EVALUATION OF MICROBIOLOGICAL QUALITY OF FRESHWATER FISH IN USMA LAKE

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Microorganisms may colonize the skin, gills and the gut of fish reflecting the bacterial load of aquatic environment. Altogether 22 samples of freshwater fish, including European perch (*Perca fluviatilis* n=7), silver bream (*Blicca bjoerkna* n=4) and European eel (*Anguilla anguilla* n=11) were collected during September 2014 from Usma lake in Latvia. Samples of gills, skin and gut of each fish were examined for the Total Bacterial Count (TBC), *Enterobacteriaceae*, fecal coliforms and psychrotrophic bacteria count. Pooled samples of skin, muscles, gut and internal organs from each fish were investigated for the presence of *Salmonella* spp., *Listeria* spp. and *Yersinia* spp. The highest count of TBC was found on skin of silver bream -7.96 $\log_{10}$ cfu cm^{-2}, while the lowest in gut of European eel - 2.16 $\log_{10}$ cfu g^{-1}. Also the highest count of *Enterobacteriaceae* was found on European perch skin (6.63 $\log_{10}$ cfu cm^{-2}). The lowest count of *Enterobactericeae* was found on skin of European eel (0.41 $\log_{10}$ cfu cm^{-2}). Number of fecal coliforms was higher on skin of silver bream - 5.43 $\log_{10}$ cfu cm^{-2}. The lowest number of fecal coliforms was found in gills of European perch (3.42 $\log_{10}$ cfu g^{-1}), while fecal coliforms were not isolated from European eel gut. Number of psychrotrophic bacteria was the highest in gills of European perch (6.23 $\log_{10}$ cfu g^{-1}), but the lowest psychrotrophic bacteria counts were observed in gills of European eel (2.24 $\log_{10}$ cfu g^{-1}). All samples were *Salmonella* spp., *Listeria* spp., *Yersinia* spp. negative. Contamination rates with TBC, *Enterobacteriaceae*, fecal coliforms and psychrotrophic microorganisms were significantly lower in eels ($p<0.05$) than in silver bream and European perch. Differences in microbiological contamination of tested samples could be linked to different ecology of fish.

Key words: freshwater fish, microbiological quality, *Salmonella*, *Listeria* spp., *Yersinia* spp.

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INTRODUCTION

Microbiological quality and safety of freshly caught fish depends on the hygienic quality of the surrounding aquatic environment. Fish are susceptible to contamination, especially those from freshwater environments characterized by slow water exchange and high anthropogenic contamination (Orban et al. 2007). In the aquatic environment microorganisms spread easily between habitats and hosts. Fish may obtain bacteria from water, sediment and food organisms, therefore the microbiological contamination of fish species may be affected by its feeding behavior, available food and environmental pollution (Olafsen 2001, Geldreich & Clarke 1966).

The European perch and silver bream are common fish species in inland waters in Latvia. The European perch, a predatory freshwater fish, has traditionally been the main target species for recreational fishermen. In Latvia the commercial value of European perch has increased in recent years. According to Birzaks (2007) the European perch caught in inland waters in Latvia comprised more than 80% among fish obtained by recreational fishermen. Despite silver bream has a relatively low commercial value, it is widely distributed in water environment and is popular fish among fishermen for angling and consumption. The European eel (*Anguilla anguilla*) is facultatively catadromous fish, living in fresh, brackish and coastal waters but migrating to pelagic marine waters to breed (Tsukamoto et al. 2002). The various life stages of European eel, ranging from glass eel to silver eel, are of great importance for human consumption and for stocking purposes (Crook & Nakamura 2013).

Total bacterial count (TBC), *Enterobacteriaceae* and fecal coliforms are frequently used as criteria to assess the quality and safety of foods. TBC is used to assess the general microbiological quality of fish and can be a useful indicator to predict the shelf life of raw fish (Huss et al. 1974). Psychrotrophic bacteria may contribute to fish spoilage during storage, while contamination with *Salmonella* spp., *Listeria monocytogenes* and *Yersinia* spp. are related to safety of fish (Huss 1995).

A number of studies have been carried out on microbial flora associated with marine (Alexopoulos et al. 2011, Gennaria et al. 1999) or aquacultured fish (Al-Harbi & Uddin 2005, Gonzalez et al. 1999, Austin 1983). However, information on microbiological contamination of freshwater fish from natural population is scarce. The aims of this study were to evaluate the microbiological quality of European perch, silver bream and European eel caught in Usma lake by analysis of the contamination of fish gills, skin and gut, and to determine the presence of *Salmonella* spp., *Listeria* spp. and *Yersinia* spp. as an indicator of fish safety.

