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Identification of Intestinal Microflora in Rainbow Trout

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Although trout farming is well established in Korea, very little information is available on the composition of intestinal microflora in rainbow trout (*Salmo gairdnerii*). In 1994, from October through November, we investigated the composition and succession of intestinal bacteria. As fish grew, total viable counts increased dramatically until 45 days after fertilization when anaerobes started to appear on the media. After this time, they increased steadily. Ten aerobic genera were identified and Gram negative bacteria constituted 85% of total isolates. Among these, *Pseudomonas*, *Eikenella*, and *Alcaligenes* were the three major genera. Six anaerobic genera were isolated and identified. The ratio of anaerobes to aerobes was about 1 : 1 in adult trout and the composition of genera was changed under different conditions.

Key words: intestinal microflora, *Salmo gairdnerii*, aerobe, anaerobe

We can make a diagnosis of ill fish by observing decreased appetite, lack of vitality, different swimming pattern, change of color, protrusion of eye, ulcer, etc. However, it takes time and money to treat the ill fish once a disease has been detected. If the onset of disease could be detected as early as possible even before the symptoms appeared, it will save a lot of time and labor. One of the possible detection methods would be to observe the intestinal microflora in fish.

Intestinal microflora of animals can be changed by age, disease, nutritional condition, and stress, and the changes can induce disease, and vice versa (6, 8, 11), so it may be possible to assay the susceptibility of fish to disease by observing the composition of microflora. To do this, it is essential to know the normal intestinal microflora to compare with. There have been several researches about fresh water fish, but most of them were about microorganism associated with a specific disease (8, 11). Even though many reports concerning the normal microflora in marine (3, 10) and fresh water fish (6, 7) have been published, but trouts in a fishery has not been reported, yet. In this report, we identified the intestinal microflora of the rainbow trout (*Salmo gairdnerii*) and the time of establishment during growth.

Materials and Methods

Fish

Rainbow trout used in this study were provided from Wonbok fishery (Kangwon Province, Korea). Fish were cultured at 14°C in the fishery and transported to the laboratory about 3 hours away from the fishery. During transportation, fish were wrapped in a plastic bag and placed in an ice box.

Media and reagents

Homogenized samples were inoculated and spread on Brain Heart Infusion Agar (BHIA) with selective media. *Pseudomonas* isolating agar (for isolating *Pseudomonas*), Phenylethanol Agar (for isolating *Staphylococcus* and *Streptococcus*), Lactobacilli MRS (for isolating *Lactobacillus*) and BHIA were all purchased from Difco (Detroit, U. S. A.). Other reagents were purchased from Sigma (Saint Louis, U. S. A.).

Enumeration of viable bacteria

Eggs and fry : To remove bacteria from the external surfaces, eggs or fry were washed three times with sterile water. Tens of eggs or fry were broken up in 5 ml of sterile saline solution using a sterile glass homogenizer, and an aliquot of 10 μl of homogenate was spread on the solid media.

Adult trout : The surfaces of trout were sterilized with 70% alcohol, and intestines (from stomach to anus) were obtained asceptically from five trout and homogenized with 100 ml of sterile water in a glass homogenizer.

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After 10 μl of homogenate was inoculated and spread on the solid media, inoculated plates were incubated at 30℃ under aerobic, microaerophilic (5% CO_2), and anaerobic conditions. Colonies were counted after 3 days.

Isolation and identification of bacteria

Aerobic and facultatively anaerobic bacteria : A plate with about 100 colonies was selected and every colony was isolated and subcultured on new BHIA. Bacteria were identified to the genus level following the scheme of Baron *et al.* (5). The tests employed for the identification were done as described in Laboratory Manual of Experimental Microbiology including colony morphology, Gram staining, cell morphology, pigment production, oxidase reaction, motility, catalase reaction, oxidation-fermentation, growth on blood agar, growth on MacConkey agar, indole formation, H_2S formation, urease test, arginine dihydrolase test, fermentation of glucose, maltose, sorbitol, sucrose, and lactose, nitrate reduction, spore formation, and acid fast staining (Table 3) (4).

Anaerobic bacteria : Plates inoculated as above were kept at 30℃ in an anaerobic jar (anaerobic BBL GasPak jar, Becton Dickenson Microbiology Systems, U. S. A.) for one week. Bacteria grown under anaerobic condition were identified using API ID 32A Kit (BioMrieux Vitex Inc., U. S. A.).

