

BACTERIAL MICROFLORA OF FRESHWATER FISH ORIGINATED FROM USMAS LAKE IN LATVIA

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ABSTRACT

The aim of the present study was to evaluate the bacterial contamination of gills, skin and content of gastrointestinal tract of freshwater fish. Altogether 31 fish samples, including European eel (n=11), Silver bream (n=3) and European perch (n=17) were collected between April and July in 2014 from Usmas lake located in West part of Latvia. For evaluation of bacterial load the number of TBC (total bacterial count) and *Enterobacteriaceae* genera in gills, skin and intestinal tract was enumerated. Presence of *Salmonella* spp., *L. monocytogenes* and *Y. enterocolitica* was analyzed according to the ISO methods. Among different fish species investigated TBC in gills, skin and intestinal samples ranged from 1.89 to 7.47 log CFU.g⁻¹, from 0.66 to 8.47 log CFU.cm⁻² and from 0.30 to 8.25 log CFU.g⁻¹ accordingly. Number of *Enterobacteriaceae* genera in gills, skin and intestinal tract ranged from 0.7 to 5.78 log CFU.g⁻¹, from 4.91 to 7.12 log CFU.cm⁻² and from 0.70 to 7.47 log CFU.g⁻¹, respectively. Number of TBC and *Enterobacteriaceae* in European eel samples of gills, skin and intestinal tract was significantly less than in Silver bream and European perch samples (P<0.05). *Salmonella* and *Y. enterocolitica* were not found in fish, while *L. monocytogenes* was isolated from one perch (1/ 17/ 6). Results of study indicate that microbiological quality of eel was better than of perch and bream, which found to be extensively contaminated with TBC and *Enterobacteriaceae* making fish unacceptable for human consumption. Presence of *L. monocytogenes* in perch exhibits public health concerns, indicating that fish could be a vector for transmission of pathogen to consumers.

Keywords: Freshwater fish, *Salmonella*, *L. monocytogenes*, *Y. enterocolitica*, *Enterobacteriaceae*, Total bacterial count, Latvia

INTRODUCTION

Fish meat is a significant part of diet of consumers in Latvia and average consumption of fish per capita was 6.6 kg in 2013 (Latvijas statistika, 2013). Freshwater fish comprise the important part of total fish and fish products available on market, because of the widespread occurrence in environment. Recreational fishing is very popular in Latvia, there fishermen use self-caught fish for meal preparation and this is a specific pattern of consumption of fish. Since recreational fishing for consumption is common among the inhabitants the microbiological assessment of freshwater fish is necessary to get information about fish are available for fishermen for consumption.

Fish microbiological contamination depends on the status of water source. Microflora of surfaces and skin of fish are continuously affected by aquatic environment including bacteria present in water, sediment and contamination from polluted wastewater. Since the changes in microflora of surrounding environment have an impact on number and composition of bacterial microflora fish may serve as the indicator of pollution of aquatic environment (Ribeiro et al., 2010). Despite of this the evaluation of microbiological quality of fish should address not only environmental health aspect, but also the consumers' safety. The environment health and water hygiene may be affected by various factors, e.g. human and animal wastewater or weather condition resulting in contamination of freshwater source and fish with pathogens of public health significance as *Salmonella*, *Listeria* and *Yersinia* and leading to alteration of bacterial microflora of it. Consumption of contaminated fish and fish products can be potentially hazardous to consumers and lead to foodborne infections thus, the prevalence of pathogens in fish raise concerns about product safety issues (Miettinen and Wirtanen, 2006, Junior et al., 2014).

Limited studies on bacterial contamination of fish and the occurrence of pathogens are available on freshwater fish and usually they are covering limited geographical area (Davies et al., 2001, Yücel and Balci, 2010). Such studies are very valuable to identify the problem and to implement the preventive measures among consumers, retailers and fishermen. Previous studies also mostly were

more focused on retail, but not fishermen level, so it is valuable to estimate the situation on freshly caught fish obtained from fishermen to recognize the situation at the beginning of a food chain.

Therefore, the aim of the present study was to detect Total Bacterial Count (TBC) and *Enterobacteriaceae* in fish skin, gills and gut and to evaluate the presence of *Salmonella* spp., *Y. enterocolitica* and *L. monocytogenes* on freshly caught fish samples obtained from fishermen.

MATERIAL AND METHODS

Selection of samples

All samples were obtained from Usmas lake located in West part of Latvia during April, 30 to July, 30 in 2014. During sampling water temperature of lake was from 11.8°C up to 19.6°C (Latvijas vides, ģeoloģijas un meteoroloģijas centrs, 2014) Altogether, 31 fish samples were collected, including 11 samples of European eel (*Anguilla anguilla*) and 3 and 17 samples of silver bream (*Blicca bjoerkna*) and European perch (*Perca fluviatilis*), respectively. Samples were obtained directly from fishermen, put in sterile bag and transported to the laboratory on ice immediately after sampling. Testing was initiated within 2 hours after collection of samples.