MATERIAL AND METHODS

Collection of fish samples

Fish samples were collected during September 2014 from Usma lake in Latvia. Overall, 22 fish were caught: four silver bream (*Blicca bjoerkna*), seven European perch (*Perca fluviatilis*) and 11 European eel (*Anguilla anguilla*). Samples were placed in sterile plastic bags and transported to the laboratory in ice boxes. All samples were analyzed within 24 h after time of caught, keeping them under refrigerated conditions in the meantime.

Sample preparation

Fish skin, gill and gut samples of each fish were investigated separately for TBC, *Enterobacteriaceae*, fecal coliforms and psychrotrophic bacteria count. Surface samples of fish skin were collected with abrasive sponge moisturized with 0.1% peptone water by covering a 25 cm^2 area of fish skin. The gill and gut samples from each fish were removed aseptically with sterile forceps. An amount not less than 1 g of gill and gut from each fish was investigated. A pooled sample of 25 g of skin, musculature and intestinal tract of each fish was used for *Salmonella* spp., *Listeria* spp. and *Yersinia* spp. detection.

Bacteriological analysis

For bacteriological analysis 10-fold dilutions of initial gill, skin and gut samples were prepared. Total bacterial count and psychrotrophic bacteria were determined according to ISO 4332 (Anonymous 2004b) and ISO 17410 (Anonymous 2001), respectively. An amount of 1 ml of each dilution transferred into duplicate plates of Plate Count Agar (PCA, Biolife Italiana S.r.l, Milan, Italy). For TBC, the plates were placed in an incubator at 30 °C for 72 h. For psychrotrophic bacteria enumeration, the PCA plates were incubated at 21 °C for 3 to 5 days. The numbers of *Enterobacteriaceae* were determined by inoculating 1ml of each sample in Violet Red Bile Glucose Agar (VRBG, Biolife). The inoculated VRBG plates were incubated at 37 °C for 24 h according to the ISO 21528-2 (Anonymous 2004a). PCA and VRBG plates were examined to evaluate bacterial colonies morphology, followed by manual counting of colonies according to the ISO requirements.

Fecal coliform count was determined on Violet Red Bile Agar (VRBLA, Biolife) using a plate-pouring technique (ISO 4832, Anonymous 2006). The plates were incubated at 30 °C, and after 24 h typical purplish red colonies were enumerated. The detection of *Salmonella* spp. was done according to ISO 6579 (Anonymous 2002). Briefly, an amount of 25 g of fish was homogenized with 225 ml of buffered peptone water (Biolife) in stomacher for 60 seconds and incubated at 37 °C for 18 h. An amount of 0.1 ml of peptone buffered water was transferred into 9 ml of Rappaport Vassilliadis broth (RV, Biolife) and 9 ml of Muller-Kaufmann Tetrathionate Novobiocin broth (MKTTn, Biolife). The RV broth was incubated at 41.5 °C for 24 h, while MKTTn broth at 37 °C for 24 h. An amount of 0.1 ml of enriched suspension from each broth was plated onto two selective solid media: Xylose-Lysine-Desoxycholate agar (XLD, Biolife) and Brilliant Green agar (BGA, Biolife) and inoculated agars were incubated at 37 °C for 24 h and examined for the presence of presumptive colonies.

For *L. monocytogenes* detection (ISO 11290-1, Anonymous 1996) an amount of 25 g of sample was added to 225 ml Half-Fraser broth (HF, Biolife) followed by homogenization for 60 seconds in a stomacher and incubation for 24 h at 30 °C. Thereafter 0.1 ml aliquots of the HF broth were transferred to 9 ml of Fraser broth (Biolife) and incubated at 37 °C for 48 h. An amount of 0.1 ml of both, Half-Fraser and Fraser broths after incubation were streaked onto Agar *Listeria* according to Ottaviani and Agosti (ALOA, Biolife) and Oxford agar (Biolife). After an incubation period of 24-48 h at 37 °C, the selective agar plates were examined for the presence of the colonies resembling *L. monocytogenes* morphology. Suspicious colonies on ALOA agar were Gram stained, tested for hemolysis, motility and catalase activity followed by biochemical identification with the API *Listeria* system (BioMérieux, Mancy l'Etoile, France).

For detection of *Yersinia* spp., an amount of 25 g 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. 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 analysis

Statistical analyses were performed on log-10 transformed data. The probability level at which statistical analyses were accepted as significant was < 0.05. Data were analyzed (means, standard deviations, Student's *t* test) using the software Microsoft Office Excel 2010.

