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Comparative study on bacterial flora of the intestine in Persian sturgeon (*Acipenser persicus*) fingerlings reared in fiberglass tanks and earthen ponds

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Abstract

In this study, random sampling conducted and 90 individuals of Persian sturgeon having 3-5g weight, collected by trawling in 3 earthen ponds and 15 individuals having 10-20g weight, collected by small net in 3 fiberglass Vans. After recording of biometrical characteristics, the intestine and rearing water were cultured on TSA medium. Gram staining and complementary tests were conducted. In order to identify the bacterial species, various biochemical experiments and diagnosis kit of API 20E (special for gram negative bacteria) applied. According to biometrical recording, in earthen ponds, the mean weight and length of juveniles were 5.59±3.18 and 11.4±2.9, respectively. The results showed that the mean facultative aerobic and anaerobic bacterial counts in intestine were 5.59±0.92 (log CFU g^{-1}) and 6.67±0.34 (log CFU Ml^{-1}) in *Acipenser persicus* juveniles and rearing water, respectively. Furthermore, the following bacteria determined in intestine of Persian sturgeon: *Aeromonas* sp., *A.sorbia*, *A.hydrophyla*, Entrobacteriaceae and Corynebacterium. The bacteria in the rearing water were *Aeromonas* sp. and A.*soberia*. According to biometrical recording, in fiberglass Vans, the mean weight and length of juveniles were 12.13±2.51g and 15.11±1.13 cm, respectively. The results showed that the mean facultative aerobic and anaerobic bacterial counts in intestine were 4.77±0.04 (Log CFU g^{-1}) and 4.81±1 (Log CFU mL^{-1}) in *A. persicus* juveniles and rearing water, respectively. Furthermore, the following bacteria determined in intestine of Persian sturgeon: *Aeromonas* sp., *Micrococcus*, *Staphylococcus*. The bacteria in the rearing water were *Aeromonas soberia, Acinetobacter*,*Moraxella, Micrococcus* and *Staphylococcus*.

Keywords: Earthen ponds, Intestine, Persian sturgeon, *Acipenser persicus*, Bacterial flora

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Introduction

Sturgeons are one of the oldest vertebrates on the earth and the Caspian Sea is their main habitat where 90% of the world caviar production belongs (Moghim, 1992; Pourkazemi, 1997). Regarding biodiversity and gene resources, sturgeons are considered to be the so called "fossils" of aquatic organisms living from million years ago (Sudagar, 2005). There are 27 sturgeon species worldwide which exclusively inhabit the northern hemisphere, five species of which inhabit the Caspian Sea (Bahmani, 1998). Persian sturgeon, *Acipenser persicus*, lives only in the Iranian waters in the southern part of the Caspian Sea, where different factors have reduced its stocks. Artificial rearing and propagation of sturgeons is of commercial importance and necessary for the conservation of these stocks, so Iran like other European and North American countries began artificial reproduction. We need comprehensive information on fish diseases and health because this data can improve the quality and health of sturgeon rearing in fiberglass tanks and earthen ponds for restocking and marketable rearing. Brun (1991) isolated the bacteria *Yersinia ruckeri*, *Vibrio anguillarum* and *Flexibacter culmnaris* from *A. baeri.* Also, Lartseva (1992) studied bacterial flora of sturgeons in the Volga River and observed bacteria such as *Aeromonas,Acinetobacter, Alcaligenes, Citrobacter, Escherichia, Enterobacter, Flavobacterium, Moraxella, Morganella, Klebsiella, Micrococcus, Hafnia, Proteus, Providencia, Pseudomonas, Salmonella* and *Vibrio.* Moreover, Francis-Floyd (2000), reported *A. hydrophila, A. sobria*, *Pseudomonas, Edwardsiella tarda*, *Yersinia ruckeri*, *Streptococcus* and *Flavobacterium columnaris*. Bauer (2002) investigated juvenile (3-4g) sturgeons and reported *Flavobacterium jansen*. Recently, the quality and health condition of the water environment and fishes are limiting factors in the development of freshwater fish species (Svobodova and Vykusova, 1995). In sturgeon hatcheries, one of the most important issues is the high mortality in larvae and fingerlings which should be considered carefully. The fish intestine can harbor 10^7 to 10^{11} bacteria g^{-1} intestinal content, with aerobes and facultative anaerobes being more abundant than obligate anaerobes, and these bacteria are divided into the autochthonous species which are attached to the intestinal mucosa, and the allochthonous which do not attach either due to lack of ability or outcompeted by mucous-attached bacteria (Nayak, 2010, Navarrete *et al*., 2012; Llewellyn *et al*.; 2014, Givens *et al*., 2015). Stability of bacterial flora in fish intestines is very significant, because the intestine is an important place for the outbreak of microbial infections and many fish diseases spread especially when the fish have not been vaccinated (Birkbeck and Ringo, 2005). The gut microbiota can be viewed as an "extra organ" due to their key role in intestinal development, homeostasis and protection and it is now becoming clear that the microbiota have major influences on growth, health

