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Distribution of Microflora in the Intestinal Tract of Carp *Cyprinus carpio*

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The present study was undertaken to investigate the localization of microflora in the intestinal tract of carp *Cyprinus carpio*. *Aeromonas*, Enterobacteriaceae, *Pseudomonas* and *Bacteroides* type A were predominantly detected in almost all intestine segments, although they did not necessarily increase gradually during the passage of contents from the fore- to post-intestine. Furthermore, the phenomenon was found that the percentage of each aerobe/facultative anaerobe dropped and that of each obligate anaerobe raised with the increase of total viable count (TVC). No antagonistic interaction, mediating the growth-inhibiting substance, was observed between the aerobes/facultative anerobes and the obligate anaerobes. Therefore, the above phenomenon may be attributed to the Eh or O_2 concentration in each site of intestines because the growth of obligate anaerobes and aerobes is inhibited by the O_2 at high and low concentrations, respectively.

In a previous paper,[1] we reported that *Aeromonas*, Enterobacteriaceae, *Pseudomonas*, *Bacteroides* type A and other Bacteroidaceae predominated in the intestinal tract of carp *Cyprinus carpio*, though there were significant day-to-day and individual-to-individual variations of microflora. During the last two decades, along with the above result, much have been clarified about how endogenous and exogenous factors influence the intestinal microflora of fish: that is, the developmental stage[2-4] and structure of the fish gastrointestine,[5,6] rearing conditions,[7-10] salinity[9,11,12] and temperature[13,14] of ambient water, handling stress[15] and oral administration of antibiotics.[16,17] Although the site in intestinal tracts also may be important, as it is recognized in mammals,[18-20] a detail study about this problem has yet to be preformed. The present study, therefore, was undertaken to investigate the distribution of microflora in the intestinal tract of carp.

Materials and Methods

Fish

Carp (984–1288 g body weight and 375–410 mm body length) were supplied from a fish culturist and fed *ad libitum* with pelleted diets (Nihon Haigo Shiryo) for one month prior to the experiment, in 600 *l*-plastic tanks equipped with recirculating water system.

Bacteriological Sampling

Intestinal tracts (485–680 mm in length) were aseptically removed from five specimens of carp, and were evenly divided into four parts: the 1st, 2nd, 3rd and 4th intestine segments in the order from the fore- to post-intestine. Intestinal contents squeezed from each segment were liquefied in a nine-fold volume of a diluent of phosphate buffer (pH 7.6) containing 0.05% L-cysteine hydrochloride and 0.1% Bacto-agar (Difco). Each sample thus prepared was serially diluted and plated onto seven different media: Trypticase soy blood agar [TS] (BBL), phenylethyl alcohol blood agar [PEA] (BBL), MacConkey agar (Eiken Chemical Co.), Eggerth-Gagnon blood agar [EG] (Nissui Seiyaku), Fradiomycin-*Clostridium welchii* blood agar [FM-CW] (Eiken Chemical Co.), *Bacteroides* type A-selective blood agar (AS) and *Bacteroides* type B-selective blood agar (BS).[21] The inoculated TS, PEA and MacConkey agar plates were incubated aerobically, and the EG, FM-CW, AS and BS agar plates were incubated anaerobically, both at 25°C for 7 days. Anaerobiosis was established by evacuating the atmosphere of an anaerobic jar containing steel wool, which was activated by acidic cupric sulfate, and replacing it with 20% CO_2–80% N_2 gas mixture.[22]

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

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random from each plate for further characterization. Gram-negative aerobes and facultative anaerobes isolated aerobically were identified to the genus level using a modified scheme[23] of Shewan *et al.*[24] Gram-positive bacteria were identified according to the procedure of Cowan.[25]

The obligate and facultative anaerobes isolated anaerobically were classified on the basis of Gram reaction, cellular morphology and arrangement, spore formation, and the ability to grow aerobically. Bacteroidaceae strains were further classified into three types: *Bacteroides* type A, *Bacteroides* type B, and other Bacteroidaceae since the first two types can be identified according to their specific morphology[26] and ability to grow on the AS or BS medium.[21]

After identification of bacteria, suitable dilutions of colonies of each bacterial group on the original plates were counted and expressed in CFU (colony forming unit) per gram dry weight of material. For each bacterial group, the highest count obtained on the different agar media was regarded as the estimated viable count for that group. The TVC (total viable count) was obtained by summation of viable counts of all bacterial genera.

The linear regression analysis was performed to investigate the relationship between the TVC and the viable count of each bacterial component in the intestinal segment of carp, using a Apple Macintosh Plus personal computer.

