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Full Research Article

MICROBIOLOGICAL CHARACTERISTICS OF FISH REARED IN PURIFIED WASTEWATER FROM AN ABATTOIR

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Abstract

Wastewater from abattoirs in some countries is disposed of into water bodies without adequate removal of contaminants. Therefore, the use of wastewater in fish production could pose a serious risk for humans, fish and other aquatic organisms due to possible transfer of pathogenic bacteria in aquatic culture environments. The aims of the present study were to assess the levels of individual microorganisms in different tissues of common carp and to determine any correlation with the season of sampling and the type of analysed sample in common carp reared in an integrated production system that used purified water from an abattoir. A fish pond was filled mostly with purified water from an abattoir, but also partly with well water. Carp fingerlings were stocked in the earthen fishpond in March and reared in ambient conditions. Fish were collected in the

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spring and autumn of the following year and the microbiological quality was assessed. Carp fillets with skin, gills and digestive tract samples were collected individually under aseptic conditions. All analyses were performed according to standard procedures. The levels of all the examined bacteria in the fish were under prescribed hygiene norms. Also, Listeria spp., sulphite-reducing clostridia and Staphylococcus aureus were not found in the samples. Furthermore, pathogenic bacteria, Salmonella and Listeria monocytogenes were not isolated from the samples. The hygienic quality of the fish produced in purified waste water from an abattoir was acceptable, and the common carp meat was safe for human consumption.

Key Words: food safety, hygiene norms, integrated system, sustainability, wastewater, fish microbiology

INTRODUCTION

Wastewater from abattoirs in some countries is still disposed of into water bodies without adequate removal of contaminants. Such wastewater contains an abundance of organic matter that is an ideal nutrient source for fish, but also for the growth of various bacteria. Therefore, the use of wastewater in fish production could be a serious risk for humans and various aquatic organisms due to possible introduction of pathogenic bacteria in the aquatic environment (Sapkota et al., 2008). Aquacultured fish can be vehicles for various pathogens (Ljubojević et al., 2016). Thi Phong Lan et al. (2007) and Pelić et al. (2020) stressed that consuming fish from wastewater-fed ponds is of questionable safety. Scallan et al. (2011) stated that more foodborne outbreaks are caused by pathogens than by chemical or physical hazards. In the existing literature, there is insufficient information on meat safety of fish produced in treated wastewater-fed earthen ponds, which makes this research a novelty in the field of integrated fish production systems. Therefore, the aims of the present study were to assess the levels of individual microorganisms in different tissues of common carp and to determine any correlation with the season of sampling and the type of analysed sample in common carp reared in an integrated production system that used purified water from an abattoir.

MATERIALS AND METHODS

Fish pond

A 1-ha fish pond with an average depth of 1.2 m was set up in the vicinity of an abattoir facility, in Pećinci, Serbia. The fish pond was filled mostly with treated wastewater directly from the abattoir and partly with well water. Pond aeration was provided by continuous use of an aerator. A channel, 2 m wide, was dug around the pond for filtering the water from the pond, thus significantly reducing the required amount of water. The wastewater treatment system, measuring 25.50 x 16.15 m, consisted of a pumping station and several purification components located in separate tanks/reservoirs: accumulation tank, grease separation system, denitrification treatment system, equalization tank and biological oxidation tank for biological treatment.

Fish species and supplementary feed

Two-year-old fingerlings of common carp (*Cyprinus carpio*) obtained from a commercial fish farm were stocked in the pond at a density of 2,500 individuals/ha. The fish pond was stocked in March 2017. The mean initial live weight of the fish was 200 g. Relatively low cost, accessible nutrients were used as supplementary feed and industrial feed for fish was added.