and development in all vertebrates (O'Hara and Shanahan, 2006; Dhanasiri *et al.*, 2011). The host may also exert a direct control on community assembly by physiological and lifestyle forces, offering a niche for specialized bacteria that are beneficial for their dietary life style (Roeselers *et al*., 2011). The aim of this study was to identify the bacterial flora of the intestine in sturgeon fingerlings and rearing water obtained from fiberglass tanks and earthen ponds. The total count of bacteria in rearing conditions were determined in order to identify facultative anaerobic and aerobic bacteria in the intestine of sturgeon fingerlings and rearing water and also to calculate the total count of bacteria. So, the results can be used for sturgeon health management and prevention of diseases during the rearing period which is commercially important for the restocking of the sturgeons.

Materials and methods

This study was conducted to determine the bacterial flora of sturgeon fingerlings in fiberglass tanks and earthen ponds in Shahid Beheshti Hatchery Facilities and also in Dadman International Sturgeon Research Institute, Rasht, Iran, during 2008-2009.

Ninety fingerlings of Persian sturgeon, *A. persicus* (3-5g in weight) were collected from three earthen ponds using trawl nets, and 15 fingerlings were randomly sampled from 3 fiberglass tanks. Laboratory devices were sterilized and rearing water was sampled. The fingerlings were transferred live to the Fish Health Laboratory, Dadman International Research Institute of Sturgeons, Rasht, Iran. After morphometric measurements and disinfection of abdominal surface with 70% alcohol, fish were gutted and the intestines were separated. The contents of the intestine were washed three times with saline serum, weighed, placed in sterilized glass dishes and then saline serum was added for dilution. The dilutions were prepared in the order of 10^{-1} to 10^{-6}, so 6 vials each containing 9 mL saline serum were prepared, tagged and then 1 mL of the initial solution was added to the first vial and shaken very well to homogenize it (10^{-1}). Then 1 mL of each vial was transferred to the 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} vial (Ringo *et al*., 1995). Thereafter, the required dilutions were cultured in TSA medium, transferred to an incubator at 20 ^{o}C (Memert Germany) for 24-48h and then the number of bacteria was calculated as CFU (colony forming unit). All the colonies were observed for bacterial growth and examined according to color, shape and size in order to enumerate and prepare pure cultures (Lartseva, 1992). After verifying the purity of colonies, gram staining and supplementary tests were performed consisting of oxidase, catalase, motility, oxidation, fermentation, hydrolyzing tests and etc. All stages of the experiment were also performed on the rearing water. Different biochemical tests were employed to determine bacterial species based on Holt method (Newman *et al*., 1972) and diagnostic kits of API 20E (specific for gram-negative bacteria). Physicochemical

factors of pond water including temperature, dissolved oxygen and pH were measured two times a day with an oxygen meter and pH meter (WTW-Multi 3401). Electric conductivity was measured two times a week with an EDT unit (EDT BA 300). Solid materials were measured weekly using the gravimetric method. Nitrite, ammonium and orthophosphate were measured weekly using standard kits. One-way analysis of variance (ANOVA), Tukey and HSD were employed to analyze the results of enumeration, identification of bacteria as well as the data from water physicochemical factors (Microsoft Office, SPSS 22).

Results

The results of morphometric measurements including the mean weight and length of fingerlings in earthen ponds were 5.59±3.81 g and 11.4±2.9 cm, respectively. Physicochemical factors of pond water during the rearing period were as follows: mean temperature=25.6±0.5˚C, dissolved oxygen=4.76±1.48 mg L^{-1}, pH=8.05±0.5, NO_2=0.06±0.01 mg L^{-1}, NH_4=0.24±0.80 mg L^{-1}, PO=0.17 mg L^{-1}, and water clarity= 66±7.5 cm (Table 1).

