in the dried daphnia, also occurred in the fecal pellets of the goldfish fed on the same diet. These fecal bacteria may derive from the diets. It was already reported that bacterial components found in the intestines of goldfish in the culture pond should be divided into three categories, (1) transient type, consisting of *P. shigelloides*, *Enterobacteriaceae*, *Moraxella* and *Bacteroidaceae*, (2) permanently indigenous type of *A. hydrophila*, *A. punctata*, *Pseudomonas* and *Clostridium*, and (3) adult type of *Bacteroides* type A.[7] Of the bacteria detected in the fecal pellets, therefore, the *Bacillus*, *Enterobacteriaceae*, *Flavobacterium* and coryneforms which were common to the pelleted diets, tubifex worms or dried daphnia, may be corresponding to the transient type whereas it remains unclear whether the *A. hydrohila*, *Pseudomonas* and *Clostridium* in the intestines derived from those diets.

The present study, nevertheless, suggests that those bacteria contained in usually used diets does not disturb the dominance of *A. hydrophila* and *Bacteroides* type A. Sera *et al.*[8] demonstrated that the bacteria invading into the gastrointestinal tract of fish were partly selected by the action of gastric and bile acids excreted, resulting in the intestinal microflora specific to each fish species. Sugita *et al.*[9] suggested that the ability to attach on the inside wall of intestinal tracts is also important for indigenous bacteria. Therefore, the bacteria which were contained in the diet seem to be either washed out from the intestines or restricted to low densities by those mechanisms even though they invaded into the fish intestines.

On the other hand, the phenomenon that the microflora once established in the intestinal tract could not be easily changed by the diets or feeds used usually, is also recogenized in the human and livestock,[17] although the reason for it could not be clearly explained. Recently, Satoh *et al.*[12] and Yano *et al.*[13] individually reported that the composition of diets affected the fish physiology, especially complement activity. These results suggest that there are complicated relationships among the fish host, intestinal bacteria and diets. Therefore, the approach from immunology and physiology of fish, along with microbiological aspect, may be hereafter desirable to analyze it.

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