Sampling

The first fish sampling was conducted in April 2018 (spring). The fish then had an average weight of 820 g. The study lasted until the beginning of October (autumn), when the second fish sampling was conducted. The average fish weight then was 1950 g. All fish were fit for human consumption. Fish were sacrificed by a quick blow to the head. Seven individual fish were collected from the pond in each sampling season. Each fish was placed in a sterile plastic bag. All fish were kept at 4 °C during the transportation to the laboratory and analysed within four hours of sampling.

Microbiological analysis

Samples of common carp fillets with skin, gills and digestive tract were collected individually under aseptic conditions. All analyses were conducted according to standard procedures. The total aerobic counts of bacteria were enumerated by standard plate count (SRPS EN ISO 4833-1:2014) method. Total *Enterobacteriaceae* and coliform counts were determined according to SRPS EN ISO 21528-2:2017 and SRPS ISO 4832:2014, respectively. *Escherichia coli* enumeration was performed according to SRPS ISO 16649-2:2008. Sulphite-reducing clostridia were analysed according SRPS EN ISO 7937:2010. *Staphylococcus aureus* counts were determined according to ISO 6888-1:2009. *Listeria* spp. enumeration was according to ISO 11290-2:2017. *Salmonella* spp. were detected following the standard procedure of ISO 6579-1:2017 and *Listeria monocytogenes* according to ISO 11290-1:2017.

Statistical analysis

The Student t-test was used to determine if the means of the results from the samples that were taken in spring and in autumn were significantly different. The data were analysed in Excel 2013. The duplicate plate counts for all examined bacteria were averaged. Average counts for total aerobic counts, *Enterobacteriaceae*, coliforms and *E. coli* were analysed following log transformation of the data. All data are shown as means±S.D. The differences were significant when $p<0.05$. Significant effects of type of analysed sample were further assessed using analysis of variance (ANOVA). A p-value of 0.05 or lower was considered as statistically significant.

RESULTS

The results of the microbiological analysis of fish grown in a pond supplied with purified wastewater from the abattoir are presented in Table 1. No significant differences ($p>0.05$) in the total aerobic counts were observed that might be attributed to seasons.

The total bacteria count, the number of *Enterobacteriaceae*, the number of coliforms and the number of *E. coli* depended on the type of analysed sample. The differences in the counts of these bacteria were statistically significant in the fillets with skin, gills and digestive tract ($p<0.001$) (Table 2). The total bacteria counts were highest in the digestive tract and lowest in fillets with skin. The numbers of bacteria present in the fillets with skin versus digestive tract and gills versus digestive tract were also significantly different. Statistical analysis also showed significant differences in the number of all tested bacteria between fillets with skin and gills.

DISCUSSION

An important health concern in aquaculture is the possible contamination of fish with faecal coliforms in wastewater (El-Shafai et al., 2004). The presence of faecal coliforms in fish intended for human consumption is a potential risk and could cause disease in humans (Swartz, 2002). Moreover, faecal coliforms could transfer antibiotic resistance from bacteria related to aquatic environments to bacteria that infect human populations (Rizzo et al., 2013). In our work, *E. coli* was present only in the digestive tract, in average levels of 1.45 log CFU/g and 1.5 CFU/g in spring and autumn, respectively, while *E. coli* was not isolated from fillets with skin nor from the gills. Fernandes et al. (1997) observed significant differences in the number of *E. coli* in fish in different seasons. They observed that the numbers of *E. coli* were the highest during the summer but the bacterium was not detected in spring or in winter. Sources of contamination of fish flesh with *E. coli* can be the intestinal tracts of fish and humans.