Sampling

For detection of TBC and *Enterobacteriaceae* genera skin, gill and intestinal tract samples were investigated separately. Surface samples of fish skin were collected with abrasive sponge moisturized with 0.1% peptone water by covering a 25 cm² or 100 cm² area of fish skin depending on fish size. For collection of intestinal tract samples the abdomen was incised and the intestinal tract containing both the intestines with the content was separated from surrounding tissues. For gill sampling the gills were aseptically separated from surrounding tissues. Skin, gills and intestinal tract of each fish were investigated separately and not less than 1 g

of gills and intestinal tract and a sponge covering 25 cm² of skin were used for testing.

Samples for detection of *Salmonella* spp. *L. monocytogenes* and *Y. enterocolitica* were prepared with pooled sample of skin, musculature and intestinal tract of each fish. In total 25g of material was used for detection of each pathogen.

Bacteriological analyses

For detection of TBC and *Enterobacteriaceae* 0.1% peptone water was added to each gill, skin and intestinal tract sample to make a 1:10 dilution. After this the serial dilutions were prepared for the investigation of samples. For TBC 1 ml of each serial dilution was transferred onto plate count agar (PCA, Biolife, Milan, Italy) using two plates for each dilution, and incubated for 72 h at 30°C. After incubation bacterial colonies were enumerated.

For detection of *Enterobacteriaceae* 1 ml of suspension from each serial dilution was transferred onto violet-red bile glucose agar (VRBA, Biolife) using two plates for each dilution and incubated for 24 h at 37°C. Typical colonies of *Enterobacteriaceae* were tested from oxidase activity and inoculated into Glucose agar (Biolife) with subsequent incubation for 24 h at 37°C. Only oxidase negative and glucose positive colonies were confirmed as *Enterobacteriaceae*. For *Enterobacteriaceae* count typical bacterial colonies were enumerated.

For detection of *Salmonella* a total amount of 25g of fish samples were transferred into 225 ml of buffered peptone water and incubated for 18±2 h at 37°C. After pre-enrichment 0.1ml of suspension was transferred in Rappaport-Vassiliadis broth (Biolife) and Mueller-Kauffmann Tetrathionate-Novobiocin broth (Biolife) for enrichment for 24 h at 41.5°C and 37°C accordingly. Enriched suspension was plated out onto brilliant green (Biolife) and XLD (Biolife) agar for subsequent incubation for 24 h at 37°C and plates were examined for the presence of presumptive colonies.

For detection of *L. monocytogenes* an amount of 25g of sample was transferred into Half-Fraser broth and incubated for 24 h at 30°C. After incubation 0.1ml of suspension was transferred into Fraser broth and incubated for 48 h at 37°C. An amount of 0.1ml of enriched material of Half-Fraser and Fraser broth was plated

out on Oxford (Biolife) and ALOA (Biolife) agar plates and incubated for 24-48 h at 37°C. After incubation agar plates were screened for the presence of presumptive colonies, which were selected for further confirmation. Presumptive colonies were stained according to Gram, checked for catalase activity, mobility and β-hemolysis. Suspicious *Listeria* spp. isolates were confirmed with API *Listeria* (BioMérieux, France).

For detection of *Yersinia* spp. an amount of 25g of sample was transferred into peptone sorbitol bile salt broth and incubated 24 h at 22°C. After incubation 0.1 ml of suspension was plated out onto CIN agar (Biolife) with and without treatment with 0.5% KOH prior to plating. CIN plates were incubated at 30°C for 24-48 h and examined for the presence of typical colonies after incubation. Selected colonies were screened for oxidase activity and urea hydrolysis and oxidase activity negative and urea hydrolysis positive cultures were confirmed with API 20E (BioMérieux, France).

Statistical analyses

All microbial counts data were transformed to decimal logarithms. For evaluation of differences between means of microbial counts for TBC and *Enterobacteriaceae* genera in eel, bream and perch samples a Student's *t* test analysis was applied.

RESULTS AND DISCUSSION

Among different fish species investigated TBC in gills ranged from 1.89 to 7.47 log CFU.g⁻¹ and the highest number of TBC was observed in Silver bream gill samples, while the lowest – on eels gill samples. Number of TBC was the highest in silver bream and the lowest in eel in intestinal samples too and ranged from 0.30 up to 8.25 log CFU.g⁻¹. TBC in skin samples ranged from 0.66 up to 8.47 log CFU.cm⁻², there the highest number of TBC was originated from European perch samples, while the lowest from eel samples (Table 1).