Table 1: Physicochemical factors of rearing water for sturgeon fingerlings in earthen ponds.

Water condition / Pond number	Temperature (°C)	Oxygen Mg L^{-1}	pH	depth cm	Transparency cm	NO_2 mg L^{-1}	NH_4 mg L^{-1}	PO_4 mg L^{-1}	TSS mg L^{-1}	EC Ms cm^{-1}
1	25.6	3.1	7.61	195	57.5	0.06	0.15	0.17	0.03	1250
2	25.6	5.22	7.95	202.5	72	0.05	0.31	0.17	0.03	1305
3	25.7	5.95	8.6	206.7	68.3	0.07	0.25	0.17	0.03	1288
Mean	25.63	4.76	8.05	201.4	66	0.06	0.24	0.17	0.03	1281
Standard Deviation	0.05	1.48	0.5	5.92	7.5	0.01	0.08	0	0	28.1
Standard Error	0.03	0.85	0.29	3.42	4.34	0.005	0.04	0	0	16.25

According to One-Way ANOVA test, there were no significant differences in the physicochemical factors between earthen ponds. So similar physicochemical conditions were observed in all the three ponds.

In all the fiberglass tanks, physicochemical factors of water included mean temperature (25.8±2.02^{0}C), oxygen (4.55±0.52 mg L^{-1}), pH (7.8±0.13), nitrite (0.07±0.02 mg L^{-1}) and CO_2 (0.6±0.14 mg L^{-1}) (Table 2).

According to the one-way ANOVA test, no significant differences were found among fiberglass tanks in temperature (F=0.41, p=0.7), dissolved oxygen (F=0.38, p=0.71) and pH (F=2.1, p=0.27) (p>0.05).

Table 2: Physicochemical parameters of water in fiberglass tanks.

Factor / Rearing Period	Temperature (°C)	Oxygen mg L^{-1}	Oxygen saturation (%)	CO_2	pH	Nitrite
First week	22.3	5.1	61	-	7.9	0.02
Second week	26.9	3.8	42	0.5	7.7	0.03
Third week	25.3	4.8	50	0.7	7.8	0.02
Fourth week	27.2	4.9	61	-	8	0.01
Fifth week	27.9	4.7	58	-	-	-
Sixth week	25.2	4	49	-	7.7	0.02
Mean	25.8	4.55	53.5	0.6	7.8	0.02
Standard Deviation	2.02	0.52	7.7	0.14	0.13	0.007

The results of bacterial counting in the intestine of fingerlings and rearing water showed that in each pond there are two

species bacteria in intestine and there are three species in the rearing water. The number of bacteria was 5.13-5.97 Log CFU mL^{-1} in the pond rearing water. The mean count of aerobic and anaerobic facultative bacteria in the fish intestines and rearing water were 5.59±0.92 Log CFU g^{-1} and 6.67 ± 0.4 Log CFU mL^{-1}, respectively (Table 3). The results of one-way ANOVA test showed that there were no significant differences in the diversity and number of the bacteria among the earthen ponds ($p \geq 0.05$) (Fig. 1).

Table 3: The diversity and number of bacteria counted in the earthen ponds.

Result / Pond Number	Bacterial Variation in fish intestine (species)	Total Count of bacteria in intestine Log CFU mL^{-1}	Bacterial Variation in Pond water (species)	Total Count of bacteria in Water Log CFU mL^{-1}
1	2	5.13	3	7.01
2	2	5.93	3	6.67
3	2	5.97	3	6.33
Mean	2	5.59	3	6.67
Standard Deviation	0	0.92	0	0.34

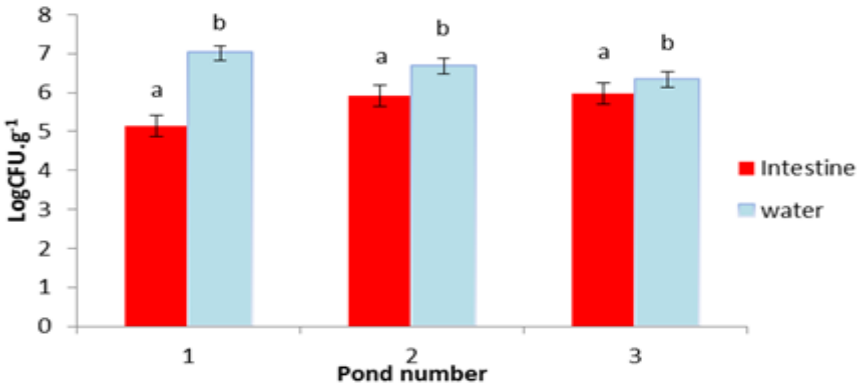

Figure 1: Total count of bacteria in the fish intestine and water of earthen ponds.