The lower number of bacteria during winter and fall consequently leads to reduced bacteria numbers in fish meat. Coliform counts could be used for assessing the efficiency of safety procedures during fish manipulation and processing. According to Andrews et al. (1977), total coliform counts in fish were in the range from <3 to 2.4×10^6 CFU/g. Trust and Sparrow (1974) stated that the presence of coliforms in fish digestive tracts depends on water quality and that fish are only carriers of these bacteria. According to Kay et al. (2008), the presence of faecal indicator bacteria in pond water can be a consequence of pond fertilization with manure applied directly to the pond or faeces excreted by fish in the ponds. Moreover, faecal indicator bacteria could originate from abattoir wastewater. Dang and Dalsgaard (2012) examined the level of *E. coli* in fish meat and digestive tract contents of grass carp, silver carp and rohu obtained from five fish ponds with and without pig excrement in Hanoi, Vietnam. The number *E. coli* in fish muscle was <10, 320 and 820 CFU/g in grass carp,

Table 1. Spring and autumn microbial loads in fillets with skin, gills and digestive tract from common carp produced in a pond fed with purified wastewater

Bacterial group log	Fillets with skin			Gills			Digestive tract		
	Spring	Autumn	p-value	Spring	Autumn	p-value	Spring	Autumn	p-value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Total bacteria count	5.74±0.14	5.76±0.16	0.64	6.86±0.06	6.83±0.08	0.39	7.87±0.12	7.78±0.16	0.09
Enterobacteriaceae	<10	<10	/	2.69±0.11	2.55±0.23	0.05	4.52±0.18	4.69±0.35	0.14
Coliforms	<10	<10	/	2.77±0.07	2.76±0.11	0.95	4.64±0.06	4.73±0.15	0.07
E. coli	<10	<10	/	<10	<10	/	1.45±0.19	1.5±0.29	0.60

All values are mean±S.D. (n=7); Sulphite-reducing clostridia; Coagulase positive staphylococci; *Listeria*, enumeration = < 10; *Salmonella* spp. and *Listeria monocytogenes* – not detected

Table 2. Microbial loads in fillets with skin, gills and digestive tract from common carp produced in a pond fed with purified wastewater, in spring and in autumn

Bacterial group log	Spring				Autumn			
	Fillets with skin	Gills	Digestive tract	p-value	Fillets with skin	Gills	Digestive tract	p-value
Total bacteria count	5.74±0.14	6.86±0.06	7.87±0.12	p<0.001	5.76±0.16	6.83±0.08	7.78±0.16	p<0.001
Enterobacteriaceae	<10	2.69±0.11	4.52±0.18	p<0.001	<10	2.55±0.23	4.69±0.35	p<0.001
Coliforms	<10	2.77±0.07	4.64±0.06	p<0.001	<10	2.76±0.11	4.73±0.15	p<0.001
E. coli	<10	<10	1.45±0.19	p<0.001	<10	<10	1.5±0.29	p<0.001

All values are mean±S.D. (n=7); Sulphite-reducing clostridia; Coagulase positive staphylococci; *Listeria*, enumeration = < 10; *Salmonella* and *Listeria monocytogenes* – not detected

silver carp and rohu, respectively, and the authors did not find any significant effect of the presence of pig excrement. They further reported that the number of *E. coli* in intestinal contents of fish raised in ponds with pig excrement was 4.75, 5.25, and 5.07 log CFU/g for silver carp, grass carp, and rohu, respectively. These levels were approximately 100 times higher than in fish raised in ponds without pig excrement. The *E. coli* count in the digestive tract of our fish was about 2000 times lower than the counts reported by other authors in fish muscle obtained from in fish cultured in ponds fertilized with pig excrement (Dang and Dalsgaard, 2012), which indicates the efficacy of our abattoir's wastewater purification. Furthermore, our results are in accordance with the results obtained by Dang and Dalsgaard (2012) showing that fish muscle from fish produced in ponds fertilized with pig excrement has a low number of faecal indicator bacteria even when the fish have high *E. coli* counts in their guts. The same authors suggested that the prevention of faecal cross-contamination during fish processing at home or at markets is a critical point for food safety control.

