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IDENTIFICATION AND ANTIBIOTIC SUSCEPTIBILITY OF BACTERIAL MICROBIOTA OF FRESHWATER FISH

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ABSTRACT

The fish meat is an essential part of human diet. However, fish may be contaminated with different microorganisms, including pathogens. Antimicrobial resistance of fish microbiota may facilitate the spread of resistant microorganisms causing serious consequences for human health. The aim of the present study was to detect bacterial contamination in fish gill, gut and skin and to determine antimicrobial susceptibility of the bacterial isolates. Rainbow trout (*Oncorhynchus mykiss*) and bream (*Abramis bram*) were obtained from the market in Jelgava city. Chub (*Leuciscus cephalus*), crucian carp (*Carassius carassius*) and tench (*Tinca tinca*) were collected from fishermen. Fish samples were examined for the total bacterial count (TBC), coliforms, *Enterobacteriaceae*, *Pseudomonas* spp. and *Aeromonas* spp. Testing was done in accordance with International Organization for Standardization (ISO) standards. Identification of all bacteria was accomplished with the Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) method. The disc diffusion method was used for the detection of antibiotic susceptibility of isolated bacterial species. TBC ranged from 2.70 to 7.00 log CFU.g^{-1}, coliforms from 0 to 2.67 log CFU.g^{-1}, *Enterobacteriaceae* from 0 to 2.85 log CFU.g^{-1}. The highest contamination with *Pseudomonas* spp. and *Aeromonas* spp. was observed in chub gut samples with 1.60 log CFU.g^{-1} and 2.23 log CFU.g^{-1}, respectively. Altogether, 16 microbial genera and 31 bacterial species were identified. The dominant bacterial species belonged to *Pseudomonas* spp. (54%) and *Enterobacteriaceae*. *Pseudomonas* spp. were resistant to ticarcillin, susceptibility to ciprofloxacin showed 88% of isolates. All *Enterobacteriaceae* isolates were susceptible to imipenem. The microbial quality of the fish was acceptable, but the presence of antibiotic resistant bacteria may further cause a negative impact on public health.

Keywords: bacteria; freshwater fish; MALDI-TOF MS; antibiotic

INTRODUCTION

The bacterial contamination of freshly caught fish depends to a great extent on the quality of surrounding water. The microbiota of fish is very diverse, its composition and amount can be influenced by many different factors, such as microbial population of water and bottom mud, water source type, fish species and the conditions of their habitat. The majority of microorganisms in fish were located in gills, gut and in mucus on the fish skin, while the internal organs and muscle tissue were relatively sterile **(Austin, 2006; Cwiková, 2016)**.

Enterobacteriaceae and coliforms are indicator microorganisms that are permanently present in the intestine of humans and animals. They are not harmful to the host's organism, but under the appropriate conditions can cause a disease. The presence of indicator microorganisms in external environment indicates the contamination with human or animal faeces and could be detected with quantitative isolation of indicator microorganisms. The contamination rates are important for microbiological safety because of ability to indicate the presence of human and animals' pathogens of intestinal origin. *Enterobacteriaceae* are widespread in the nature and can be found in soil, water, fruits, vegetables, grains, flowering plants and trees **(Tosun, Üçok Alakavuk and Mol Tokay, 2016)**. *Salmonella*, *Shigella*, *Yersinia*, *Escherichia* of *Enterobactericeae* can cause intestinal foodborne infections and are transmitted by the fecal-oral or oral route with contaminated water, food or due to direct contact. Other *Enterobactriaceae* may serve as an opportunistic pathogens and *Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Proteus*, *Providencia*, *Serratia* were reported to cause infections with non-intestinal clinical signs – bacteraemia, meningitis, wound infections, genitourinary and respiratory tract injuries **(Flores-Tena et al., 2007; Dekker and Frank, 2015)**. The studies on composition of fish microbiota has been conducted in different countries but mostly the sea fish were studied **(Yagoub, 2009; Oliveira, Oliveira and Pelli, 2017)**.

Antimicrobial resistance is one of the most serious threats to public health, environmental health and food safety. Antimicrobial resistance is increasing to threateningly high levels throughout the world.

Antimicrobial resistance reduces the effectiveness of treatment of infections in humans and animals, contributes to the increase in morbidity, mortality and leads to significant economic losses **(Petersen et al., 2002; Wamala et al., 2018)**. The use of antibiotics in aquaculture for therapeutic and preventive purposes is a growing problem for world livestock and environmental health. From aquaculture, the antimicrobials may be spread in the environment in the concentration sufficient to cause microbial imbalance in animals and human with the emergence of antimicrobial resistance in the society. It has been proved that the antibiotic-resistant bacteria may be present in fish with their subsequent transfer to humans via the consumption of fish or contaminated environment **(Heuer et al., 2009)**.

