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preliminary communication

Differences in Bacterial Population in Rainbow Trout (*Oncorhynchus mykiss Walbum*) Fry after Transfer from Incubator to Pools**

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Summary

The microflora of rainbow trout (O*ncorhynchus mykiss Walbaum*) fry from a commercial freshwater hatchery, along with important water quality parameters such as temperature, dissolved oxygen and pH, was analysed. Samples for bacteriological analysis were taken from gill, heart and kidney, from the third to the eighth week after hatching. Pure bacterial colonies were examined macroscopically, with Gram staining and biochemical tests. For final identification, the APILAB Plus programme (bioMérieux, France) was used. The bacterial populations of rainbow trout fry changed depending on age. Most of the bacterial colonies were cultured from the gills (64.4 %), rather than the heart (21.8 %) and kidney (13.8 %). The bacterial community of fry gills from an incubator was composed mostly of Gram-positive bacteria such as *Renibacterium salmoninarum*, *Lactobacillus* spp., *Staphylococcus* spp. and *Corynebacterium aquaticum*. After the transfer of fry from incubator into the pools the Gram-negative bacteria increased in number and became the dominant microflora of rainbow trout fry and comprised more than 95 % of its bacterial flora. *Flavibacterium*, *Acinetobacter* and *Yersinia* were the predominant Gram-negative genera in fry in the incubator, whereas *Aeromonas* and *Pseudomonas* were the main isolates from rainbow trout fry until the end of the experiment.

Key words: fry, rainbow trout, bacterial flora

Introduction

In the aquatic environment, hosts and microorganisms share a similar ecosystem where bacteria can either colonise the host (intestinal tract, gills, or skin) or not (*1*). The primary microbiota of the early stages of aquatic larvae and fry depend partly on the bacterial community in the rearing water (*2*,*3*). Therefore, the properties of the bacteria in the ambient water are of some importance for the development of the fry microbiota (*4*).

Disease resistance in fish under farming conditions may be affected by stress (*5*). In this context subclinical infections occur, often unrecognized and ignored, manifesting themselves as lower production potential. Economic consequences can be reduced growth, increased mortality and high costs of treatment with antibiotics. Many reports describe *Yersinia ruckeri*, *Flavobacterium psychrophilum* and *Aeromonas salmonicida* as causal agents of diseases (enteric redmouth disease, rainbow trout fry syndrome and furunculosis) with major significance in the culture of salmonids (*6*,*7*).

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In fish health management the most useful index of success is prevention of disease, and systematic physico-chemical analysis of water and monitoring of the microorganisms in the aquaculture system, which should be routine practice in hatcheries.

A few reports on the intestinal microflora of rainbow trout (O*ncorhynchus mykiss*) are available (*8*,*9*), and also about the microflora associated with gills, intestine, liver and kidney, with main bacterial groups of *Aeromonas*, *Pseudomonas*, enterobacteria and Gram-positive cocci (*10*). However, very little information is available on the composition of the rainbow trout fry microbiota in the first weeks of posthatching. The major aim of the present study was therefore to isolate and characterize the bacteria of the gills of rainbow trout during early feeding. This evaluation is of great importance as the gills are one of the most probable routes of primary infection, being somehow analogous to inhalation as a way of transmission of bacteria to the bloodstream (*11–13*). Accordingly, the present work studies the effects of the gill microbiota on the health and survival of rainbow trout fry by comparing the composition of the gill flora of rainbow trout with the heart and kidney microbiota from the third to the eighth week after the hatching.

Materials and Methods

Fish samples

Samples of rainbow trout fry were taken from one commercial freshwater fish farm in Zagreb. The third week after hatching the fry (200 000) were transferred to four outdoor rearing pools with a continuous through-flow of water. The fry (40) were randomly selected from two of four pools in the same developmental and environmental conditions, and were sampled twice a week (on Tuesday and Friday) for six weeks.

Samples of rainbow trout fry (10) were taken under aseptic conditions from the pool and collected in sterile plastic bags. Live samples were immediately transferred to the laboratory and subjected to analysis within 30 min of collection. A total of 220 rainbow trout fry were killed by severing the spine just behind the cranium with a scalpel.

Physicochemical analysis

Temperature, dissolved oxygen and pH were measured on site using a digital thermometer, dissolved oxygen meter (UC-12 Kagaku, Japan) and digital pH meter (UC-23 Kagaku, Japan).