S. aureus in the fish could result from animal or human contamination of the pond and was not detected in our research. *S. aureus* can grow and produce enterotoxin in fish meat if fresh fish are improperly handled, if the temperature exceeds 10 °C, and especially when total aerobic counts are low. If total aerobic counts are high, *S. aureus* is unable to compete with these bacteria, and the fish meat spoils before *S. aureus* can multiply. *S. aureus* is an important factor in the microbiological safety of fish since *S. aureus* enterotoxin is thermostable and is not destroyed during thermal treatment.

Listeria spp., and sulphite-reducing clostridia were not found in the examined fish samples. Furthermore, pathogenic bacteria, *Listeria monocytogenes* and *Salmonella* were not found in tested samples. The incidence of salmonellosis related to consumption of fish is relatively low in comparison with the incidence of infection due to consumption of meat from terrestrial animals. Considering that *Salmonella* is the leading causative agent of foodborne diseases, causing gastroenteritis with diarrhoea, nausea, vomiting, fever and abdominal cramps, *Salmonella* detection remains important for food safety. This pathogen could spread to aquatic environments via faecal contamination, and it can be present in fish and fish products. Iwamoto et al. (2010) reported that *Salmonella* was isolated in 18 outbreaks, with 374 cases and 28 hospitalizations associated with seafood consumption during the period 1973 to 2006. Hassan et al. (2018) reported that about 62 people were infected with *Salmonella* Java linked to eating raw or frozen tuna. It should be emphasized that wastewater treatment processes do not eliminate all pathogenic and indicator microorganisms present in wastewater. A proportion of pathogenic and indicator organism loads remained in digested sludge even after treatment processes. According to Sidhu and Toze (2009), the percentage of removal of *Salmonella* during primary treatment is 95.8-99.8%, during secondary treatment is 98.65-99.996% and during tertiary treatment is 99.99-99.9999995%.

The results obtained in the current study showed bacteria levels were dependent on the type of analysed sample, which is in accordance with results obtained by Zmyslowska et al. (2002). They reported that the highest count of microorganisms was in the gastric

contents, lower counts were obtained in the mucus on the surface of the skin and the lowest counts (for some bacteria, absent, i.e., not detected) were in the muscle tissue of fish. According to their results the total count of microorganisms in the digestive tract ranged from 5.8×10^5 to 6.5×10^5, levels below the levels in our fish digestive tracts. In the current study, the highest counts of *Enterobacteriaceae* and coliforms were in the digestive tract, followed by gills, while in fillets with skin, *Enterobacteriaceae* and coliforms levels were below the limit of detection.

The relatively low bacteria count in fish meat compared to the gills and digestive tract and the absence of pathogenic bacteria in our study is a consequence of aseptic and proper sampling, handling and fish processing. Emipke et al. (2011) suggested that the high number of total aerobic bacteria in fish meat could be due to proliferation after harvesting and during transport or to improper handling by contaminated hands. Also, common carp mucus is bactericidal and efficiently reduces the number of microorganisms, so initially, skinless carp meat is mostly without bacteria.

The total bacteria counts in fish samples reported by Sanjee and Karim (2016) ranged from 2.8 to 4.9×10^5 CFU/g and were lower than counts in our study, where the total bacteria counts in fillets with skin ranged from 3.5 to 8.47×10^5 and 3.16 to 9.87×10^5 CFU/g in spring and in autumn, respectively. Zmyslowska et al. (2003) reported that the average bacteria counts of fish reared in electric power plant cooling water were 1.8×10^5 CFU/g in digestive tract contents, and $1{,}06\times10^3$ CFU/cm^2 in mucus.

The presence and survival of bacteria in digestive tracts of fish strongly depend on environmental conditions, mostly temperature (Zmyslowska et al., 2001). On both our sampling days, the water temperature was 21-25 °C, the same as in the study conducted by Zmyslowska et al. (2003). Importantly, Zmyslowska et al. (2003) examined sturgeon, while common carp is a bottom feeder and so could be exposed to bacteria in pond sediment (Thi Phong Lan et al., 2007), which would not affect sturgeon.