The consumption of the fish in the EU is continuously increasing, however, the fish products were frequently associated with gastrointestinal infections in humans **(EFSA and ECDC, 2017)**. Nowadays, people tend to follow a healthy lifestyle by choosing fresh foods or food with optimal nutritional value like fish and fish products, which are known as the source of protein, contain omega-3 amino acids, vitamins and minerals such as phosphorus. However, the risk for consumers related to the eating of fish contaminated with non-pathogenic and pathogenic microflora with variable antimicrobial resistance rates is concerning **(Da Silva et al., 2010; Cwiková, 2016)**. Therefore, the aim of the present study was to detect bacterial contamination in fish gill, gut and skin and to determine antimicrobial susceptibility of the bacterial isolates.

Scientific hypothesis

The scientific hypothesis of this study was that the freshwater fish were contaminated with bacteria, and the bacterial isolates are resistant to antibiotics.

MATERIAL AND METHODOLOGY

Selection of samples

Altogether, 15 fish samples were collected, including 2 rainbow trout (*Oncorhynchus mykiss*) and 3 bream (*Abramis brama*) samples were obtained from the market and 3 chub (*Leuciscus cephalus*), 4 crucian carp (*Carassius carassius*) and 3 tench (*Tinca tinca*) were collected from the fishermen in June 2017 in Jelgava. Fish were caught in the river Lielupe. All fish were placed in the sterile polyethylene sampling bags, and transported n ice to the laboratory. The investigations were initiated within 2 h after deliver to the laboratory.

Sampling

Aseptically, 1 g of sample was taken from each fish skin, gills and gut. Skin samples were taken from the lateral line of the fish. Operculum was opened, and gills were dissected for preparation of gill samples. Gut samples were taken by cutting of the abdomen till the anal fin and after opening the body cavity.

Bacteriological analyses

The sample suspension of 1:10 with 0.1% peptone water (OXOID, UK) was used for bacteriological testing. An amount of 1 mL was plated onto Tryptone Soy Agar (TSA, OXOID, UK) for detection of total bacterial counts. After incubation at 30 °C for 72 h, all colonies were counted. For the detection and enumeration of coliforms, the sample suspension was plated onto Violet Red Bile Lactose Agar (VRBL, OXOID, UK). Inoculated agars were incubated at 37 °C for 24 h and all typical colonies from dark red to deep purple coloured colonies were counted **(ISO 4832:2006)**. MacConkey agar (MAC, OXOID, UK) were used for detection of *Enterobacteriaceae* and inoculated agars were incubated at 37 ºC for 24 h, and after that the typical colonies were enumerated - lactose fermenting bacteria produced red to pink and non-lactose fermenting bacteria have colourless and transparent colonies **(ISO 21528:2017)**. Pseudomonas CFC Selective Agar (OXOID, UK) were used for detection and enumeration of *Pseudomonas* spp. **(ISO 13720: 2010)**. Agar plates were incubated at 30 °C to 48 h and examined for the presence of colonies. All grown colonies were counted. Aeromonas Agar (Ryan, OXOID, UK) was used for detection of *Aeromonas* spp. The plates were incubated at 37 °C for 24 h, after that were examined for the presence of dark green, opaque colonies with dark centres.

Identification of bacterial species with MALDI-TOF Biotyper MS

MALDI-TOF Mass Spectrometry model Microflex LT/SH biotyper (Bruker Daltonics, Germany, Bremen) was used for identification of bacteria species isolated from fish. Typical bacterial colonies were selected from all agars, picked up and suspended in 300 µL of sterile distilled water and mixed. Then, a 900 µL of absolute ethanol (99%, Sigma-Aldrich, USA) was added. The solution was centrifuged at 13 000 × g for 2 min. Supernatant was removed, ethanol pipetted, and the pellet was allowed to dry at a room temperature. At first, the pellet was added and mixed with formic acid (10 µL, 70%) and then with acetonitrile (10 µL, 100%). The solution was centrifuged at maximum speed for 2 min. The supernatant was placed on a polished MALDI plate (Bruker Daltonics, Germany) and after drying a 1 µL of the matrix solution (HCCA: α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany), 50% acetonitrile with 0.025% trifluoroacetic acid (TFA) (100%, Sigma-Aldrich, USA)) was added in the spots. Sample processing was performed with MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics) using the MALDI Biotyper software package (version 3.0) and data were obtained with Realtime Classification software (RTC) (Bruker Daltonics). Data processing was carried out by Biotyper software, where sample mass spectrum was compared with the reference mass spectrum and the score values were calculated.

Antibiotic susceptibility testing of bacterial isolates

Antimicrobial resistance of *Pseudomonas* and *Enterobacteriaceae* bacteria confirmed with the MALDI TOF MS Biotyper (Brucker Daltonics) was tested with the disc diffusion method. Suspension of bacterial isolates in Mueller Hinton broth was placed onto Mueller Hinton agar (MHA, OXOID, UK). The antimicrobial discs were placed on the MHA (OXOID, UK) surface after the inoculation, agars were allowed to dry out at room temperature and

were incubated at 37 °C for 24 h. *Pseudomonas* spp. isolates were tested against ticarcillin (75 μg), cefotaxime (30 μg), ciprofloxacin (10 μg), imipenem (10 μg) and doripenem
(10 μg) (Oxoid, UK). *Enterobacteriaceae* were tested against ticarcillin (75 μg), cefepime (30 μg), ciprofloxacin (10 μg), imipenem (10 μg) and tobramycin (10 μg) (Oxoid, UK). Antimicrobial susceptibility testing was performed according to the CLSI guidelines and the results were interpreted in accordance with EUCAST breakpoint tables **(EUCAST, 2018)**.