Table 1. Total bacterial count (TBC) in European perch (*Perca fluviatilis*), silver bream (*Blicca bjoerkna*) and European eel (*Anguilla anguilla*) from Usma lake in Latvia

Fish	No. of samples	Gills		Skin		Gut	
		mean ±SD[c]	range	mean ±SD	range	mean ±SD	range
European perch (*Perca fluviatilis*)	7	6.69 ±1.45	3.78-8.00	7.56 ±0.40[b]	7.04-8.18	6.27 ±1.02	4.62-7.36
Silver bream (*Blicca bjoerkna*)	4	6.40 ±1.21	5.41-8.08	7.96 ±0.76	6.89-8.61	7.02 ±0.36	6.58-7.36
European eel (*Anguilla anguilla*)	11	2.40 ±0.64[a]	1.26-3.76	2.51 ±0.81	1.04-3.93	2.16 ±0.42	1.45-2.78

[a] TBC in European eel samples was significantly less than in silver bream and European perch samples ($p<0.05$)
[b] TBC on European perch skin was significantly higher ($p<0.05$) than in gut
[c] The unit of number is $\log_{10}$ cfu g^{-1} or $\log_{10}$ cfu cm^{-2}

RESULTS AND DISCUSSION

In fish samples analyzed, TBC in gills ranged from 1.26 to 8.08 $\log_{10}$ cfu g^{-1} in European eel and silver bream, respectively (Table 1). TBC on skin of fish was from 1.04 to 8.61 $\log_{10}$ cfu cm^{-2} and the highest count was recorded on skin of silver bream. Also in gut, TBC was from 1.45 to 7.36 $\log_{10}$ cfu g^{-1} in European eel and both, European perch and silver bream, respectively.

In general, TBC in gills, skin and gut samples of European eel was significantly lower than in silver bream and European perch ($P<0.05$). The mean value of TBC was significantly ($p<0.05$) higher on European perch skin (7.56 $\log_{10}$ cfu cm^{-2}) than in gut (6.27 $\log_{10}$ cfu g^{-1}). 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 European eel microflora (Terentjeva et al. 2015). In our study TBC in gills of European perch and silver bream were higher in comparison with Mudarris and Austin (1988) study, who demonstrated that contamination of turbot gills did not exceed 5.84 $\log_{10}$ cfu g^{-1}. However, Al-Harbi and Uddin (2005) reported that TBC in fresh tilapia (*Oreochromis niloticus*) from brackish pond ranged from 7.44 to 8.00 $\log_{10}$ cfu g^{-1} in gut and from 5.93 to 6.32 $\log_{10}$ cfu g^{-1} in gills and these results are comparable with our findings regarding European perch and silver bream.

Enterobacteriaceae count for the fish samples examined ranged from 0.00 to 7.67 $\log_{10}$ cfu g^{-1} in European eel and silver bream, respectively. The lowest *Enterobacteriaceae* count was on European eel skin – from 0.0 $\log_{10}$ cfu cm^{-2}, while European perch skin samples contained the highest number of *Enterobacteriaceae* count. *Enterobacteriacae* count in gut was from 0.00 $\log_{10}$ cfu g^{-1} in European eel to 6.88 $\log_{10}$ cfu g^{-1} in European perch (Table 2).

Enterobacteriaceae count in European eel samples was significantly lower ($p<0.05$) than in European perch and silver bream. There were significant ($p<0.05$) differences in *Enterobacteriaceae* count revealed between skin and gut of European perch. High contamination rates of fish skin may be attributed to skin contamination from surrounding environment. *Enterobacteriaceae* counts in gut were higher than in study presented by Manzano et al. (2013) who found *Enterobacteriaceae* of 3.7 $\log_{10}$ cfu g^{-1} in the gut of brown trout fed by routine feed. Exceed contamination of gut

Table 2. *Enterobacteriaceae* count in European perch (*Perca fluviatilis*), silver bream (*Blicca bjoerkna*) and European eel (*Anguilla anguilla*) from Usma lake in Latvia

