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## THE FIRST OUTBREAK OF LACTOCOCCOSIS CAUSED BY LACTOCOCCUS GARVIEAE IN SERBIA

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### Abstract

The outbreak of lactococcosis affecting rainbow trout, weighing 70 - 120 g, occurred at a trout aquaculture facility in Central-West Serbia during July, 2018. This outbreak lasted for three weeks, and cumulative mortality attributed to the disease was around 40%. The diseased fish showed erratic swimming, dark discoloration and exophthalmia, with the cumulative mortality of around 40%. A pure Gram-positive cocci isolates were obtained from the eye and kidney samples. Based on colony morphology, phenotypic and biochemical characteristics, the isolated bacterium was presumably identified as *Lactococcus garvieae*. Using the BBL CRYSTAL GP ID system, the isolate was identified as *L. garvieae* and the identity of the isolate was confirmed with MALDI-TOF Mass Spectrometry. Blast analysis of 16S rRNA sequence of our isolate had a 99.4 to 99.6% similarity to the *L. garvieae* strains previously isolated from diseased fish. The antibacterial activity of 15 antimicrobials against *L. garvieae* was evaluated using disc diffusion. In this paper, we report the first case of lactococcosis in rainbow trout in Serbia, isolation and characterization of causative agent, *Lactococcus garvieae* from diseased rainbow trout.

**Key words:** Lactococcosis, *Lactococcus garvieae*, rainbow trout

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## PRVI SLUČAJ LAKTOKOKOZE IZAZVANE SA *LACTOCOCCUS GARVIEAE* U SRBIJI

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### Kratak sadržaj

Pojava laktokokoze u zapatu kalifornijske pastrmke, težine 70 - 120 g, je utvrđena na pastrmskom ribnjaku u zapadnoj Srbiji u julu 2018. godine. Bolest je trajala tri nedelje, a kumulativni mortalitet nastao kao posledica oboljenja je iznosio oko 40%. Klinička slika se karakterisala promenama u plivanju, tamnom pigmentacijom kože i izraženim egzoftalmusom. Na osnovu morfoloških i biohemijskih karakteristika, izolovana bakterija je identifikovana kao *Lactococcus garvieae*. Korišćenjem BBL CRYSTAL GP ID sistema, izolat je identifikovan kao *L. garvieae*, a identitet izolata je potvrđen pomoću MALDI-TOF masene spektrometrije. Analiza 16Sr RNK sekvence našeg izolata pokazala je sličnost od 99,4 do 99,6% sa sekvencama sojeva *L. garvieae* prethodno izolovanih iz obolelih riba. Antibakterijska aktivnost 15 antimikrobnih lekova protiv *L. garvieae* procenjena je metodom disk difuzije. U radu je opisan prvi slučaj pojave laktokokoze kalifornijske pastrmke u Srbiji i izolacija i karakterizacija uzročnika bolesti.

**Ključne reči:** laktokokoza, *Lactococcus garvieae*, kalifornijska pastrmka

### INTRODUCTION

Lactococcosis is a significant fish disease caused by the *Lactococcus garvieae* bacteria. It is a systemic hyper-acute infection with the occurrence of widespread haemorrhaging (Austin & Austin 2016), described for the first time at the end of the 1950s in Japan, where the first cases were diagnosed in rainbow trout (Vendrell et al. 2006). Now, the disease is present in many parts of the world, affecting sea fish and freshwater fish in aquaculture (Meyburgh et al. 2017). Rainbow trout is an important species for aquaculture in Serbia,