Statistical analyses

Statistical analyses were performed by R software, version 3.4.3, for data management RStudio was used. Bacterial counts were expressed in decimal logarithms. T-test was used for calculating differences among the total bacterial count (TBC), coliforms and *Enterobacteriaceae* in fish gills, gut and skin samples. The one-way analysis of variance (ANOVA) was used to detect significant differences between the bacterial contamination rates of fish gills, gut and skin samples (p <0.05).

RESULTS AND DISCUSSION

TBC ranged from 2.70 to 7.00 log CFU.g^{-1} in all fish samples. The highest TBC was detected in gut of bream and tench, while the lowest in rainbow trout skin (Table 1). Coliforms were not detected in crucian carp gill and tench skin samples, while the highest numbers of coliforms were found in crucian carp and tench gut with 2.67 and 2.51 log CFU.g^{-1}. The highest *Enterobacteriaceae* counts of 2.85 and 2.75 log CFU.g^{-1} were identified in bream gill and crucian carp gut, while *Enterobacteriaceae* were not isolated from the chub gill, bream skin and tench gill and skin samples. *Pseudomonas* spp. and *Aeromonas* spp. were not found in rainbow trout, crucian carp and tench samples. The highest contamination with *Pseudomonas* spp. was observed in chub gill and gut samples with 1.46 and 1.60 log CFU.g^{-1}. The most contaminated fish species with *Aeromonas* spp. was the chub – 1.93 log CFU.g^{-1} in gill, 2.23 log CFU.g^{-1} in gut and 0.60 log CFU.g^{-1} in skin.

The microbiological criteria as total bacterial count (TBC) and *Enterobacteriaceae* are applied widely to ensure the microbiological quality and safety of foods. The TBC shows the general level of contamination and the shelf-life stability while the coliforms and *Enterobacteriaceae* indicate the presence of faecal contamination and possible pathogens in foods **(Tortorello, 2003)**. In the present study, the TBC were in line with **Eizenberga et al. (2015)** reported for freshwater fish from Usmas lake in Latvia with gill, skin and gut contamination rates from 1.26 to 8.08 $\log_{10}$ CFU.g^{-1}, from 1.04 to 8.61 $\log_{10}$ CFU.g^{-1} and from 1.45 to 7.36 $\log_{10}$ CFU.g^{-1}, respectively. However, our results were lower than **Terentjeva et al. (2015)** stated for bream obtained from Usmas lake in Latvia with 5.48 log CFU.g^{-1} for skin contamination with TBC and 7.24 log CFU.g^{-1} with *Enterobacteriaceae*.

Aeromonas spp. are distributed in freshwater habitats worldwide. **Stratev, Vashin and Daskalov (2015)** found the contamination with *Aeromonas* spp. in cooled rainbow trout and trout fillets at retail markets. **Abd-El-Malek (2017)** reported that the raw fish in Egypt were contaminated with *Aeromonas* spp. and majority of the isolates were identified as *A. hydrophila*. This bacterium is pathogenic not only for humans but also for fish and is commonly found in water. Inhabited in an aquatic environment, *A. hydrophila* is reported to be the cause of secondary infection of wounds, sepsis, cellulitis, pneumonitis, gastroenteritis and necrotizing fasciitis. **(Hassan and Farag, 2006)**. High prevalence psychrophilic bacteria, especially proteolytically active microorganisms

Table 1 Bacterial contamination of freshwater fish.

Fish species	Sampling site	TBC log CFU.g^{-1}	Coliforms log CFU.g^{-1}	*Enterobacteriaceae* log CFU.g^{-1}	*Pseudomonas* spp. log CFU.g^{-1}	*Aeromonas* spp. log CFU.g^{-1}
Rainbow trout (*Oncorhynchus mykiss*)	Gills	3.16[a]	1.52	2.03	0	0
	Gut	3.37[b]	1.04	1.68	0	0
	Skin	2.70[a]	1.43	2.25	0	0
Chub (*Leuciscus cephalus*)	Gills	3.58[a]	2.11	0	1.46[e]	1.93[e]
	Gut	2.97[b]	1.77	2.35[d]	1.60	2.23
	Skin	3.18[a]	0.30	0.78	0	0.60
Bream (*Abramis bram*)	Gills	3.15[a]	0.60	2.85	0.3	0
	Gut	7.00[b]	1.78[c]	2.36[d]	0	0
	Skin	3.23[a]	0.30	0	0	1.38
Crucian carp (*Carassius carassius*)	Gills	3.08[a]	0	1.18	0	0
	Gut	3.18[b]	2.67[c]	2.75[d]	0	0
	Skin	2.74[a]	0.30	1.30	0	0
Tench (*Tinca tinca*)	Gills	3.39[a]	1.04	0	0	0
	Gut	7.00[b]	2.51[c]	2.60[d]	0	0
	Skin	2.79[a]	0	0	0	0