Fish	No. of samples	Gills		Skin		Gut	
		mean ±SD[c]	range	mean ±SD	range	mean ±SD	range
European perch (*Perca fluviatilis*)	7	5.28 ±1.58	2.32 -7.36	6.63 ±0.52[a]	5.64 -7.00	5.31 ±1.37[a]	3.25 -6.88
Silver bream (*Blicca bjoerkna*)	4	4.34 ±2.35	2.46- 7.67	5.67 ±0.46	5.15 -6.17	5.81 ±0.99	4.74 -6.86
European eel (*Anguilla anguilla*)	11	1.32 ±0.50[b]	0.48 -2.23	0.41 ±0.38	0.00 -1.08	1.21 ±0.76	0.00 -2.49

[a] *Enterobacteriaceae* count on European perch skin was significantly higher ($p<0.05$) than in gut
[b] Number of *Enterobacteriaceae* in European eel samples was significantly lower than in silver bream and European perch samples ($p<0.05$)
[c] The unit of number is $\log_{10}$ cfu g^{-1} or $\log_{10}$ cfu cm^{-2}

in European perch and silver bream could be linked to consumption of microflora with feed from surrounding environment that reflected in increased *Enterobacteriaceae* contamination rate. Number of fecal coliform varied from 0.00 to 6.04 $\log_{10}$ cfu g^{-1} in different sampling sites in European eel and silver bream, respectively. Fecal coliform count in gills was from 0.00 to 4.23 $\log_{10}$ cfu g^{-1}, and the highest value was identified in European perch, while the lowest in European eel. Fecal coliform count ranged from 0.00 to 1.74 $\log_{10}$ cfu cm^{-2} on skin of European eel. Skin of European perch and silver bream was more contaminated than gills, where the count of fecal coliform was from 4.00 to 6.04 $\log_{10}$ cfu cm^{-2}. Fecal coliform count in gut was from 0.00 to 5.38 $\log_{10}$ cfu g^{-1}, and the lowest count was identified in European eel, while the highest in European perch (Table 3).

The fecal coliform count in European eel samples was lower ($p<0.05$) in comparison with European eel and silver bream. Also contamination of skin with fecal coliform was significantly higher in silver bream than in European perch ($p<0.05$). In silver bream significant ($p<0.05$) differences between gills and skin were detected.

High counts of fecal coliforms in fish could be linked to contamination from surrounding water environment. Similar to our study, gills, skin and gut of pond reared tilapia (*Oreochromis niloticus*) were contaminated with fecal coliforms in previous studies (Geldreich & Clark 1966, El-Shafai et al. 2004). In El-Shafai et al. study tilapia from treated sewage-duckweed-fed pond and settled sewage-fed pond contained higher number of fecal coliforms in gills and gut compared to skin, which could be attributed to the gills high specific surface area for bacterial attachment and, and to the high water flow passing through them. Study of Geldreich and Clark (1966) reveals that the fecal coliform densities in gut varies from 1.37 $\log_{10}$ cfu g^{-1} in bluegills (*Lepomis macrochirus macrochirus*) to 6.04 $\log_{10}$ cfu g^{-1} in channel catfish (*Ictalurus lacustris punctatus*) caught in moderately polluted sections of the Little Miami river in Ohio, USA, at the water temperature between 13 to 18 °C. Authors concluded that rate of contamination of fish with fecal coliform depends on the intake and degree of contamination of food ingested by fish.

Psychrotrophic bacteria count varied from 1.51 to 7.64 $\log_{10}$ cfu g^{-1}, and the lowest contamination rate was observed in gills of European eel, but

Table 3. Fecal coliform in European perch (*Perca fluviatilis*), silver bream (*Blicca bjoerkna*) and European eel (*Anguilla anguilla*) from Usma lake in Latvia

Fish	No. of samples	Gills		Skin		Gut	
		mean ±SD[d]	range	mean ±SD	range	mean ±SD	range
European perch (*Perca fluviatilis*)	7	3.42 ±1.10	1.48 -4.23	4.20 ±0.52[a]	4.00 -5.38	4.34 ±0.88	3.18-5.38
Silver bream (*Blicca bjoerkna*)	4	3.38 ±0.76[b]	2.43 -4.20	5.43 ±0.49[a,b]	4.94 -6.04	4.20 ±0.87	3.11 -5.20
European eel (*Anguilla anguilla*)	11	0.54 ±0.68[c]	0.00-1.92	0.34 ±0.57	0.00 -1.74	0.00	0.00

[a] Fecal coliform count on silver bream skin was significantly higher ($p<0.05$) than on European perch skin

[b] Fecal coliform count on silver bream skin was significantly higher ($p<0.05$) than in gills

[c] Number of fecal coliforms in European eel samples was significantly lower than in silver bream and European perch samples ($p<0.05$)

[d] The unit of number is $\log_{10}$ cfu g^{-1} or $\log_{10}$ cfu cm^{-2}

the highest in gills of European perch. The skin of silver bream and European eel was more contaminated with psychrotrophic bacteria (6.91 $\log_{10}$ cfu g^{-1}), than skin of European perch (6.81 $\log_{10}$ cfu g^{-1}). In gut the contamination was from 1.73 $\log_{10}$ cfu g^{-1} in European eel to 7.43 $\log_{10}$ cfu g^{-1} in European perch.