Results and Discussion

The intestinal microflora in fish can be used as an indicator for the degree of stress and the susceptibility of fish to disease. The first thing was to determine the composition of the intestinal microflora in a normal trout to use as a standard. Often people have reported quite different generic compositions of the bacterial microflora in a specific animal. The differences mainly resulted from the two following factors (3); (1) changes in the views of microbial classification; and (2) differences in methods of bacterial isolation. Concerning the latter factor, special consideration should be given to the composition of the media used for isolating bacteria and incubation temperature. For the isolation of fish pathogens, BHIA, Nutrient agar (NA), or Tryptic Soy Agar (TSA) has been commonly used. However, when we used BHIA and NA, BHIA gave more various types of bacteria than NA (data not shown). So we used BHIA in this study for the isolation and culture of bacteria. One other thing to be considered was the temperature. Since the optimum growth temperature for trout is 14℃, intestinal bacteria in trout are expected to grow best at 14℃. But when plates were incubated at different temperatures (14℃, 30℃, and 37℃), the plates incubated at 30℃ gave the largest number of viable counts (data not shown). So we chose 30℃ for the incubation temperature in this study. To calculate the total viable counts, the number of colonies appeared on Lactobacilli MRS was added to those on BHIA because *Lactobacillus* does not grow well on BHIA and needs microaerophilic condition for better

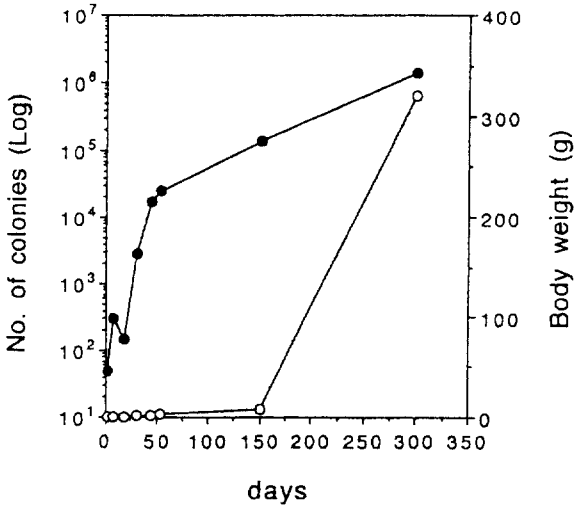

Fig. 1. Total viable counts at various developmental stages. Eggs, fry and adult trout were broken in sterile saline solution using a sterile glass homogenizer. Homogenate was inoculated with spreading on BHIA and Lactobacilli MRS. BHIA were incubated under aerobic and anaerobic conditions and Lactobacilli MRS plates were incubated under microaerophilic condition (5% CO_2). Total viable counts was calculated by adding the number of colonies on BHIA to that on Lactobacilli MRS. ●—●, the total number of colonies; ○—○, the body weight

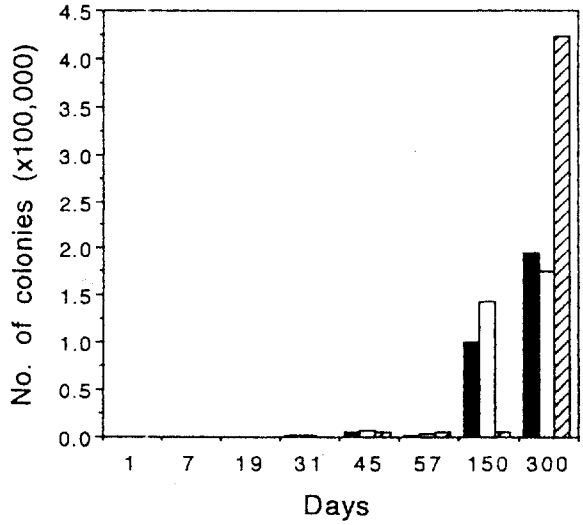

Fig. 2. The distribution of *Pseudomonas, Staphylococcus/Streptococcus,* and *Lactobacillus* at various developmental stages. Trout at various developmental stages were treated as described in Materials and Methods. The homogenate was inoculated on each selective medium for *Pseudomonas, Staphylococcus/Streptococcus,* and *Lactobacillus.* ■ *Pseudomonas*; □, *Staphylococcus/Streptococcus*; ▨, *Lactobacillus.*

growth. There might be a failure to detect additional bacteria due to unsuitable bacterial culture condition for each specific bacterium.