Note: [a] no significant differences between TBC in tested fish gill and skin samples were observed (p >0.05);
[b] TBC in bream and tench gut was significantly higher than in gut of rainbow trout, chub and crucian carp (p <0.05);
[c] coliform counts in bream, crucian carp and tench gut were significantly higher than in gill and skin samples (p <0.05);
[d] *Enterobacteriaceae* counts in chub, bream, crucian carp and tench gut samples were significantly higher than in gill and skin samples (p <0.05);

– *Pseudomonas* spp. and *Aeromonas* spp. can predispose fish to a more rapid process of microbiological deterioration of fish meat quality **(Cipriano and Dove, 2011; Larsen, 2014)**.

Tested fish bacterial microbiota contained 16 microbial genera, from which the most abundant were *Pseudomonas* spp. (54%) (Figure 1). *Shewanella* spp. (5%) and *Serratia* spp. (5%) were also among the most frequently isolated. Other genera isolated were *Escherichia* spp., *Lelliottia* spp., *Leclercia* spp., *Bacillus* spp., *Citrobacter* spp. and *Rahnella* spp., *Morganella* spp., *Acinetobacter* spp., *Achromobacter* spp., *Aromatoleum* spp., *Burkholderia* spp., *Arthrobacter* spp. and *Staphylococcus* spp.

Our results agreed with **Kim, Brunt and Austin (2007)**, who found *Aeromonadaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* to be the most abundant microflora in rainbow trout intestine samples. Our study confirms the findings of **Yagoub (2009)** who isolated similar bacteria genera from fish samples.

Bacterial species isolated from freshwater fish gill, gut and skin samples are shown in Table 2. Overall, 31 bacterial species were identified with MALDI-TOF mass spectrometry. The gill microbiota was more diverse than the microbiota of gut and skin with only some *Pseudomonas* spp. were present in fish skin. *Pseudomonas proteolytica*, *P. brenneri*, *P. cedrina*, *P. veronii* and *Lelliottia amnigena* were dominant bacteria species isolated from gills and skin. Intestinal bacterial microbiota consisted of species, which were specifically found in gut – *Arthrobacter monumenti*, *P. grimondii*, *P. gessardii*, *P. synxantha*, *P. libanensis*, *P. tolaasii*, *Achromobacter xylosoxidans*, *Aromatoleum alkani*, *Burkholderia thailandensis*.

In previous studies, the skin and gill microbiota were evaluated together due to influence of water pollution on the fish microflora **(Larsen, 2014)**. **Fernandes (2009)** found that the Gram-negative bacteria were predominant. Several genera as *Bacillus* spp., *Pseudomonas* spp., *Leclercia* spp., *Acinetobacter* spp., *Citrobacter* spp., *Achromobacter* spp., *Escherichia* spp., *Serratia* spp., *Rahnella* spp. and *Staphylococcus* spp. found in our study, have been previously associated with microbiota of gill, gut and skin **(Austin, 2006)**.

Pseudomonas spp. are widespread in nature and have been isolated from aquatic environment and fish. Some species can cause a negative effect on animal and human health, eg. *P. fluorescens* is a potential pathogen that can influence the physiological processes of neurons **(Picot et al., 2001)**. Other pseudomonas can cause diseases in fish and contribute the spoilage processes of fish meat. *Pseudomonas* spp. were considered to be relatively resistant to antibiotics **(Kačániová et al., 2017)**. The members of the *Shewanella* spp., specially *S. baltica* were described in spoilage of chilled marine products **(Ge et al., 2016)**. In agreement with **Starliper (2001)** and **Aydin, Erman and Bilgin (2011)**, *Serratia liquefaciens* is a potential fish pathogen, which can lead to high mortality, causing economic losses. **Altun et al. (2013)** refers about pathogenicity of *Citrobacter braakii* isolated from rainbow trout in Bursa. *Leclercia adecarboxylata* infection was rarely reported in humans and the infection were described in patients with impaired immunity. *L. adecarboxylata* is expected to be the pathogen related to the aquatic environment **(Keren et al., 2014)**. *R. aquatilis* is prevalent in the environment and has previously been isolated from various water reservoirs, soil, clinical specimens and foodstuffs. *R. aquatilis* is an opportunistic pathogen that can cause a variety of serious gastrointestinal, urinary, respiratory and cardiovascular diseases. The consequences can be even dangerous for human life **(Alikunhi et al., 2017)**. *Bacillus cereus* is widespread in nature, it causes two types of food-borne diseases: toxicosis caused by previously produced toxin and toxicity caused by bacterial cells that produce enterotoxins in the small intestine