The results of bacterial identification in earthen ponds showed that the bacteria existing in intestine of Persian sturgeon fingerlings include Enterobacteriaceae, *Corynebacterium, Aeromonas* sp., which were similar to *A. sorbia* and *A. hydrophila. Aeromonas* sp., were dominant in the intestine (47.7%). The bacteria in rearing water were *Aeromonas* sp. and *A. sorbia,* the former being the most dominant (87.5%). Also, the total frequency of the bacteria in fish intestine and rearing water were found to be in the range of 2.6-52.2%; the lowest value belonged to *A. hydrophila,* while the highest belonged to *Aeromonas* sp. (Table 4).

Table 4: The frequency of the bacteria observed in the rearing water and intestine of Persian sturgeon fingerlings.

Bacteria	Frequency in intestine (%)	Frequency in water (%)	Total Frequency (%)
A. sobria	26.3	13.5	23.9
Aeromonas sp.	44.7	87.5	52.2
A. hydrophila	2.6	-	2.2
Corynebacterium sp.	13.2	-	10.9
Enterobacteriaceae	13.2	-	10.9

The results of bacterial count in the intestine of fingerlings and rearing water showed that the bacterial diversity in the first tank was two species, while in the second and third tanks it was one, and in the rearing ponds it was two; based on Log in range, 4.3-5 CFU g^{-1} were found in the intestine and 3.78-5.81 CFU mL^{-1} in the rearing water. The mean counts of facultative aerobic and anaerobic bacteria in the intestine of fingerlings and the rearing water were 4.77±0.4 Log CFU g^{-1} and 4.8±1 Log CFU mL^{-1}, respectively (Table 5).

The results of one-way ANOVA showed that there are no significant differences in the number of bacteria in the water of fiberglass tanks and intestines of fingerlings ($F=0.004$, $p=0.95$).

Table 5: The diversity and number of bacteria counted in fiberglass tanks.

Count / Basin Number	Bacterial Variation in intestine	Total Count Log CFU g^{-1})	Bacterial Variation in Pond water	Total Count Log CFU mL^{-1})
1	2	4.3	2	5.81
2	1	5	2	4.85
3	1	5	2	3.78
Mean	-	4.77	2	4.8
Standard Deviation	0	0.4	0	1

The bacteria identified in the fiberglass tanks belonged to 6 genera as follows: *Aeromonas* sp., *A. sobria, Acinetobacter, Micrococcus, Moraxella* and *Staphylococcus* which included 4 isolates in the intestine and 6 isolates in water. Of these bacteria, *Aeromonas* sp., *Staphylococcus* and *Micrococcus*

were recovered from fish intestines, while the other five genera, except *Aeromonas* sp., were recovered from water. The results showed that in the fiberglass tanks, the most frequent bacteria was *Micrococcus* (60%), while the least were *Aeromonas* sp. and *Staphylococcus*, (each at 20%) in the gut of fingerlings. In the water of fiberglass tanks, the genera *Moraxella*, *Aeromonas* sp., *Acinetobacter* and *A. sobria* were found at the lowest frequency (16.5%) and *Micrococcus* at a higher frequency (34%) (Table 6).

Table 6: The amount (%) of identified bacteria in fiberglass tanks 1, 2 and 3.

Bacteria	Intestine		Water		Total	
	Number	Frequency (%)	Number	Frequency (%)	Number	Frequency (%)
Acinetobacter	-	-	1	16.5	1	9.1
A. sobria	-	-	1	16.5	1	9.1
Aeromonas sp.	1	20	-	-	1	9.1
Micrococcus	3	60	2	34	5	45.4
Moraxella	-	-	1	16.5	1	9.1
Staphylococcus	1	20	1	16.5	2	18.18

