Variations in the Intestinal Microflora of *Tilapia* Reared in Fresh and Sea Water*1

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The composition and characteristics of microflora isolated from the intestine of *Tilapia zillii* were determined for fishes reared in fresh and sea water. Intestinal microflora of fresh or sea water fishes consisted mainly of *Aeromonas* or *Vibrio* and *Aeromonas* respectively; while the predominant bacteria obtained from fresh or sea water samples were *Flavobacterium* or *Pseudomonas*, respectively. The intestinal bacteria isolated from sea water fish were slightly halophilic and lower in the percentage of casein hydrolytic strains compared with those from fresh water fish. Chitin hydrolytic bacteria were abundant in the intestines of both fresh and sea water fishes in contrast with water samples. The change of intestinal microflora of *Tilapia zillii* happened for a fairly short term depending on the change of environment.

Many investigators reported the existence and composition of gastrointestinal microflora of various fishes. Studies by Liston*1) and Sera *et al.*1) indicated the existence of *Vibrios* specific in the digestive tract of marine fishes. On the other hand, Trust*1) and Yoshimizu and Kimura*1) showed that the intestinal microflora of salmonids are mainly composed of the genus *Aeromonas* and the family *Enterobacteriaceae* when living in fresh water. However, little is known about its physiological roles and the interactions between microbes and host animals. More attention should be given to the significance of intestinal microflora in nutrition and immunological systems of fishes as well as mammals.

*Tilapia*, which lives in fresh water and river estuary of Africa originally, has been expected to be one of the most useful culture fishes in Japan. Moreover, *Tilapia* can be appreciated as an experimental fish which requires further investigation on intestinal microflora because of its feeding habit and adaptability in the various environments such as fresh water and sea water. *Tilapia* is considered to be omnivorous and can digest phytoplankton, algae and sometimes water plants. The digestive tract of *Tilapia* is 5–7 times as long as its body length and abundant in commensal microbes.

This paper describes the influence of environmental salinity, namely, fresh water and sea water on intestinal microflora and characteristics of isolates from the intestine of *Tilapia zillii*.

### Materials and Methods

**Fish Studied**

Fish (*Tilapia zillii*) used in this study had been reared at 20–23°C for 7–181 days in plastic tanks containing 50 l of tap water or natural sea water. If they were housed in sea water, tap water of tank was replaced with 1/2, 2/3 and finally full strength of sea water at intervals of two days. They were fed with commercial pellets twice a day. The data of fish samples are listed in Table 1.

### Table 1. Samples of *Tilapia zillii* used

<table>
<thead>
<tr>
<th>Samples</th>
<th>Sampling date</th>
<th>Feeding period days</th>
<th>Body length cm</th>
<th>Weight g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh water</td>
<td>1: '77, 6, 3.</td>
<td>43</td>
<td>17.0</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>2: '77, 10, 31.</td>
<td>19</td>
<td>18.5</td>
<td>135.0</td>
</tr>
<tr>
<td></td>
<td>3: '78, 5, 26.</td>
<td>7</td>
<td>18.5</td>
<td>89.0</td>
</tr>
<tr>
<td>Sea water</td>
<td>1: '77, 11, 17.</td>
<td>15</td>
<td>20.0</td>
<td>160.0</td>
</tr>
<tr>
<td></td>
<td>2: '77, 12, 7.</td>
<td>35</td>
<td>16.0</td>
<td>81.0</td>
</tr>
<tr>
<td></td>
<td>3: '78, 4, 21.</td>
<td>181</td>
<td>14.0</td>
<td>62.5</td>
</tr>
</tbody>
</table>

**Preparation of Samples**

Fish was sampled before the morning feeding. Immediately, the abdomen of fish was opened and the gastrointestinal tract was removed aseptically. The section of intestine with the contents was cut to small pieces and homogenized with mortar. The homogenized specimen was weighed and transferred to a bottle containing a diluted solution of artificial sea water (ASW, Herbst’s formula). Sequential...

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ten fold dilutions were prepared and 0.1 ml of the appropriate dilutions were smeared on agar plate.