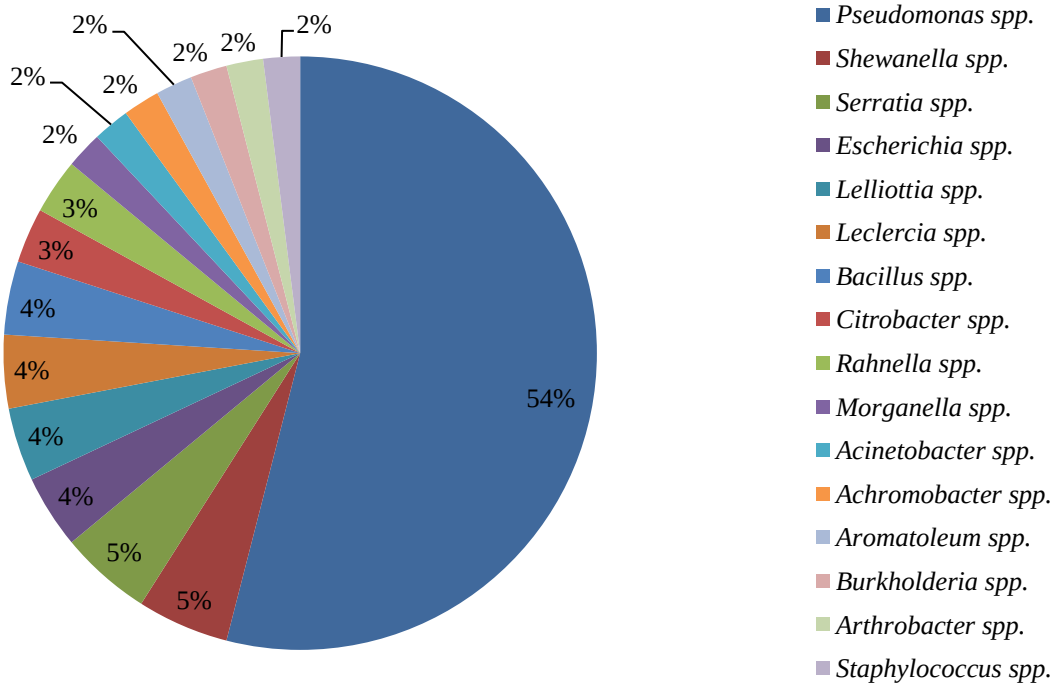

Figure 1 Microorganisms isolated from freshwater fish.

Table 2 Microorganisms wre isolated from gills, gut and skin of freshwater fish.

Bacterial species		
Gills	Gut	Skin
Lelliottia amnigena *Escherichia vulneris* *Pseudomonas proteolytica* *Pseudomonas rhodesiae* *Pseudomonas fluorescens* *Pseudomonas gessardii* *Bacillus cereus* *Pseudomonas putida Pseudomonas brenneri Pseudomonas cedrina* *Pseudomonas koreensis* *Pseudomonas veronii* *Shewanella profunda* *Shewanella baltica* *Rahnella aquatilis* *Leclercia adecarboxylata* *Citrobacter braakii*	*Arthrobacter monumenti* *Pseudomonas grimondii* *Pseudomonas fluorescens* *Pseudomonas gessardii* *Pseudomonas synxantha* *Pseudomonas libanensis* *Pseudomonas tolaasii* *Achromobacter xylosoxidans* *Aromatoleum alkani* *Burkholderia thailandensis*	*Pseudomonas brenneri* *Pseudomonas proteolytica* *Pseudomonas cedrina* *Pseudomonas orientalis* *Acinetobacter tjembergiae* *Morganella morgani* *Serratia rubidaea* *Staphylococcus equorum* *Lelliottia amnigena* *Serratia liquefaciens* *Pseudomonas veronii*

Table 3 Antibiotic susceptibility of *Pseudomonas* spp. isolates from fish samples.

Bacterial isolates	No. of isolates	TIC	FEP	CIP	IMP	DOR
		No. of resistant isolates (%)				
Pseudomonas grimondii	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Pseudomonas putida	1	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)
Pseudomonas fluorescens	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
Pseudomonas synxantha	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Pseudomonas libanensis	2	2 (100)	2 (100)	0 (0)	2 (100)	2 (100)
Pseudomonas tolaasii	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Pseudomonas taetrolens	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)

Note: TIC: ticarcillin; FEP: cefepime; CIP: ciprofloxacin; IMP: imipenem; DOR: doripenem.

Table 4 Antibiotic susceptibility of *Enterobacteriaceae* isolates from fish samples.

Bacterial isolates	No. of isolates	TIC	CTX	CIP	IMP	TOB
		No. of resistant isolates (%)				
Lelliotia amnigena	2	2 (100)	0 (0)	1 (50)	0 (0)	1 (50)
Escherichia vulneris	2	2 (100)	1 (50)	0 (0)	0 (0)	1 (50)
Staphylococcus equorum	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Rahnella aquatillis	1	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)

Note: TIC: ticarcillin; CTX: cefotaxime; CIP: ciprofloxacin; IMP: imipenem; TOB: tobramycin.

(Rasool et al., 2017). *B. cereus* was isolated from fish in India and tropical fish from Shivajinagar area of Bangalore, India **(Rasool et al., 2017; Prasad, 2014)**.