Surendraraj et al. (2009) reported that the total plate count for carp flesh ranged from 4.19 to 4.85 log CFU/g, which is also below the numbers we detected. It should be highlighted that the high number of microorganisms detected in the current study was present in the inedible portions (the fillets examined were with the skin) of common carp, i.e., the skin, gills and intestines. These parts are separated during processing, and consequently, the microbiological quality of the fish is improved. Zmyslowska et al. (2002) noted that the bacteria count in fish mucus is usually 10^2 to 10^7 per cm^2 of skin, and the bacteria count in the digestive tract reaches 10^8 per g of gastric content. Furthermore, Sterniša et al. (2016) reviewed literature data and reported that bacteria loads ranged from 2.0 log CFU/cm^2 to 7.0 log CFU/cm^2 for skin and from 3.0 to 9.0 log CFU/g for gills and intestine of different fish species at the time of capture, which is in good agreement with our results. Harnisz and Tucholski (2010) noted that the microorganisms present in the digestive tract and mucus on the skin of fish play a role as indicators of the microbiological quality of water used for fish production.

Ogbondeminu (1993) noted that the occurrence of *Enterobacteriaceae* was much lower in conventional aquaculture production than in integrated aquaculture production. Ogbondeminu and Okoeme (1989) noted that 50% of the bacteria isolated from fish reared in ponds fertilized with animal faeces belonged to the family *Enterobacteriaceae*. Zmyslowska et al. (2002) reported that the highest count of *Enterobacteriaceae* was in the gastric contents, and they found *Enterobacteriaceae* in autumn and spring but not in winter. Also, the level of *Enterobacteriaceae* in fish examined in the current study was far below levels measured by Ogbondeminu and Okoeme (1989) and Njoku et al. (2015).

Healthy fish meat is generally sterile at the time of harvest. However, fish meat is highly susceptible to microbiological contamination due to its high content of unsaturated fatty acids and water (Sterniša et al., 2016). On the contrary, a wide range of microorganisms can be present on fish skin, gills and in the digestive tract, and this depends mainly on the microbial load of the aquatic environment. Indigenous bacteria are normally present on fish while nonindigenous bacteria are considered as a result of contamination of water and fish due to poor hygienic and processing practices. Non-indigenous bacteria include *Escherichia coli, Staphylococcus aureus, Salmonella*, *Enterobacteriaceae* and *Shigella*, while *Listeria monocytogenes* is classified as both indigenous and non-indigenous (Feldhusen, 2000). We did not find non-indigenous bacteria in fillets with skin. On the other hand, Njoku et al. (2015) reported that the counts of total aerobic bacteria and non-indigenous bacteria were high both in concrete and in earthen ponds, mainly due to water temperature which ranged from 26 to 29 °C and was optimal for bacterial growth. Also, the organic matter load as result of the fish feed was very high. They found that concrete and earthen ponds were contaminated with pathogenic and commensal microorganisms and that this would produce a risk to public health. Fafioye (2011) also found that fish cultivated in controlled environments, in concrete ponds in Nigeria, were contaminated with pathogenic and opportunistic microorganisms. They suggested the contamination could be related to high fish population density, but also to water quality. However the results obtained by Slabbert et al. (1989) were consistent with our results and showed that fish fillets reared in wastewater ponds were microbiologically safe for human consumption. The levels of all the tested bacteria were acceptable and were lower than the maximum allowable microbiological count limits.