Biometric analysis

Prior to bacteriological analysis the collected rainbow trout fry were weighed and their length was measured. Total length was measured in centimeters from the top of the head to the end of the tail. Mass was measured in grams on a digital scale. The total body condition factor was calculated according to Fulton (*14*).

Microbiological sampling

The first sample of 10 fry was collected from the incubator prior to transfer of fry from incubator to pools. Subsequent sample of 10 fry was taken 45 min later after the transfer of fry to the pools. During the next five weeks of the experiment, samples of 200 fry were collected from two pools.

Samples for bacterial analysis were collected from gill, heart and kidney. All the samples were placed onto tryptic soy agar plates (BD-BBL) for isolation. The plates were incubated for 24 to 48 h at 22 °C. After that, colonies, which represent different morphologies per plate, were randomly picked (to pick many different phenotypes) from each sample and restreaked onto nutrient agar plates three times to obtain pure culture.

All the purified isolates were observed and characterized according to cell morphology, motility, Gram staining, oxidase and catalase activities. Phenotypic characterisation of the isolates was carried out using the APILAB Plus program (bioMérieux, France) according to the manufacturer's instructions.

Results

Physicochemical analysis

Data on physicochemical characteristics of water at the time of sampling are presented in Table 1. There was no significant difference in physicochemical values of rearing pools, which were within the optimum range for rainbow trout fry rearing.

Table 1. The physicochemical characteristics of pool water at the time of sampling (N=2)

Day	t/°C	pH	γ(dissolved oxygen)/(mg/L)
1	7.3	7.54±0.007	10.8±0.354
4	7.3	7.54±0	10.4±0.919
8	7.4	7.55±0.014	11.6±0.071
11	7.4	7.59±0.007	11.9±0.141
15	7.5	7.28±0.007	12.1±0.212
18	7.6	7.29±0.007	12.2±0.283
22	8.5	7.23±0.007	12.0±0.566
25	8.0	7.31±0.007	12.3±0.354
29	8.7	7.27±0.028	12.4±0.212
32	8.7	7.27±0.014	12.1±0.071
35	8.5	7.36±0.028	12.5±0.354

Biometric analysis

Biometric analysis showed an exponential relationship between the length and mass. Average body length generally increased during sampling period. Average body mass also increased during all sampling periods, except on the eight day of the experiment when a slight decrease was measured, which also reduced the condition factor.

Bacterial flora

The microbiota detected in rainbow trout gill, heart and kidney before (sample 1; N=10) and after (sample 2; N=10) the transfer of fry from the incubator to the pool are shown in Table 2.

Table 2. Microbiota of gills, heart and kidney of rainbow trout (*Oncorhynchus mykiss*) on the first day of the experiment (*N*=20)

Bacterial group		Gram-positive bacteria				Gram–negative bacteria				
Total		11				6				17
%		64.7				35.3				100
Species		*Corynebacterium aquaticum*	*Lactobacillus* spp.	*Renibacterium salmoninarum*	*Staphylococcus* spp.	*Acinetobacter* sp.	*Aeromonas hydrophila*	*Flavobacterium psychrophilum*	*Yersinia* sp.	Total
Sample 1 from incubator	Gill	1	2	2	1	1		1	1	9
	Heart	1		1						2
	Kidney				1					1
Sample 2 from pool	Gill							2		2
	Heart			1						1
	Kidney				1		1			2
Total		2	2	4	3	1	1	3	1	17

On the first day of the experiment a total of 17 strains of bacteria were isolated from gills (11), heart (3) and kidney (3), and identified from the samples 1 and 2.

Of the 9 strains isolated from the gill of fry at the beginning of the experiment (sample 1), 6 strains were Gram-positive and 3 strains were Gram-negative. *Lactobacillus* spp. (2), *Renibacterium salmoninarum* (2), *Corynebacterium aquaticum* (1) and *Staphylococcus* sp. (1) were detected as Gram-positive strains isolated from the gills. Gram-negative strains from the gills were identified as *Acinetobacter* sp. (1), *Aeromonas hydrophila* (1) and *Yersinia* sp. (1). Gram-positive strains (*Corynebacterium*, *Renibacterium* and *Staphylococcus*) were also detected as the only organisms from the heart and kidney of the fry in incubator.