Emetic disease caused by *B. cereus* associated with tuna fish consumption was reported **(Doménech-Sánchez et al., 2011)**. *Escherichia vulneris* was originally isolated from human skin lesions, but recently the microorganism was isolated from water and fish. *E. vulneris* is present in the intestinal tract of animals, so the bacterium can cause environmental pollution **(Aydin, Celebi and Akyurt, 1997)**. Proportion of the microorganisms isolated in this study can contribute to the spoilage processes of fish and also act as infectious agents for fish and consumers.

All *Pseudomonas* species isolated from freshwater fish were resistant to ticarcillin (Table 3). *P. grimondii*, *P. fluorescens*, *P. synxantha*, *P. tolaasii*, *P. taetrolens* and *P. gessardii* were susceptible to cefepime (64%). Susceptibility to ciprofloxacin showed 91% of the isolates. Resistance to imipenem demonstrated 73% of the isolates. Most of the isolates (91%) except *P. taetrolens* were resistant to doripenem. *P. taetrolens* was sensitive to antibiotics including cefepime, ciprofloxacin, imipenem and doripenem. This study shows the high prevalence of antibiotic-resistant *Pseudomonas* spp. strains in fish. Our results agreed with **Kačániová et al., 2017** about occurrence of antibiotic-resistant *Pseudomonas* spp. in freshwater fish. Some antimicrobials were effective against *Pseudomonas* spp. in **Kholil et al. (2015)** study, who showed *Pseudomonas* spp. susceptibility to ciprofloxacin. Oxytetracycline, tetracycline and ciprofloxacin were found to be effective against *Pseudomonas* spp. also by **Mastan (2013)**.

Results on antimicrobial resistance of *Enterobacteriaceae* from fish samples are shown in Table 4. *L. amnigena* isolates were resistant to ticarcillin, however, showed susceptibility against other antibiotics. *E. vulneris* showed 100% resistance to ticarcillin and 50% to cefotaxime and tobramycin. *Staphylococcus equorum*

were resistant only to cefotaxime. *R. aquatillis* showed resistance to ticarcillin, cefotaxime, imipenem and tobramycin. Totally, 83% of isolates expressed susceptibility to ciprofloxacin with only one isolate of the *L. amnigena* was resistant. Susceptibility to imipenem was 83%, but resistance to cefotaxime was 50% among *Enterobacteriaceae* isolates. Resistant *Enterobacteriaceae* were found in water sources previously **(Guyomard-Rabenirina et al., 2017)**. **Stock and Wiedemann (1999)** reported *E. vulneris* was the species the most susceptible to ticarcillin. The antimicrobial resistance become emerging problem with international organizations and the EU institutions have recognized that the development of antimicrobial resistance raises severe consequences for human and animal health and well-being **(Singer et al., 2016)**. The present study showed the occurrence of resistant bacteria in freshwater fish.

CONCLUSION

The results of our study confirm that freshwater fish are contaminated with bacteria, but in general, the microbial quality of freshly caught fish is acceptable. Fish gill, gut and skin microflora was very diverse with *Pseudomonas* spp. and *Enterobacteriaceae* were the most abundant. Groups of microorganisms. Our results confirm that antibiotic resistant bacteria could be found in fish that represents possible public health consequences.

REFERENCES

Abd-El-Malek, A. M. 2017. Incidence and virulence characteristics of *Aeromonas* spp. in fish. *Veterinary World*, vol. 10, no. 1, p. 34-37. https://doi.org/10.14202/vetworld.2017.34-37

Alikunhi, N. M., Batang, Z. B., AlJahdali, H. A., Aziz, M. A. M., Al-Suwailem, A. M. 2017. Culture-dependent bacteria in commercial fishes: Qualitative assessment and molecular identification using 16S rRNA gene sequencing. *Saudi Journal of Biological Sciences*, vol. 24, no. 6, p. 1105-1116. https://doi.org/10.1016/j.sjbs.2016.05.017

Altun, S., Duman, M., Büyükekiz, A. G., Özyiğit, M. O., Karataş, S., Turgay, E. 2013. Isolation of *Citrobacter braakii* from rainbow trout (*Oncorhynchus mykiss*). *The Israeli Journal of Aquaculture – Bamidgeh*, vol. 65, p. 915-924.

Austin, B. 2006. The bacterial microflora of fish, revised. *The Scientific World Journal*, vol. 6, p. 931-945. https://doi.org/10.1100/tsw.2006.181

Aydin, S., Celebi, S., Akyurt, I. 1997. Clinical, haematological and pathological investigations of *Escherichia vulneris* in rainbow trout (*Oncorhynchus mykiss*). *Fish Pathology*, vol. 32, no. 1, p. 29-34. https://doi.org/10.3147/jsfp.32.29

Aydin, S., Erman, Z., Bilgin, O. C. 2011. Investigations of *Serratia liquefaciens* infection in rainbow trout (*Oncorhynchus mykiss Walbaum*). *Turkish Journal of Veterinary and Animal Sciences*, vol. 25, no. 5, p. 643-650.