Antibacterial Activity Test

Antibacterial activity of the aerobes/facultative anaerobes against obligate anaerobes and the obligate anaerobes against aerobes/facultative anaerobes was tested by the disc-plate method.[27] A total of 30 strains of carp intestinal bacteria, including *Aeromonas* (6 strains), *Plesiomonas* (2), Enterobacteriaceae (2), *Pseudomonas* (2), *Flavobacterium* (2), *Acinetobacter* (2), *Moraxella* (2), *Micrococcus* (2), *Staphyloccocus* (2), *Streptococcus* (2), *Bacillus* (2), *Bacteroides* type A (2) and other Bacteroidaceae (2), were incubated in GAM broth (Nissui Seiyaku) at 25°C for 2 days under aerobic or anaerobic conditions. After being centrifuged at 10,000 rpm for 20 min (at 5°C), 50 μl of the supernatant of each culture was spotted onto a paper disc for antibiotic examination (Toyo), and then freeze-dried. A test plate was prepared by overlaying the GAM agar (20 m*l*) with a thin layer of the GAM agar (5 m*l*) containing the tested bacteria at final concentration of about 10^6 cells/m*l*. The disc was put on the test plate, incubated at 25°C for 1–2 days aerobically or anaerobically, and then examined for the clear zone around the disc.

Results

Bacteria Isolated

A total of 1,200 strains of aerobic and facultatively anaerobic bacteria were isolated aerobically from the carp's intestine. These were composed of *Aeromonas* (561 strains), Enterobacteriaceae (157), *Pseudomonas* (132), *Streptococcus* (127), *Micrococcus* (34), *Acinetobacter* (29), *Moraxella* (26), *Flavobacterium* (25), *Staphylococcus* (24), *Bacillus* (16), *Plesiomonas* (7), and others (62) which lost viability during subculture.

On the other hand, a total of 1,600 strains of anaerobic bacteria were isolated anaerobically. There were 1,176 obligate anaerobes and 424 facultative ones. The former consisted of *Bacteroides* type A (1,062 strains), other Bacteroidaceae (107) and *Clostridium* (7).

Distribution of Microflora in Carp's Intestine

The TVC of each intestine segment of carp varied from 3.1×10^6 to 6.0×10^{10} CFU/g dry wt., and increased gradually during the passage of contents from the fore- to post-intestine of only the specimen No. 1 of carp (Fig. 1). As shown in Fig. 2, *Aeromonas*, Enterobacteriaceae, *Pseudomonas* and *Bacteroides* type A were predominantly detected in almost all intesine segments, as previously reported,[1] whereas *Plesiomonas*, other Gram-negative aerobes (including *Moraxella*, *Acinetobacter* and *Flavobacterium*), aerobic Gram-positive cocci (including *Staphylococcus*, *Micrococcus* and *Streptococcus*), *Bacillus*, other Bac-

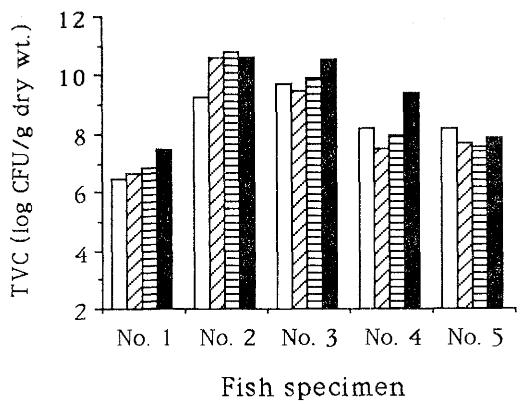

Fig. 1. The TwC (CFU/g dry wt.) in the content of the 1st (□), 2nd (▨), 3rd (▤) and 4th intestine segements (■) of carp.