Of the 5 strains isolated after the transfer of fry from the incubator to the pool (sample 2), 2 strains were isolated from the gills and identified as Gram-negative (*Flavobacterium psychrophilum*). One strain was isolated from the heart and identified as Gram-positive (*R. salmoninarum*), whereas 2 strains were isolated from the kidney and identified as *Staphylococcus* sp. and *A. hydrophila*.

A total of 239 strains of bacteria (Table 3) were isolated during the five weeks of sampling of 200 fry from the two pools.

Of the 154 strains isolated from the gills, 30.52 % (47 strains) and 26.62 % (41 strains) were *Aeromonas* and *Pseudomonas*, respectively. Of the 52 strains isolated from the heart, 32.69 % (17 strains) and 21.15 % (11 strains) were *Aeromonas* and *Pseudomonas*, respectively. Of the 33 strains isolated from the kidney, 33.33 % (11 strains) and 30.30 % (10 strains) were non-fermenter spp. and *Pseudomonas*, respectively. *Aeromonas* (4 strains) were also detected as minor organisms from the kidney. From these findings, the dominant genera in the gills and heart were *Aeromonas* and *Pseudomonas*, while non-fermenter bacteria and *Pseudomonas* were dominant in the kidney.

Discussion

Of the 256 strains isolated in the present study, 165 strains were isolated from the gills, 55 strains from the heart and 33 strains from the kidney. These results confirmed a previous report (*10*) about the distribution and diversity of the microbiota among the organs, with decreasing diversity and count from the gills to the internal organs.

The general composition of bacterial population encountered in the gill of rainbow trout is in agreement with other studies performed on this fish species (*10*). The present work shows that *Aeromonas* and *Pseudomonas* were the dominant bacteria on the gills, which are also represented as dominant in the heart flora. These results confirmed previous findings (*11,12*) that the gills are the route of infection. In this context, the results of lower bacterial count obtained from the heart seemed to be determined by the gills, which are not only a route of infection but also the immune response tissue (*13*).

Gram-positive bacteria show weak colonizing potential. Out of the 18 Gram-positive isolates obtained during the experiment, 11 isolates (61.11 %) were identified from the sample 1 from the incubator on the first day of the experiment and the majority of isolates (6 strains) were isolated from the gills. The dominant Gram-positive bacteria on the gills belonged to *Renibacterium salmoninarum* (2 strains), *Lactobacillus* spp. (2 strains), *Staphylococcus* sp. (1 strain) and *Corynebacterium aquaticum* (1 strain). Transfer of the fry to the pools reduced bacterial flora of the gill and most strains belonged to the predominating Gram-negative bacteria. Likewise, the majority of the Gram-positive and Gram-negative isolates on the first day of the experiment were recognized as fish pathogenic species (*5–7*). However, we also identified *Lactobacillus* sp., which was previously reported as bacteria associated with postspawning mortality of rainbow trout (*15*).

From the 33 isolates obtained from the kidney, 10 belong to the *Pseudomonas*. *Aeromonas* were also detected as microbiota from the kidney of the rainbow trout fry, but not as dominant microbiota like in previous observations (*10*). Identifications could not be determined for 11 of the isolates from the kidney. These unidentified isolates were all Gram-negative rod-shaped non-fermenter bacteria (+/- *Pseudomonas* spp. according to bioMérieux, France).

Condition factor reflects the nutritional state of the fish and it was used in the experiment as a health index of the fry collected during this study. Results do not suggest systematic changes in the health index, except a reduction in mass that was seen on the 8th day of experiment. These results suggest that during the yolk-sac stage, *i.e.* yolk resorption, the fry lose mass.

The results presented here indicate that the presence of bacteria is not the only factor for the outbreak of disease. The absence of the mortality and abnormalities

Table 3. Bacterial flora of rainbow trout fry in the pools during the experiment (N=200)