with more than 100 farms in business. Trout farms are mainly concentrated in South-East part of the country. In Europe, the first outbreak of this disease in rainbow trout was reported in Spain in 1989 (Palacios et al., 1993). After that, *L. garvieae* was isolated in Italy (Reimundo et al. 2011), the UK (Algöet et al. 2009), France (Eynngor et al. 2004), Portugal (Pereira et al. 2004), Greece (Savvidis et al. 2007), Spain (Aguado-Urda et al. 2011), Turkey (Diler et al. 2002) and Bulgaria (Eynngor et al. 2004). Lactococcosis is the single most important risk factor in the European trout industry, with losses approximating 50% of the total annual production (Eynngor et al., 2004). The impact of lactococcosis is particularly significant as losses often occur when fish reach market size (Ceschia et al. 1998). The disease causes significant losses at temperatures above 15°C. The oral route is the main route of *L. garvieae* transmission (Nakai et al. 1999), but the results of a study conducted by Avci et al. (2010) suggest that the gills and eyes are major spots of attachment and the proliferation of *L. garvieae* during infection period. Infected fish exhibit a variety of clinical signs, such as anorexia, exophthalmia, melanosis, conjunctivitis, erect swimming, severe internal haemorrhage and congestion of blood vessel, peritonitis, abscess of spleen and liver, meningoencephalitis, and bacterial septicemia (Eldaret al.1999, Evans et al.2009, Pereira et al. 2004).

The causative agent, *L. garvieae* is one of the most important bacterial fish pathogens indifferent freshwater and marine fish species in many countries (Vendrell et al. 2006; Evans et al. 2009, Meyburgh et al. 2017), with the highest economic impact in rainbow trout aquaculture. *L. garvieae* is a non-motile, non-sporulating, facultative anaerobic, catalase and cytochrome oxidase negative, Gram-positive coccus. It is a lactic acid bacterium, first isolated from a case of bovine mastitis it the UK (Collins et al. 1983), and later from other animal hosts, such as cows, buffalos, pigs, dolphins, water buffalos, cats and dogs (Aguado-Urda et al. 2013, Tejedor et al. 2011). The bacterium was isolated from rivers and sewage waters, vegetables, meat and dairy products (Aguado-Urda et al. 2013). *L. garvieae* strains of dairy origin have been found to be free from virulence determinants, such as haemolysins and gelatinase (Fortina et al., 2007), suggesting that *L. garvieae* dairy strains are unrelated to the pathogenic ones (Foschino et al., 2008). Also, *L. garvieae* was involved in an increasing number of human clinical cases including infective endocarditis, septicemia, urinary and skin infections (Aguado-Urda et al. 2011), giving rise to the status of an emerging zoonotic pathogen. Although *L. garvieae* is a well-known fish pathogen, human infections are usually related to a contact with raw fish. It has gained recognition as an emerging zoonotic opportunistic pathogen, with the ingestion of contaminated foodstuffs being a likely route

of infection. Handling and of raw fish is reported as a source or risk factor in the majority of clinical cases (Gibello et al. 2016). High levels of antibiotic resistance and resistance genes in *L. garvieae* strains should be considered as a potential danger for trout culture as well as for public health (Raissy and Moumeni, 2016).

In this paper, we report the first case of lactococcosis in rainbow trout in Serbia, isolation and characterization of causative agent, *L. garvieae* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum).

## **MATERIAL AND METHODS**

A total of 20 rainbow trout (weighing 70-120g) that showed the clinical signs of the disease were collected from a trout aquaculture facility in Central-West Serbia in July, 2018. The samples for bacterial isolation were obtained from the kidney, liver, spleen and eye of the diseased fish. They were streaked on blood agar plates containing 10% defibrinated sheep blood (BA), Mueller-Hinton (MH) agar and trypticase-soy agar (TSA) plates, and incubated at 20°C for 48h. Single colonies were restreaked on the same media to obtain pure isolates. Pure colonies were subjected to Gram staining, followed by catalase and oxidase tests. The routine tests for the determination of biochemical characteristics were carried out as previously described (Austin & Austin 2016). Additionally, the BBL CRYSTAL™ Gram-Positive (GP) Identification (ID) system was used for the biochemical identification of isolated bacterium.