Discussion

The results showed that the physiochemical parameters of water in the rearing ponds were almost similar to each other and there were no significant differences between them. The most important parameters were temperature, oxygen and pH. The immune response caused by temperature changes was considerable in fishes (Ellis, 1982). When immune response was stopped by a decrease in temperature, the fish could not defend themselves against the bacterial pathogen mechanisms in the living environment (Cahill, 1990). The bacteria that exists and move from the gut should be able to resist low pH, enzymatic digestion, lysosome effects and immunoglobulin in the intestine (Cahill, 1990). Like higher vertebrates, the microflora in fish gut should be adapted to different conditions like food compounds, pH, anaerobic conditions, concentration of bile salts and digestive tract enzymes, host immunity system and interaction of bacteria in the gut (Hansen and Olafsen, 1999). Fishes are aquatic organisms which are in direct contact with the water environment and this does not mean that the microbial flora in water can affect the fish body, because different mechanisms in fish lead to a decrease in fish microbial intensity compared to water. Mucous secretions, lysosomes and externally secreted antibodies as well as cell defense barriers can prevent direct entry of bacteria into the fish body. Microbial flora of the digestive tract in human and inland creatures are nearly the same (Moriarty, 1990), while in aquatic organisms that are cold-blooded and the body temperature is affected by the environment, the microbial flora changes constantly (Lesel, 1990). According to previous studies, the optimum rates of dissolved oxygen, temperature and pH for rearing sturgeon fingerlings are 5mg L^{-1}, 19-24 $^{\circ}C$ and 7-8, respectively (Cahill, 1990). In this study these parameters were in the temperature range

mentioned above. In another research, 10^8 heterotrophic bacteria were separated from each gram of fish intestine (Brun, *et al.*, 1991). There are evidences which show seasonal changes in the bacterial numbers, in that the maximum and minimum bacterial flora were observed in summer and winter, respectively. Differences in the number of bacteria can be related not only to physicochemical factors, but also to season, type of feeding, sampling method, fresh or frozen digestive tract, methods of culture, homogenization of gut contents, etc. (Brun, *et al.*, 1991). In the present study, the results of bacterial count in the intestine of fingerlings and water of rearing ponds showed no significant differences. Also, no significant differences were observed in the results of bacterial assays in fiberglass tanks, which could be due to the similar physicochemical conditions in the tanks. In this study, the number of facultative aerobic and anaerobic bacteria was 10^5-10^6 or 5.13-5.97 Log CFU g^{-1} in the intestine and 10^6-10^7 or 6.33-7.01 Log CFU mL^{-1} in the water of rearing ponds. In fiberglass basins the bacterial count in gut was 10^4-10^5 or 4.3-5 Log CFU g^{-1} and 10^3-10^5 or 3.78-5.81 Log CFU mL^{-1} in the rearing water. The bacterial count in the gut of farmed *Huso huso, A. gueldenstaedtii* and *A. stellatus* were 1.4×10^2, 1.5×10^6 and 2.9×10^6 CFU g^{-1}, respectively, while it was 2.75×10^4-4.93×10^5 CFU.g^{-1} in farmed Sterlet (*Acipenser ruthenus).* Furthermore, the number of bacteria in water of rearing ponds was 9.75×10^5 CFUmL^{-1} (Lartseva and Bormotova, 1998). In the study on farmed *H. huso* fingerlings by Moazzenzadeh (2008), the number of facultative aerobic and anaerobic bacteria in the gut was 6.07-7.11 Log CFU g^{-1}. These rates are higher than in the present study in fiberglass tanks and earthen ponds. Moreover, the bacterial count in the was 4.87±1.54 Log CFU mL^{-1} in the rearing water which was higher than that in fiberglass tanks and less than the count in earthen ponds compared to the present study. This can be due to variation in water physicochemical factors, type of diet, difference in bacterial flora of live food and the source of water. The rate of bacteria in the gut is indicative of fish habitat and type of food consumed (Cahill, 1990). Mostly, there are 10^3-10^9 bacteria per gram in fish intestine (Poursafar Tabalvandani, 2009). The present study showed that the bacterial counts in the intestine of fingerlings in fiberglass tanks and earthen ponds were in this range. Trust *et al*. (1979) observed a number of bacteria such as *Proteus, Bacteroides sp. Moraxella. A. hydrophila, Acinetobacter, Clostridium* and *Micrococcus* present in the gastrointestinal tract of grass carp, goldfish, and rainbow trout.