Regarding the number of psychrotrophic bacteria count in gills and gut samples of European eel, it was significantly ($p<0.05$) lower than in European perch and silver bream samples. With respect to psychrotrophic bacteria, the differences between counts of psychrotrophic bacteria in gills, skin, gut of European perch and silver bream were not significant ($p>0.05$).

Counts of psychrotrophic bacteria on fish in the present study were high and this could be explained with relatively low temperature of ambient environment, which favors growth of psychrotrophic bacteria (Dalgaard 2003). Scherer et al. (2006) showed that grass carp (*Ctenopharyngodon idella*) raised in an earth pond, in the temperature range between 15-29 °C, had a slightly lower psychrotrophic bacterial count (3.0 $\log_{10}$ cfu g^{-1}) in their skin with muscles.

None of the European perch and silver bream samples examined were found positive for *Salmonella* spp., *Listeria monocytogenes* or *Yersinia* spp.

Negative results on the prevalence of zoonotic pathogenic bacteria in the present study indicate that the microbiological quality of freshly caught fish in Latvia was good. However, contrary results were reported in study of Gaertner et al., 2008 in the USA, who found the prevalence of 33% of *Salmonella* spp. in the guts of different fish (largemouth bass *Micropterus salmoides*, channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, and suckermouth catfish *Hypostomus plecostomus*) caught at the downstream site of San Marcos river in Texas (Gaertner et al. 2008). Also Miettinen and Wirtanen (2005) studied 510 rainbow trout from fish farms in lakes and sea areas around Finland and in their study the prevalence of *Listeria* spp. in fish gill, viscera and skin varied greatly between fish farms from 0 to 75%. In the investigation by Davies et al. (2001), 23 % of fresh salmon (*Salmo salar*) and trout (*Oncorhyncus mykiss*) from Great Britain contained *Y. enterocolitica*. Conversely, absence

Table 4. Psychrotrophic bacteria in European perch (*Perca fluviatilis*), silver bream (*Blicca bjoerkna*) and European eel (*Anguilla anguilla*) from Usma lake in Latvia

Fish	No. of samples	Gills		Skin		Gut	
		mean ±SD[b]	range	mean ±SD	range	mean ±SD	range
European perch (*Perca fluviatilis*)	7	6.23 ±1.01	4.38 -7.64	6.15 ±0.41	5.50 -6.81	5.60 ±1.24	4.50 -7.43
Silver bream (*Blicca bjoerkna*)	4	5.49 ±1.01	4.58 -6.79	6.34 ±0.44	5.87 -6.91	6.13 ±0.47	5.56 -6.68
European eel (*Anguilla anguilla*)	11	2.24 ±0.64[a]	1.51 -3.82	6.22 ±0.39	5.51 -6.91	2.76 ±0.89[a]	1.73 -5.08

[a] Number of psychrotrophic bacteria in European eel gills and gut was significantly lower than in Silver bream and European perch samples ($P<0.05$)
[b]The unit of number is $\log_{10}$ cfu g^{-1} or $\log_{10}$ cfu cm^{-2}

of *Salmonella* spp., *L. monocytogenes* and *Y. enterocolitica* in aquacultured rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis* spp.), hybrid striped bass (*Morone saxatilis* × *M. chrysops*), and pacu (*Piaractus mesopotamicus*) have also been reported in survey by Pullela et al. (1998), that is in agreement with our results.

CONCLUSIONS

The results of our study showed that *Enterobacteriaceae,* TBC, fecal coliforms and psychrotrophic bacteria could contaminate the gills, skin and gut of freshly caught European perch and silver bream in high numbers in comparison with European eel. Significant differences in the counts of TBC, *Enterobacteriaceae* and fecal coliforms show that different parts of fish are subjected to the contamination from ambient environment, but contamination of skin with TBC, *Enterobacteriaceae* and fecal coliform indicates to contamination of fish from outside environment. All fish samples tested were negative for *Salmonella* spp., *Listeria* spp. and *Yersinia* spp, indicating that pathogens of zoonotic significance had been not isolated from the tested samples.

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