Cipriano, R. C., Dove, A. 2011. Far from superficial: microbial diversity associated with the skin and mucus of fish. In *Bridging America and Russia with Shared Perspectives on Aquatic Animal Health, Third Bilateral Conference between Russia and the United States* : proceedings. Maryland, USA : Khaled bin Sultan Living Oceans Foundation, p. 156-167. ISBN: 9780983561101.

Cwiková, O. 2016. Microbiological evaluation of fish. *Potravinarstvo*, vol. 10, no. 1, p. 407-412. https://doi.org/doi:10.5219/617

Da Silva, M., Matte, G., Germano, P., Matte, M. 2010. Occurrence of pathogenic microorganisms in fish sold in Sao Paulo, Brazil. *Journal of Food Safety*, vol. 30, no. 1, p. 94-110. https://doi.org/10.1111/j.1745-4565.2009.00192.x

Dekker, J., Frank, K. 2015. *Salmonella, Shigella*, and *Yersinia*. Clinics in Laboratory Medicine, vol. 35, no. 2, p. 225-246. https://doi.org/10.1016/j.cll.2015.02.002

Doménech-Sánchez, A., Laso, E., Perez, M. J., Berrocal, C. I. 2011. Emetic disease caused by *Bacillus cereus* after consumption of tuna fish in a beach club. *Foodborne Pathogens and Disease*, vol. 8, no. 7, p. 835-837. https://doi.org/10.1089/fpd.2010.0783

EFSA, ECDC. 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal*, vol. 15, no. 12, p. 1-228. https://doi.org/10.2903/j.efsa.2017.5077

Eizenberga, I., Terentjeva, M., Valciņa, O., Novoslavskij, A., Ošmjana, J., Strazdiņa, V., Bērziņš, A. 2015. Evaluation of microbiological quality of freshwater fish in Usma lake. *Acta Biologica Universitatis Daugavpiliensis*, vol. 15, no. 1, p. 65-73.

EUCAST (The European Committee on Antimicrobial Susceptibility Testing). 2018. Breakpoint tables for interpretation of MICs and zone diameters. Version 8.1. Available at: http://www.eucast.org/clinical_breakpoints/.

Fernandes, R. 2009. *Microbiology handbook – fish and seafood*. UK : Leatherhead Food International, 256 p. ISBN: 978-1-905224-76-0.

Flores-Tena, F. J., Guerrero-Barrera, A. L., Avelar-González, F. J., Ramírez-López, E. M., Martínez-Saldaña, M. C. 2007. Pathogenic and opportunistic Gram-negative bacteria in soil, leachate and air in San Nicolás landfill at Aguascalientes, Mexico. *Revista Latinoamericana de Microbiología*, vol. 49, no. 1-2, p. 25-30.

Ge, Y., Zhu, J., Ye, X., Yang, Y. 2016. Spoilage potential characterization of *Shewanella* and *Pseudomonas* isolated from spoiled large yellow croaker (*Pseudosciaena crocea*). *Letters in Applied Microbiology*, vol. 64, no. 1, p. 86-93. https://doi.org/10.1111/lam.12687

Guyomard-Rabenirina, S., Dartron, C., Falord, M., Sadikalay, S., Ducat, C., Richard, V., Breurec, S., Gros, O., Talarmin, A. 2017. Resistance to antimicrobial drugs in different surface waters and wastewaters of Guadeloupe. *Plos One*, vol. 12, no. 3, p. 155-173. https://doi.org/10.1371/journal.pone.0173155

Hassan, EL., Farag, S. M. 2006. Incidence of haemolysin producing motile *Aeromonas* in some shellfish and their public health significance in Port-said city. *Journal of Applied Sciences Research*, vol. 2, no. 11, p. 972-979.

Heuer, O. E., Kruse, H., Grave, K., Collignon, P., Karunasagar, I., Angulo, F. J. 2009. Human health consequences of use of antimicrobial agents in aquaculture. *Clinical Infectious Diseases*, vol. 49, no. 8, p. 1248-1253. https://doi.org/10.1086/605667

ISO 13720: 2010. *Meat and meat products – Enumeration of presumptive Pseudomonas spp.*

ISO 21528: 2017. *Microbiology of the food chain – Horizontal method for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count technique*.

ISO 4832: 2006. *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coliforms – Colony-count technique*.

Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S.; Klūga, A., Tokár, M., Kluz, M., Ivanišová, E. 2017. The antioxidant and antimicrobial activity of essential oils against *Pseudomonas* spp. isolated from fish. *Saudi Pharmaceutical Journal*, vol. 25, no. 8, p. 1108-1116. https://doi.org/10.1016/j.jsps.2017.07.005

Keren, Y., Keshet, D., Eidelman, M., Geffen, Y., Raz-Pasteur, A., Hussein, K. 2014. Is *Leclercia adecarboxylata* a new and unfamiliar marine pathogen? vol. 52, no. 5, p. 1775-1776. https://doi.org/10.1128/JCM.03239-13

Kholil, Md. I., Hossain, Md. M. M., Neowajh, Md. S., Islam, Md. S., Kabir, M. 2015. Comparative efficiency of some commercial antibiotics against *Pseudomonas* infection in fish. *International Journal of Fisheries and Aquatic Studies*, vol. 2, no. 3, p. 114-117.