Bacterial group		Gram-negative bacteria													Gram-positive bacteria			
Total		232													7			239
%		97.07													2.93			100
Species		*Aeromonas* spp.	*Brevundimonas* spp. / *Pasteurella* spp.	*Burkholderia* spp.	*Citrobacter* spp.	*Empedobacter* spp.	*Flavobacterium* spp.	*Moraxella* spp.	*Myroides* spp.	Non-fermenter spp.	*Pasteurella* spp.	*Pseudomonas* spp.	*Vibrio* spp.	*Shewanella* spp.	*Bacillus* spp.	*Renibacterium* spp.	*Staphylococcus* spp.	Total
Week	Organ																	
1st	Gill	6						2		1		2	1			1		
	Heart	3					1	2				1	3			1	1	
	Kidney	1						2				3					1	
2nd	Gill	14	2				4			6	1	6						
	Heart	5								2	1	2						
	Kidney									3			1					
3rd	Gill	13					1	1	2	7	1	10						
	Heart	5								2	2	2						
	Kidney	3							1	2	1	2						
4th	Gill	8	3	1	1	1	3			6		15			2			
	Heart	3	1	1						2		4			1			
	Kidney		1							6		4						
5th	Gill	6			4		5				7	8		4				
	Heart	1			1							2		3				
	Kidney										1	1						
No. of strains from gill %		47	5	1	4	1	13	3	2	20	9	41	1	4	2	1		154
		30.52	3.25	0.65	2.60	0.65	8.44	1.95	1.30	12.99	5.84	26.62	0.65	2.60	1.30	0.65		64.43
No. of strains from heart %		17	1	1	1		1	2		6	3	11	3	3	1	1	1	52
		32.69	1.92	1.92	1.92		1.92	3.85		11.54	5.77	21.15	5.77	5.77	1.92	1.92	1.92	21.76
No. of strains from kidney %		4	1					2	1	11	2	10	1				1	33
		12.12	3.03					6.06	3.03	33.33	6.06	30.30	3.03				3.0	13.81
Total no. of strains		68	7	2	5	1	14	7	3	37	14	62	5	7	3	2	2	239
%		28.45	2.93	0.84	2.09	0.42	5.86	2.93	1.25	15.48	5.86	25.94	2.09	2.93	1.25	0.84	0.84	100

in the rainbow trout fry observed in the experiment may be attributed to the optimal water quality.

Conclusion

This study of microbiota of rainbow trout fry shows that the composition of the microflora varies between different samples obtained from the incubator and those from the pools. The bacterial flora of the gills of the rainbow trout fry in the incubator consisted mostly of Gram-positive bacteria.

Transfer of fry to the pools reduced the Gram-positive bacteria and maintained them at a low level until the end of the experiment.

Bacterial flora of rainbow trout fry in the pools consisted mostly of Gram-negative bacteria. The majority of the bacteria were isolated from the gills. *Aeromonas* and *Pseudomonas* were the predominant genera distributed among the gills and heart, whereas non-fermenter bacteria and *Pseudomonas* were predominant genera isolated from the kidney.

These results provide a basis for further epidemiological research based on fine molecular methods in exploring bacterial flora of rainbow trout fry, and their antibiotic susceptibility.

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Razlike u bakterijskoj populaciji mlađi kalifornijske pastrve (*Oncorhynchus mykiss Walbum*) nakon premještanja iz inkubatora u bazene

Sažetak

Analizirana je mikroflora mlađi kalifornijske pastrve (O*ncorhynchus mykiss Walbaum*) iz komercijalnog uzgajališta, te važni pokazatelji, kao što su kakvoća i temperatura vode, otopljeni kisik i pH. Uzorci za bakteriološku analizu uzeti su iz škrga, srca i bubrega mlađi starih tri do osam tjedana. Čiste bakterijske kolonije utvrđene su makroskopski, bojenjem po Gramu i biokemijskim testovima. Za konačnu identifikaciju upotrijebljen je APILAB Plus program (bioMérieux, France). Bakterijska se populacija mlađi kalifornijske pastrve mijenjala tijekom razvoja. Najviše bakterijskih kolonija dobiveno je iz škrga (64,4 %), a manje iz srca (21,8 %) i bubrega (13,8 %). Bakterijska se populacija iz škrga mlađi u inkubatoru većinom sastojala od Gram-pozitivnih bakterija, i to *Renibacterium salmoninarum*, *Lactobacillus* spp., *Staphylococcus* spp. i *Corynebacterium aquaticum*. Broj Gram-negativnih bakterija povećao se nakon premještanja mlađi iz inkubatora u bazene i postao dominantna mikroflora (95 %) mlađi kalifornijske pastrve. Dominantni Gram-negativni rodovi mlađi u inkubatoru bili su *Flavibacterium*, *Acinetobacter* i *Yersinia*, dok su u bazenu glavni izolati iz mlađi kalifornijske pastrve bili *Aeromonas* i *Pseudomonas.*