The number of bacteria in the digestive tract of yolk sac larvae and matured larvae is 0-10^4 n g^{-1}, while in fingerlings it is 10^3-10^7 bacteria per gram (Cahill, 1990). The normal number of bacteria in fish intestines is 10^8 aerobic heterotrophic bacteria per gram and nearly 10^5 anaerobic bacteria per gram (Ringo *et al*., 1995). In the present study, some bacteria identified in the intestine of Persian sturgeon

fingerlings inhabiting earthen ponds included Enterobacteriaceae, *Corynebacterium, Aeromonas* sp. and the bacteria similar to *A. sobria* and *A. hydrophyla* of which *Aeromonas* sp. was the dominant one. The bacteria identified in the intestine of fingerlings reared in fiberglass tanks included *Aeromonas* sp., *Micrococcus* and *Staphylococcus,* of which, *Micrococcus* was the dominant. The bacteria in rearing water included *A. sobria, Acinetobacter, Moraxella, Micrococcus* and *Staphylococcus,* with Micrococcus being the dominant species.

In the study carried out on sturgeons by Francis-Floyd (2000) in Florida, *A. hydrophyla, A. sobria, Pseudomonas, Edwardsiella tarda, Yersinia ruckeri, Streptococcus* and *Flavobacterium columnaria* were recovered. Brun *et al*. (1991) identified *Y. ruckeri, V.anguillarum* and *F. columnaris* in Siberian **sturgeon** (*A. baeri*). The bacteria identified by Lartseva and Bormotova (1998) on sturgeons in the Volga River consisted of *Aeromonas, Enterobacteriaceae* and *Pseudomonas*. Finally in a study conducted by Bauer *et al* (2002), *F. johnsonae* was recovered from the 3-5 g sturgeon juveniles. In contrast to the bacteria recovered from the wild fish in marine water, the micro flora of intestines in freshwater fish are mostly bacteria such as *Aeromonas, Plesiomonas* and Enterobacteriaceae, *Bacteriodes* and *Fusobacterium* (Hansen and Olafsen, 1999). In another study, *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp., *Moraxella* sp., *Acinetobacter* and *Enterobacteriaceae* were recovered from *A. nudiventris, A. persicus* and *A. stellatus* larvae (Shenavar Masouleh *et al.,* 2003). When bacteria enter the digestive tract by water and food, they inhabit the gut or become some part of the natural flora in the intestine or may be killed by antimicrobial factors, enzymes secreted by the digestive tract or unfavorable conditions of gut, or may be directly excreted in feces. Furthermore, sometimes when they are located on the external surface and digestive tract, they can act as primary pathogens and cause diseases (Austin and Austin, 1993). Austin and Austin (1993) reported various types of motile *Aeromonas* and *Pseudomonas* in fish rearing ponds.

In freshwater fishes Enterobacteriaceae, *Aeromonas* and *Acinetobacter* are dominant species, while in the digestive tract of marine fishes, *Vibrio, Pseudomonas, Acromobacter, Corynebacterium, Flavobacterium* and *Micrococcus* are dominant (Colwell, 1962; Newman *et al*., 1972). In the present study, some of the above-mentioned bacteria were also recovered from fingerlings and from rearing water in fiberglass tanks and earthen ponds. The microorganisms found in the rearing water can affect the bacterial flora of the fish. The rate of bacteria transferred to the water from fish body should also be considered in fish rearing facilities where the stock density is constant (Cahill, 1990). The type of bacteria isolated from the intestine changes with variation in salinity, antibiotics, chromic acid, food and diet composition as well as daily modifications. The species

Acinetobacter, Enterobacter and *Pseudomonas* are in association with each other and they are indigenous in wild salmon, while *Aeromonas, Flavobacterium, Pseudomonas* and *Lactobacillus* are indigenous in Char (*Salvelinus alpinus*) (Ringo *et al*., 1995). In freshwater salmon, fauna and flora are essentially *Aeromonas* which consist of *Enterobacteriaceae, Flavobacterium* and *Pseudomonas. Vibrio* is dominant in the intestine of marine fish species, while Pseudomonas is isolated from many fish species and therefore diet also has some influence on the microflora of the intestine in freshwater and marine fish species (Ringo and Strom, 1994). The present study showed that *Aeromonas* has the most frequency in fish intestines and rearing water. Enterobacteriaceae were also isolated at a lower frequency. With respect to identifying some bacteria such as Enterobacteriaceae, *Corynebacterium* and *Aeromonas* all of which are pathogenic in fish, it is necessary to consider health management and prevent stressors (which cause some suppression in fish immunity). During the rearing period, it is necessary to consider some predisposing factors such as handling, transporting and releasing fish fingerlings in more favorable rivers.

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