**Basal Media and Cultural Conditions**

For estimation of viable counts and isolation of aerobic heterotrophs from fish intestine, M series media were employed, which contain 1.0% of polypeptone (Daigo Eiyo), 0.3% of yeast extract (Daigo Eiyo) and 1/6 strength of ASW (M-All) or 1/2 strength of ASW (M-BII). Viable counts of water bacteria were determined on Z series media containing 0.5% of polypeptone, 0.1% of yeast extract and 1/6 strength (Z-AII) or full strength of ASW (Z-BII). The final pH of media was adjusted to pH 7.6, except for Z-AII to pH 7.2. Inoculated plates were incubated at 25°C for 6 days.

**Identification of Bacterial Isolates**

About 50 bacterial colonies were randomly picked up from each plate of suitable dilution containing between 50 and 300 colonies and subsequently purified by streaking them on agar plates. Identification of genus of isolates was made according to the scheme of Shewan et al.5) and its modification proposed by Simidu6). Especially, 0/129 sensitivity test was applied as the most important criterion for the differentiation between *Aeromonas* and *Vibrio*.

**Salt Requirement**

Salt requirement for the growth of isolates was examined according to the method proposed by Hidaka and Sakai7). The growth response of isolates to various NaCl concentrations was determined turbidometrically by measuring the absorbance of liquid cultures at 540 nm. The mean values of growth were obtained after incubated at 25°C for 3 days.

**Hydrolysis of Macromolecules by Isolated Bacteria**

The abilities of isolates to hydrolyze various macromolecules were confirmed by the appearance of clear or opaque zone around the colony on agar plate containing each substrate. Test plates for macromolecule hydrolysis consisted of a basal medium and the following substrates: casein (0.5%), gelatin (0.1%), starch (0.5%), dextrin (0.5%), chitin (0.5%), alginate (0.75%), cellulose (0.5%), tributyrin (1.5%), tween 80 (1.0%), olive oil (2.0%) and egg yolk (10%). A positive reaction of starch and dextrin hydrolysis was indicated by development of yellowish or brownish zone after addition of several drops of Gram’s iodine solution. In the case of gelatin hydrolysis clear zone was observed around colonies after flooded with mercuric chloride solution. A positive lecithinase reaction was indicated by an opaque zone in the medium around the colonies on egg yolk agar plate.

**Results and Discussion**

Viable counts on various media of heterotrophic bacteria in the intestinal tract and water samples are shown in Table 2. The total numbers of aerobic

<table>
<thead>
<tr>
<th>Samples</th>
<th>Viable counts (log No./g or ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z (1/6, 1/2 or 1)</td>
</tr>
<tr>
<td>Fresh water</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.1</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
</tr>
<tr>
<td>Fish intestine</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>6.8</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
</tr>
<tr>
<td>Sea water</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>5.6</td>
</tr>
<tr>
<td>Cultural water</td>
<td></td>
</tr>
<tr>
<td>Fresh water</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.1</td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
</tr>
<tr>
<td>3</td>
<td>4.2</td>
</tr>
<tr>
<td>Sea water</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.1</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
</tr>
</tbody>
</table>

* Number in parenthesis indicates the strength of ASW in each medium.
bacteria from fish intestine, water and diet were $7.6 \times 10^6$ to $1.7 \times 10^9$, $1.4 \times 10^4$ to $1.3 \times 10^7$ and $3.6 \times 10^9$ to $1.2 \times 10^4$ c.f.u./g or ml, respectively. The colony counts varied with the counting media used. Especially, the numbers of intestinal bacteria from fresh water fish were lower on M medium with full strength of ASW, while those from sea water fish were not significantly different on M media with three levels of ASW strength.

The generic compositions in the intestinal flora of fishes reared in fresh water and sea water, water samples and diet are given in Fig. 1. *Aeromonas* spp. (resistant to O/129) were the most abundant in the intestine of fresh water fish while *Vibrio* spp. (sensitive to O/129) and *Aeromonas* spp. were the predominant organisms recovered from that of sea water fish. It is interesting to note that most of *Aeromonas* spp. isolated from the intestine of fresh water fish produced gas in Hugh and Leifson medium while *Vibrio* spp. isolated from that of sea water fish did rarely. Furthermore, most of *Vibrio* spp. isolated from the intestine of sea water fish showed dim inhibition zone in O/129 sensitivity test compared with those of *Vibrio* standard strains (*V. parahaemolyticus*, *V. alginolyticus* and *V. fischeri*).