Kim, D. H., Brunt, J., Austin, B. 2007. Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). Journal of Applied Microbiology, vol. 102, no. 6, p. 1654-64. https://doi.org/10.1111/j.1365-2672.2006.03185.x

Larsen, A. M. 2014. *Studies on the microbiota of fishes and the factors influencing their composition* : dissertation theses. Auburn, Alabama, USA : Auburn University. 242 p.

Mastan, S. A. 2013. Pseudomonas septicemia in *Labeo rohita* (ham.) and *Cyprinus carpio* (linn.) in andhra pradesh–natural occurrence and artificial challenge. *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 5, no. 2, p. 564-568.

Oliveira, R. V., Oliveira, M. C., Pelli, A. 2017. Disease infection by *Enterobacteriaceae* family in fishes: a review. *Microbiology & Experimentation*, vol. 4, no. 5, p. https://doi.org/10.15406/jmen.2017.04.00128

Petersen, A., Andersen, J. S., Kaewmak, T., Somsiri, T., Dalsgaard, A. 2002. Impact of integrated fish farming on antimicrobial resistance in a pond environment. *American Society for Microbiology*, vol. 68, no. 12, p. 6036-6042. https://doi.org/10.1128/AEM.68.12.6036-6042.2002

Picot, L., Abdelmoula, S. M., Merieau, A., Leroux, P., Cazin, L., Orange, N., Feuilloley, M. G. 2001. *Pseudomonas fluorescens* as a potential pathogen: adherence to nerve cells. *Microbes and Infection*, vol. 3, no. 12, p. 985-995.

Prasad, M. P. 2014. Molecular characterization of enterotoxigenic *Bacillus cereus* species isolated from tropical marine fishes using RAPD markers. *International Journal of Pure & Applied Bioscience*, vol. 2, no. 4, p. 189-195.

Rasool, U., Ahmad, A., Badroo, G. A., Mudasir, M., Fayaz, S., Mustafa, R. 2017. Isolation and Identification of *Bacillus cereus* from fish and their handlers from Jammu, India. *International Journal of Current Microbiology and Applied Sciences*, vol. 6, no. 8, p. 441-447. https://doi.org/10.20546/ijcmas.2016.501.058

Singer, A. C., Shaw, H., Rhodes, V., Hart, A. 2016. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. *Frontiers in Microbiology*, vol. 7, p. 1-22. https://doi.org/10.3389/fmicb.2016.01728

Starliper, C. E. 2001. Isolation of *Serratia liquefaciens* as a pathogen of Arctic char, *Salvelinus alpinus*. *Journal of Fish Diseases*, vol. 24, p. 53-56. https://doi.org/10.1046/j.1365-2761.2001.00266.x

Stock, I., Wiedemann, B. 1999. Natural antibiotic susceptibility of *Escherichia coli*, *Shigella*, *E. vulneris*, and *E. hermannii* strains. *Diagnostic Microbiology and Infectious Disease*, vol. 33, no. 3, p. 187-199.

Stratev, D., Vashin, I., Daskalov, H. 2015. Microbiological status of fish products on retail markets in the Republic of Bulgaria. *International Food Research Journal*, vol. 22, no. 1, p. 64-69.

Terentjeva, M., Eizenberga, I., Novoslavskij, A., Strazdiņa, V., Valciņa, O., Ošmjana, J., Bērziņš, A. 2015. Bacterial microflora of freshwater fish originated from Usmas Lake in Latvia. *The Journal of Microbiology, Biotechnology and Food Sciences*, vol. 4, no. 1, p. 74-77. https://doi.org/10.15414/jmbfs.2015.4.ispecial1.74-77

Tortorello, M. L. 2003. Indicator organisms for safety and quality—uses and methods for detection: minireview. *Journal of AOAC International*, vol. 86, no. 6, p. 1208-1217.

Tosun, Ş.Y., Üçok Alakavuk, D., Mol S. 2016. Isolation of *Salmonella* spp. and other members of *Enterobacteriaceae* from horse mackerel (*Trachurus trachurus*), sold in public markets of Istanbul, Turkey. *Journal of Food and Health Science*, vol. 2, no. 2, p. 82-89. https://doi.org/10.3153/JFHS16009

Wamala, S. P., Mugimba, K. K., Mutuloki, S., Evensen, Ø., Mdegela, R., Byarugaba, D. K., Sørum, H. 2018. Occurrence and antibiotic susceptibility of fish bacteria isolated from *Oreochromis niloticus* (Nile tilapia) and *Clarias gariepinus* (African catfish) in Uganda. *Fisheries and Aquatic Science*, vol. 21, no. 1, p. 1-10. https://doi.org/10.1186/s41240-017-0080-x

Yagoub, S.O. 2009. Isolation of *Enterobacteriaceae* and *Pseudomonas* spp. from raw fish sold in fish market in Khartoum state. *Journal of Bacteriology Research*, vol. 1, no. 7, p. 85-88.

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