Table 1 The total bacterial count (TBC) in freshwater fish gills, skin and intestinal tract

Fish species	Sampling site					
	Gills		Skin		Intestinal tract	
	Mean ± SD ^d	Range	Mean ± SD	Range	Mean ± SD	Range
European eel (<i>Anguilla anguilla</i>)	2.41±0.51 ^a	1.89-3.25	1.5±0.36	0.66-1.84	1.88±1.59	0.30-5.82
Silver bream (<i>Blicca bjoerkna</i>)	7.37±0.15 ^b	7.26-7.47	7.24±0.39	6.96-7.51	7.55±0.99	6.85-8.25
European perch (<i>Perca fluviatilis</i>)	5.73±0.72 ^c	4.53-6.29	7.99±0.36	7.39-8.47	6.63±0.71	5.57-7.43

Legend: ^a TBC in European eel samples was significantly less than in Silver bream and European perch samples (P<0.05)

^b no significant differences between TBC in gill, skin and intestinal tract samples were observed in Silver bream gills, surface and intestinal tract samples (P>0.05)

^c significant differences between TBC in gills, surface and intestinal tract were observed in European perch (P<0.05)

^d CFU/cm² or CFU/g⁻¹

In general contamination of gills, skin and intestinal samples of eel was significantly less than in breams and perches (P<0.05). Eels in Latvia are introduced to lakes in elder or glass eel stage in the frame of eel breeding program and this could influence the contamination rates of eel microflora. TBC of eel gill, skin and intestinal samples is in accordance with previous reported study on number of bacteria presents on wild and farmed fish from cold and unpolluted environment (González et al., 1999). In contrast the Silver bream and European perch are wide spread in freshwater environment and belong to their native inhabitants in Latvia and therefore they can better reflect the situation with contamination of water source. In general contamination of skin, gills and intestinal tract for Silver bream and European perch was high in the present study. Nedoluha and Westhoff, 1997, Pullela et al., 1998 reported that bacterial load of freshwater fish depends on environmental influences and on type of fish production system. Thus, the high level of contamination of gills, intestinal tract and especially skin could be linked to contamination from outside environment.

Broad differences in TBC in freshwater fishes were reported previously and the number of TBC in skin samples was from 0.03 CFU.g⁻¹ in carps in fish farms in Slovakia up to 10⁷ CFU.g⁻¹ in Rainbow trout skin samples in fish farm in Turkey (Andreji et al., 2006, Diler et al., 2000). Also number of TBC in gut was higher than 8.5x10⁶ CFU.g⁻¹ in Mahmoud et al., 2004 study, however number in the intestinal content of stipped bass and salmonids could be >10⁸ CFU.g⁻¹ that is agreement with our results (González et al., 1999).

Enterobacteriaceae was not detected on European eel, but was present in Silver bream and European perch skin samples. Number of *Enterobacteriaceae* in gills were ranged from 0.7 up to 5.78 log CFU.g⁻¹, there the highest *Enterobacteriaceae* count was identified in European perch, but the lowest – in European eel samples. Number of *Enterobacteriaceae* count in skin and intestinal tract was from 4.91 to 7.12 log CFU.cm⁻² and from 0.70 to 7.47 log CFU.g⁻¹, respectively, there the wide range in *Enterobacteriaceae* count among the European perch samples was observed (Table 2).

Table 2 The number of *Enterobacteriaceae* count in freshwater fish gills, skin and intestinal tract

Fish species	Sampling site					
	Gills		Skin		Intestinal tract	
	Mean ± SD ^d	Range	Mean ± SD	Range	Mean ± SD	Range
European eel (<i>Anguilla anguilla</i>)	1.4±0.51 ^a	0.7-2.11	0±0.00	0	2.43±2.44	0.70-4.15
Silver bream (<i>Blicca bjoerkna</i>)	4.93±0.4 ^b	4.9-4.97	5.48±0.8	4.91-6.04	6.04±1.26	5.14-6.93
European perch (<i>Perca fluviatilis</i>)	4.68±0.79	3.8-5.78	7.07±0.11	6.95-7.12	5.70±1.28	3.83-7.47

^a Number of *Enterobacteriaceae* in European eel samples was significantly less than in Silver bream and European perch samples (P<0.05)

^b Number of *Enterobacteriaceae* of skin samples was significantly higher in European perch than in Silver bream (P<0.05), but there were no differences in number of *Enterobacteriaceae* between gills and intestinal tract samples of European perch than in Silver bream (P>0.05)