In contrast with intestinal flora, the predominant bacteria isolated from fresh water and sea water samples (sample A) belonged to *Flavobacterium* spp. (light orange pigmented) and *Pseudomonas* spp., respectively. Sea water sample B, in which fish had been reared for a long time (181 days) possessed more *Aeromonas* (resistant to O/129) in addition to *Pseudomonas* spp. This can happen under the influence of the feces from fish during feeding period. The bacteria isolated from diet (commercial pellet) were exclusively *Bacillus* spp.

Typing of isolates by mineral requirement according to the method by Hidaka and Sakai indicated that T type and HL type were mainly isolated from fresh water fish and sea water fish respectively (see Table 3). Fig. 2 shows the growth response of isolates to various NaCl concentrations. Intestinal bacteria from fresh water fish had 1.5% of optimal NaCl concentration and grew well at 0% NaCl in polypeptone-yeast extract medium (M medium). On the other hand, isolated bacteria from sea water fish grew well at wide range of NaCl concentrations (2–5%) but could not grow at 0% NaCl.

Table 4 gives the percentage of isolates which possess hydrolytic activities of each macromolecule.
Table 3. Typing of isolated bacteria on the basis of mineral requirement

<table>
<thead>
<tr>
<th>Five types of media</th>
<th>Isolates from (%)</th>
<th>Intestine</th>
<th>Water</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.W. 0.5% NaCl 3.0% NaCl 1/6 ASW 1 ASW Type</td>
<td>F.W.* S.W.* F.W. S.W.-A S.W.-B</td>
<td>T 100 78</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>+ + + + T</td>
<td>100 78</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- + + + HL</td>
<td>78 22</td>
<td>20</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>- - + ± HH</td>
<td>22 100</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- - - ± M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total strains tested</td>
<td>19 27 18 20 10 7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* F.W.: fresh water, S.W.: sea water.

Fig. 2. Growth response of isolated bacteria to various NaCl concentrations.
A: sea water sample-A, B: sea water sample-B.

Table 4. The abilities of isolated bacteria to hydrolyse various macromolecules

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Intestine</th>
<th>Water</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.W.</td>
<td>S.W.</td>
<td>F.W.</td>
<td>S.W.-A</td>
</tr>
<tr>
<td>Casein</td>
<td>93</td>
<td>54</td>
<td>87</td>
</tr>
<tr>
<td>Gelatin</td>
<td>93</td>
<td>77</td>
<td>89</td>
</tr>
<tr>
<td>Starch</td>
<td>90</td>
<td>98</td>
<td>71</td>
</tr>
<tr>
<td>Dextrin</td>
<td>91</td>
<td>98</td>
<td>70</td>
</tr>
<tr>
<td>Chitin</td>
<td>83</td>
<td>60</td>
<td>7</td>
</tr>
<tr>
<td>Alginate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tributyrin</td>
<td>91</td>
<td>95</td>
<td>70</td>
</tr>
<tr>
<td>Tween 80</td>
<td>63</td>
<td>50</td>
<td>69</td>
</tr>
<tr>
<td>Olive oil</td>
<td>17</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Lecithin</td>
<td>89</td>
<td>66</td>
<td>37</td>
</tr>
</tbody>
</table>

Total strains tested | 122 100 107 48 50 59 | | | |
Interestingly, most of intestinal bacteria, both from fresh and sea water fishes had chitin hydrolytic activities compared with water bacteria. On the other hand, the percentage of casein hydrolysis in the intestinal flora of sea water fish were comparatively lower. Some investigators\(^5-10\) reported previously that the predominant bacteria from the intestine of sea water fish have not the ability to hydrolyse casein or gelatin.

These results in this study reinforce the idea that the change of intestinal flora of fish can occur for a fairly short term depending on the change of environmental factors.

Isolation and identification of intestinal flora of many fishes have been made by many investigators. However, mutual relationships between fish and its associated organisms, such as infection, immunity and nutrition, are still unclear compared with mammals. In this respect we feel that *Tilapia*, which has a highly developed digestive tract and adaptability to various environments, is very useful as an experimental fish to be made further studies of intestinal microflora.

**Acknowledgements**

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**References**