Until a trout hatches from an egg, it is supposed that it has no bacteria in its body. Even after hatching, the alevins (yolk-sac fry) do not eat food until the yolk sacks are wholly absorbed. This means that the intestinal microflora in fry is supposed to come entirely from water until fry starts to eat food. As fish grows, the intestinal microflora would be established at some time, and it is critical to find out the time of this microflora establishment. The total viable counts of aerobes in trout increased rapidly up to 45 days (swim-up fry) after fertilization. But after 45 days, the increasing rate had reduced (Fig. 1), suggesting the critical period to help trout to establish intestinal microflora is before 45 days when fry starts eating food and getting bacteria from food. Every aerobic genera grown on each selective medium were shown in Fig. 2 and Table 1. As trout developed from egg to fry, *Pseudomonas*, *Staphylococcus/Streptococcus*, and *Lactobacillus* had appeared and made up the main genera in adult trout. *Staphylococcus/Streptococcus* were the first ones to appear. Then as trout grew bigger, *Lactobacillus* became prevalent making up 60% of all aerobes. So this genus was thought to have a healthy microflora. In fact, when disease occurred from water contamination during flood, the mortality was very low in fry treated with Doarase (composed of vitamins, lactic acid fermenting, saccharose hydrolyzing bacteria) compared with non-treated ones (data not shown). In an adult trout (300 days old after fertilization), aerobes took half the total viable counts in the intestinal microflora. Anaerobes started to appear at 45 days after fertilization and the number of them increased dramatically after 150 days (Fig. 3 and Table 1).

Since the frequency of disease in trout larger than 100 g became scarce, trouts with an average weight of 170 g from October to November, 1994 were chosen to study the composition of the intestinal microflora in adult trout. After plates were inoculated with serially

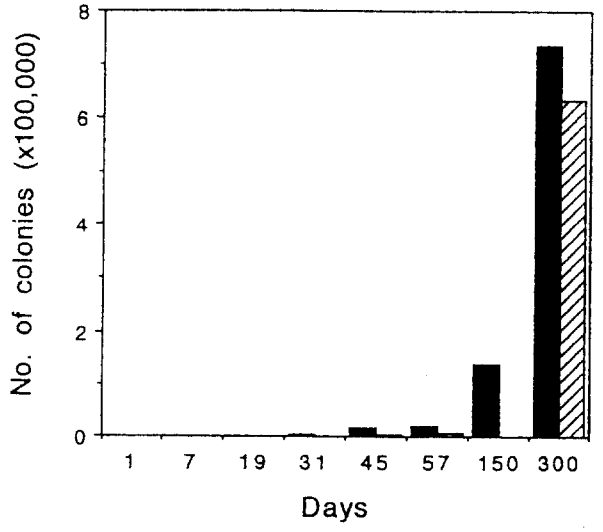

Fig. 3. Aerobes and anaerobes in the intestinal microflora in trout at various developmental stages. Eggs, fry and adult trout were broken in sterile saline solution using a sterile glass homogenizer. Homogenate was inoculated with spreading on BHIA and Lactobacilli MRS. BHIA were incubated under aerobic and anaerobic conditions and Lactobacilli MRS plates were incubated under microaerophilic condition (5% CO_2). The number of aerobes was the number of colonies on BHIA and Lactobacilli MRS. ■, aerobes; ▨, anaerobes.

Table 2. Identified genera of normal intestinal bacteria in rainbow trout

		Genera	No. (%)
Aerobe	Gram(+)	*Staphylococcus aureus*	7.1
		Non-spore forming rods	8.1
	Gram(−)	*Acinetobacter*	3.0
		Alcaligenes	13.1
		Capnocytophaga	4.0
		Eikenella	13.1
		Enterobactericeae	6.1
		Flavobacterium	9.1
		Kingella	2.0
		Pseudomonas	26.3
		Vibrio	2.0
		N.I.*	6.1
Anaerobe		*Clostridium histolyticum*	100.0

*N.I., could not be identified.