^d CFU/cm² or CFU/g¹

Regarding to number of *Enterobacteriaceae* count of gills, skin and intestinal samples of eel it was significantly less in eels than in bream and perch (p>0.05) similar to number of TBC and the explanation of overall goof microbiology quality of eels in comparison with perch and bream could be their introduction to freshwater environment under the breeding program. In general number of *Enterobacteriaceae* count of eels in the present study is in agreement with previously reported on wild and farmed fish from cold and unpolluted environment (González et al., 1999). An *Enterobacteriaceae* genus serves as a valuable indicator of hygienic status of the environment and products, related to hygiene and environmental contamination. High number of *Enterobacteriaceae* count in gills and intestinal and especially in skin samples indicates on contamination possibilities from outside environment. It is important to point out

that increased number of *Enterobacteriaceae* count along with TBC may reflect the contamination from outside environment and possible consequences for environment and for fish consumers. Results on *Enterobacteriaceae* count in the present study were higher than the previously reported by González et al., 1999 in Spain for wild and farmed fish ranged from 1.00x10¹ CFU.g⁻¹ to 4.86x10⁴ CFU.g⁻¹ and by Su et al., 2011 in China fish farms ranged from 3.3x10³ CFU.g⁻¹ to 4.4 x10⁴ CFU.g⁻¹. Skin was the most contaminated with coliforms in tilapia samples in Junior et al., 2014 study, who linked this to contamination of fish skin after fishing.

Salmonella spp. and *Y. enterocolitica* were not isolated from fish samples in the present study, while one sample of European perch was found to be *L. monocytogenes* positive (Table 3).

Table 3 Prevalence of *Salmonella* spp., *Listeria monocytogenes* and *Yersinia enterocolitica* in freshwater fish samples

Fish species	<i>Salmonella</i> spp.		<i>Listeria monocytogenes</i>		<i>Yersinia enterocolitica</i>	
	No. of positive samples/ No. of samples (%)	No. of samples (%)	No. of positive samples/ No. of samples (%)	No. of samples (%)	No. of positive samples/ No. of samples (%)	No. of samples (%)
European eel (<i>Anguilla anguilla</i>)	0/ 11 (0)		0/ 11 (0)		0/ 11 (0)	
Silver bream (<i>Blicca bjoerkna</i>)	0/ 3 (0)		0/ 3 (0)		0/ 3 (0)	
European perch (<i>Perca fluviatilis</i>)	0/ 17 (0)		1/ 17 (6)		0/ 17 (0)	
Total	0/ 31 (0)		0/ 31 (3)		0/ 31 (0)	

Absence of pathogenic bacteria in eel and bream indicates that fish are safe for consumption, while presence of *L. monocytogenes* in freshwater fish raise public health concerns. *Salmonella* was not isolated from freshwater fishes in studies across Europe and this is in agreement with our results (Davies et al., 2001, Andreji et al., 2006, Hudecová et al., 2010, Papadopoulos et al., 2010). *Salmonella* spp. can enter water sources with polluted and natural water related to human, wild and domestic animal activity. The high prevalence of *Salmonella* in fish was reported previously in Asia and Africa and usually is related to unsatisfactory hygienic condition of water sources and subsequently contamination of fish during placement on market (Budiati et al., 2013).

Our findings on the presence of *L. monocytogenes* in perch indicate that pathogen is distributed in aquatic environment in Latvia. *L. monocytogenes* is a natural inhabitant of water sources and can contaminate fish from sediment and outside environment and occasionally can contaminate fish and fish products. The prevalence of *L. monocytogenes* in previous studies in fish in Europe ranged from 0% in France, Portugal and Greece to 10% in Great Britain (Davies et al., 2001, Papadopoulos et al., 2010) and is in line with our results.

Y. enterocolitica was frequently isolated from water and terrestrial ecosystems and can be isolated also from fish (Velázquez et al., 1996). However in study of Davies et al., 2001 *Y. enterocolitica* was not found in fish samples from France, Great Britain and Portugal that is in agreement with our study.

In conclusion microbiological quality of bream and perch tested make these fishes unsuitable for consumption and therefore recreational fishing practice in Latvia and measures for prevention of cross-contamination at consumer level should be evaluated.

CONCLUSION

Microbiological quality of eels in the present study was the best comparing with bream and perch inhabiting the same lake and this finding may be attributed to different nature and biology of all fish species inhabiting Latvian freshwater sources.

L. monocytogenes is present in fish and therefore in aquatic environment in Latvia and this finding indicate that possible transmission of pathogen between environment and fish consumers exists.

Microbiological quality of perch and breams obtained from fishermen was not satisfactory and make fish unfit for human consumption therefore recreational fishing practice should be evaluated in order to prevent cross-contamination possibilities and to minimize the public health concerns.

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