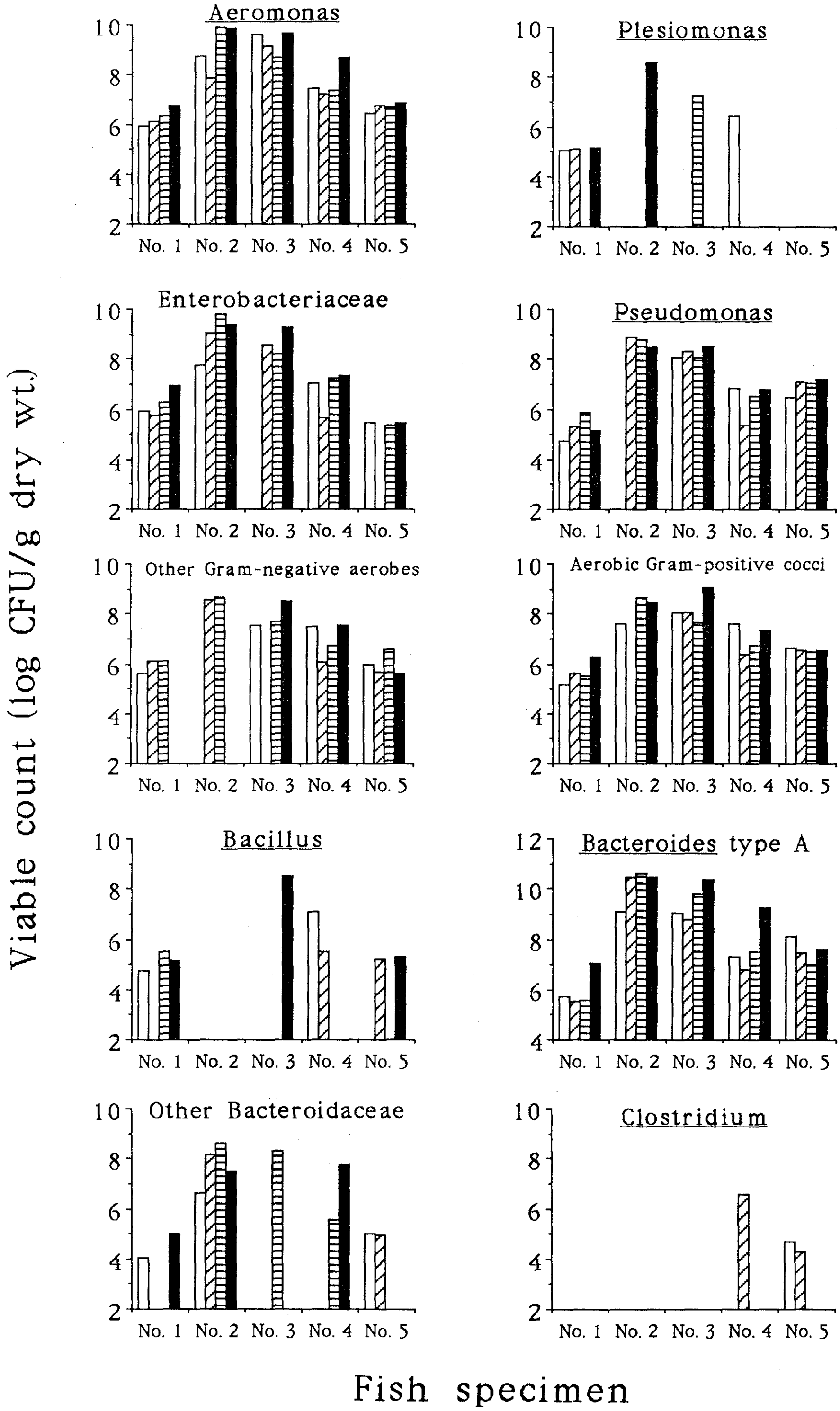

Fig. 2. The viable count of each bacterial genus in the content of the 1st (□), 2nd (▨), 3rd (▤) and 4th intestine segments (■) of carp.

Table 1. The relationship between viable count (VC) of each bacterial genus and TVC in the intestinal contents of carp, as shown in the following equation:

$$\log VC = a + b \times \log TVC$$

Genus (No. samples)	a	b	r*
Aeromonas (20)	0.254	0.874	0.917
Plesiomonas (6)	−0.492	0.823	0.969
Enterobacteriaceae (18)	−0.809	0.936	0.902
Pseudomonas (19)	−0.099	0.830	0.913
Moraxella (12)	0.168	0.774	0.929
Acinetobacter (9)	2.21	0.503	0.822
Flavobacterium (8)	2.73	0.423	0.514
Staphylococcus (10)	−1.05	0.908	0.966
Micrococcus (12)	−0.936	0.883	0.985
Streptococcus (14)	0.124	0.801	0.922
Bacillus (8)	−1.56	0.953	0.913
Bacteroides type A (20)	−1.87	1.17	0.986
Other Bacteroidaceae (11)	−3.03	1.07	0.952
Clostridium (3)	21.5	−2.10	0.602

* Correlation coefficient.

teroidaceae and *Clostridium* were isolated from the some segments or specimens. The predominant bacteria increased gradually during the passage of contents through the intestine of some specimens of carp, while any tendencies of distribution were not observed in other bacterial genera and in other specimens.

Table 1 shows the relationship between the viable count (VC) (log CFU/g dry wt.) of each bacterial genus and the TVC (log CFU/g dry wt.) in carp's intestines. The viable count of 12 bacterial genera out of 14, was closely related to the TVC with a correlation coefficient ranging from 0.822 to 0.986. Eleven genera of aerobes/facultative anaerobes possessed the slope (b value in Table 1) of less than 0.953 whereas the slope of *Bacteroides* type A and other Bacteroidaceae was 1.17 and 1.07, respectively. This result indicates that there was a close relationship between the declining percentage of each aerobe/facultative anaerobe and the increasing TVC ($b<1.00$), and conversely, between the raising percentage of each obligate anaerobe and the increasing TVC ($b>1.00$) in carp's intestines, as shown in Fig. 3.