Table 1. Viable counts of aerobes and anaerobes and the number of colonies on each selective media

Days	BHIA	PEA[1]	PI[2]	MRS[3]	Aerobe	Anaerobe
1	50	0	0	0	50	0
7	300	0	0	0	300	0
19	150	0	0	0	150	0
31	2,800	1,200	1,250	0	2,800	0
45	9,600	6,900	6,000	5,600	15,200	2,050
57	15,600	3,400	1,450	5,200	20,800	4,800
150	132,000	176,000	196,000	424,000	736,000	632,000

[1] Phenylethanol agar, [2] *Pseudomonas* isolating agar, [3] Lactobacilli MRS.

Table 3. Physiological properties of isolates obtained from aerobic condition

Characteristics	Group of isolates									
	Sta.[1]	Capno.[2]	Aine.[3]	Pseu.[4]	Vibrio	Kin.[5]	Eik.[6]	Flavo.[7]	Alkali.[8]	Entero.[9]
Gram staining	+	−	−	−	−	−	−	−	−	−
Cell shape	cocci	rod	rod	rod	rod	rod	rod	rod	rod	rod
Growth on sheep blood agar	*	+	+	+	+	+	+	+	+	+
Growth on MacConkey	*	*	+	d	*	*	*	*	*	+
Oxidation/Fermentation	*	F	O/−	O/−	F	F	−	O/−	O/−	O
Pigment	d	+	−	+	*	*	−/Y	+	−	*
Gelatin liquefaction	*	*	*	*	+	*	*	*	*	*
Growth on TCBS	−	*	*	*	+	*	*	*	*	*
Oxidase	−	d	−	+	*	+	+	+	+	−
Catalase	+	−	+	+	*	−	−	+	+	*
Acid from glucose	*	+	*	*	+	+	−	*	−	*
maltose	+	+	*	*	*	−	−	*	*	*
sorbitol	*	−	*	*	*	−	−	*	*	*
sucrose	+	+	*	*	*	−	−	*	*	*
lactose	+	d	*	*	*	−	−	*	*	*
Indole	*	−	−	−	*	−	−	*	*	*
Nitrate → Nitrite	+	d	*	*	*	d	+	*	*	*
Urease	+	*	d	d	*	*	−	−/+	d	*
H_2S in KIA	*	*	−	−	*	−	−	−	−	*
Arginine dihydrolase	+	*	−	d	*	*	−	−	−	*
Motility	−	+	−	+	+	*	*	−	+	d

Symbols: [1] *Staphylococcus;* [2] *Capnocytophaga;* [3] *Acinetobacter;* [4] *Pseudomonas;* [5] *Kingella;* [6] *Eikenella;* [7] *Flavobacterium;* [8] *Alcaligenes;* [9] *Enterobacteriaceae*; * not done; Y, yellow; F, fermentation; O, oxidation; d, different from strains.

diluted samples, a plate on which about 100 colonies appeared was selected for the identification of bacteria. Every colony appeared under aerobic condition was isolated. And 85% of all isolates was Gram negative (Table 2). Among them, *Pseudomonas, Eikenella,* and *Alcaligenes* were the three major genera. *Pseudomonas* and *Alcaligenes* were known to be harbored in many fish (1, 2, 6, 10). *Pseudomonas, Flavobacterium, Acinetobacter,* and *Enterobacteriaceae* were also found in other fish, while *Aeromonas, Bacillus,* and *Moraxella* were known to be present at significant levels in other fish but not in trouts (6, 9, 11). Randomly selected 28 colonies grown under anaerobic condition were identified as *Clostridium histoliticum* which is ubiquitous in nature (Table 2). However, different genera were identified in trouts caught during spring in 1995. These were identified as *Clostridium histoliticum, Clostridium acetobutylicum, Capnocytophaga* spp., *Gamella mobillorum, Peptostreptococcus micros,* and *Actinomyces meyeri.* In ill trouts, the majority of intestinal microflora were pathogens, which were detected in liver and kidney, too. These pathogens were identified as *Pseudomonas anguilliseptica, Pseudomonas fluoresence, Vibrio angillarum, Nocardia,* and *Streptococcus.* Treatments with ciprofloxacin, sulfonamide, erythromycin were successful but not with tetracycline (data not shown). To find out the source for the intestinal microflora in trouts, we are analyzing the food and water used in the fishery.

From this study, we suggest the following: (1) the composition of intestinal microflora can be used to detect the healthy state of trouts, (2) the use of food additives to swim-up fry before 45 days old will help the normal microflora to be established and decrease the mortality.

Acknowledgements

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