Recommendations for the microbiological quality of fish have been made by different organisation and authors. ICMSF (1986) set up limits for total bacteria counts for fish and fishery products: $m = 10^6$ and $M = 10^7$ CFU/g. The ICMSF (1986) also set up limits for counts of faecal coliforms in freshwater fish products: m = 4 and M = 400. Coliforms are considered as an insignificant health hazard because heat treatment of fish before consumption remarkably reduces the risk. The ICMSF (1986) set up limits for *S. aureus* which is also an important indicator bacterium for fresh and frozen fish products: $m = 10^3$ and $M = 2\times10^3$ CFU/g. According to Thi Phong Lan et al. (2007), Vietnam national standards prescribe that there should be ≤3 *E. coli*/g in heat

treated fish and ≤100 *E. coli*/g in frozen or raw fish. Surendran et al. (1989) stated that spoilage of food occurs when total aerobic counts are higher than 10^7 CFU/g. According to Emikpe et al. (2011), the acceptable limit for total aerobic bacteria is 10^6 CFU/g. The upper limit for the total viable counts for separation of good and bad quality fish products is 5×10^5 CFU/g (Hernández et al., 2009).

CONCLUSION

The hygienic quality of carp grown in properly purified wastewater from the abattoir was adequate, and the carp meat was safe for human consumption. Our analyses showed that all obtained bacteria counts were below the recommended limits, indicating the water was uncontaminated and that all procedures and processing were performed in aseptic conditions. Continuous monitoring of the presence and level of microorganisms in treated wastewater is of major importance, having in mind the importance of fish in human nutrition. The results obtained are very significant for the exposure assessment of microorganisms from fish cultured in treated wastewater. The microbiological quality of the fish was not affected by the season, but the microbiological count was different in different parts of the fish body. The highest microbial counts were measured in digestive tract, while counts were lower in mucus from the skin and were the lowest (or the studied bacteria were absent) in muscle tissue.

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Authors' contributions

MP, DLJP and NN made substantial contributions to the basic idea, conception and design, acquisition of samples and data, analysis of the data and interpretation of results; AG, JJD and ZM were involved in drafting of the manuscript, revising it critically for important intellectual content. MM and VĐ performed the statistical analysis and made a substantial contribution to interpretation of data. All the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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MIKROBIOLOŠKE KARAKTERISTIKE ŠARANA GAJENOG U PREČIŠĆENOJ OTPADNOJ VODI POREKLOM IZ KLANICE

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Kratak sadržaj

Otpadna voda iz klanica u zemljama u razvoju se i dalje ispušta u reke, jezera i mora bez predhodnog prečišćavanja. Stoga, korišćenje otpadne vode u akvakulturi može predstavljati rizik za zdravlje ljudi, riba i ostalih akvatičnih životinja, zbog mogućeg transfera patogenih baktreija u vodenu sredinu. Cilj ovog rada je bio da se utvrdi prisustvo i broj mikroorganizama u različitim organima šarana, kao i korelacija sa sezonom uzorkovanja i vrstom analiziranog uzorka, kod šarana koji je nasađen u integrisanom sistemu proizvodnje koji koristi prešišćenu otpadnu vodi uz klanice. Ribnjak je snabdevan najvećim delom prečišćenom otpadnom vodom iz klanice, ali uz dodatak bunarske vode. Šaranski mladunci nasađeni su u martu i bili su dobrog zdravstvenog stanja. Uzorkovanje ribe je sprovedeno u proleće i jesen sledeće godine da bi se ispitao mikrobiološki kvalitet. Uzorci mišićnog tkiva sa kožom, škrge i digestivni trakt su uzeti pojedinačno pod aseptičnom uslovima. Sve analize su sprovedene standardnim metodama ispitivanja. Broj mikroorganizama je bio zadovoljavajući i nije prelazio preporučene higijenske norme. Sulfitoredukujuće klostidije, *Staphylococcus aureus* i Listerija vrste nisu detektovane u ispitanim uzorcima. Osim toga, patogene bakterije, *Salmonella* i *Listeria monocytogenes* nisu detektovane. Mikrobiološki kvalitet šarana koji je gajen u prečišćenoj otpadnoj vodi iz klanice je bio odgovarajući i bezbedan za javnu potrošnju.

Ključne reči: bezbednost hrane, higijenske norme, integrisani sistem, održivi razvoj, otpadna voda, mikrobiologija riba