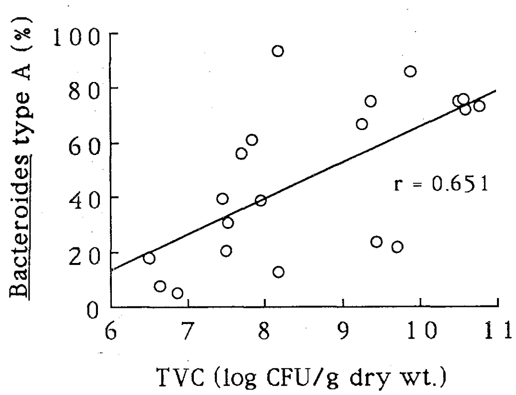

Fig. 3. The relationship between the percentage of *Bacteroides* type A and the TVC (CFU/g dry wt.) in the intestine segments of carp.

Interaction between Aerobes and Anaerobes

To provide additional insight into the above phenomenon, the interaction between aerobes/facultative anaerobes and obligate anaerobes was determined by using the disc-plate method. As a result, all the supernatant from 30 cultures of the aerobes/facultative anaerobes and the obligate anaeroes could not inhibit the growth of four obligate anaerobes or 26 aerobes/facultative anaerobes, respectively, on test plates. This result strongly suggests that the above-mentioned phenomenon was not attributed to the production of inhibitory substances by either the obligate anaerobes or the aerobes/facultative anaerobes.

Discussion

Although animals always take a large number of bacteria into their alimentary tract from their environments and diets, almost all the bacteria are temporary residents but some specific ones could stay in the intestine for a relatively long term.[4] It is known that, in mammals, the bacterial species which are capable of (a) adapting to physical and chemical conditions, such as Eh, pH, lysozyme, secretory immunogloblin, bile acids and digestive enzymes from the host; (b) overcoming competition with other bacterial species; and (c) attaching to the epithelium of intestinal tracts, could proliferate and establish in the intestinal tract of the host animal,[18-20] resulting in the colonization of microflora specific to each site of the intestinal tract.

In the previous papers,[6,12] we reported that the predominant bacteria increased prominently during the passage of contents through the intestine of tilapia *Tilapia nilotica* (=*Oreochromis niloticus*). Contrary to this, the viable counts of predominant bacteria, along with the TVC, in carp intestines did not necessarily increase during the passage of contents from fore- to post-intestine (Figs. 1 and 2). It was observed, however, that the percentage of each aerobe/facultative anaerobe dropped and that of each obligate anaerobes (including *Bacteroides* type A and other Bacteroidaceae) raised with the increase of TVC (Table 1 and Fig. 3). The following three reasons could be mainly considered for this phenomenon: (a) at higher bacterial densities, the aerobes/facultative aneaerobes consumed O_2 more rapidly in intestines, resulting in the lower Eh conditions, which is suitable for the obligate anaerobes such as the *Bacteroides* type A and other Bacteroidaceae, but is unsuitable for the aerobes; (b) the growth-inhibiting substance against the *Bacteroides* type A and other Bacteroidaceae was produced by the aerobes/facultative anaerobes; and, conversely, (c) the inhibitory substance against the aerobes/facultative anaerobes was produced by either the *Bacteroides* type A or the other Bacteroidaceae. To certify the possibilities of (b) and (c), a total of 30 intestinal bacterial strains were examined for antibacterial activity using the disc-plate method. However, no inhibitory effects were found in the supernatant of cultures in all the aerobic and anaerobic strains. This result strongly suggests that the raised percentage of the *Bacteroides* type A and other Bacteroidaceae, and the dropped percentage of aerobes at high TVCs, were attributed to the Eh and/or O_2 concentrations in carp's intestines, which unfortunately, were not measured in this study. Additionally, it was recently observed that the *Bacteroides* type A and other Bacteroidaceae could grow at high rates under low Eh conditions.* Therefore, these anaerobes may overcome the competition with aerobes/facultative anaerobes for nutrients and attaching sites on the epithelium in the intestinal environments after the low Eh condition is established, as recognized in mammals,[18-20] resulting in the raised percentage of the obligate anaerobes and the dropped percentage of aerobes/facultative anaerobes. Thus, the Eh value (O_2 concentration) should be an important factor to influence the balance of microflora at the first step in the carp's intestine. In the case of tilapia as described ealier,[12] the *Bacteroides* type A increased significantly during the passage of contents through the alimenary tract although such a trend was restricted to a part of speciments in the carp. The difference in the distribution of the *Bacteroides* type A, in the intestine, between carp and tilapia may possibly depend on the relative length of intesine (the ratio of intestine to total body length of fish) of 1.2–1.7 and 3.9–5.2, respectively. Further studies along those lines are now in progress.

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