The isolate was tested for antimicrobial susceptibility by the disc diffusion method on MH agar. After 24h of the incubation, incubation zone diameters were measured and evaluated. The isolates were classified as sensitive (S), intermediary sensitive (I), or resistant (R), on the basis of the size of the zone of bacterial growth inhibition, according to the National Committee for Clinical Laboratory Standards (CLSI, 2019).

## **MALDI-TOF**

Protein mass spectroscopy analysis was carried out by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as previously described (Heras Cañas et al. 2015) using a VITEK MS Mass spectrometry microbial identification system (VITEK MS, bioMerieux, France).

### ***16S rRNA sequence analysis***

Total bacterial DNA of the isolate was extracted using the Cadon Pathogen Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The extracted DNA from the isolate was used as a template to amplify a 1419bp segment of the 16S rRNA gene by the polymerase chain reaction (PCR) technique using the universal prokaryotic primers 27F (5'-AGAGTTTGATC-CTGGCTCAG-3') and 1492R (5' GGTTACCTTGTTACGACTT-3'). The composition of PCR mixture was as recommended by manufacturer (Hot-StarTaq Master Mix Kit, Qiagen, Germany). The PCR cycling regime was the following: one cycle of 15 min denaturation and Taq activation at 95°C, 35 cycles of 40 s at 95°C, 40 s at 55°C, and 90 s at 72°C, and final extension cycle of 10 min at 72°C. A small quantity of PCR products was verified by standard agarose gel electrophoresis. The amplicons were purified using QIAquick PCR Purification kit (Qiagen, Germany) and sequenced using the primers 27F and 1492R. The sequencing of PCR product was performed by Macrogen. The isolate sequence was compared to sequences from the NCBI database, using the BLASTn algorithm. The limit fixed for identification of a bacterial species was 98% nucleotide identity for the 16S rRNA gene. The phylogenetic relationships of the isolates were determined by comparative 16S rRNA gene sequence analysis. Genetic distances matrix was obtained using Kimura's two-parameter model, and an evolutionary tree was created using the Neighbour-Joining method with Mega X (Kumar et al.2018).

## **RESULTS**

The disease outbreak affecting rainbow trout weighing 70 - 120 g occurred at a trout aquaculture facility in Zaovine Lake (43°52'46.3"N 19°24'08.4"E) during July, 2018. This outbreak lasted for three weeks, and cumulative mortality attributed to this pathogen was around 40%. The water temperature during the outbreak was consistently higher than 14°C (with highest temperature of 20°C). The infected fish exhibited lethargy, anorexia, dark skin coloration, marked unilateral and bilateral exophthalmos with the presence of generalized hemorrhaging or blood spots in the eye, eyeball disruption and loss of eye or eyes (Figure 1).



Figure 1. The hemorrhaging in the eye and exophthalmos in diseased rainbow trout caused by lactococcosis

Macroscopic examination revealed the accumulation of fluid in the body cavity. Skeletal muscle and liver were anemic with congestion. Liver and spleen were enlarged. Hemorrhages were present in liver, adipose tissue, pyloric caeca and muscle. Enlargement of the spleen and liver, and hemorrhagic enteritis with yellow, gelatinous fluid in the lumen of intestine were observed (Figure 2).



Figure 2. Lactococcosis in rainbow trout - hemorrhages in internal organs, enlargement of the spleen and liver, and yellow, gelatinous fluid in the lumen of intestine

After 48h incubation, pure cultures of cream-colored, opaque, round and convex colonies were recovered from eye and kidney samples (Figure 3).



Figure 3. Growth of colonies of *L. garvieae* SRB NIVS-1 strain on a blood agar plate after 24 hours of incubation in aerobic conditions.

The bacterial cells were  $\alpha$ -hemolytic, non-motile, oxidase-negative, catalase-negative, Gram-positive cocci occurring in pairs and short chains (Figure 4).

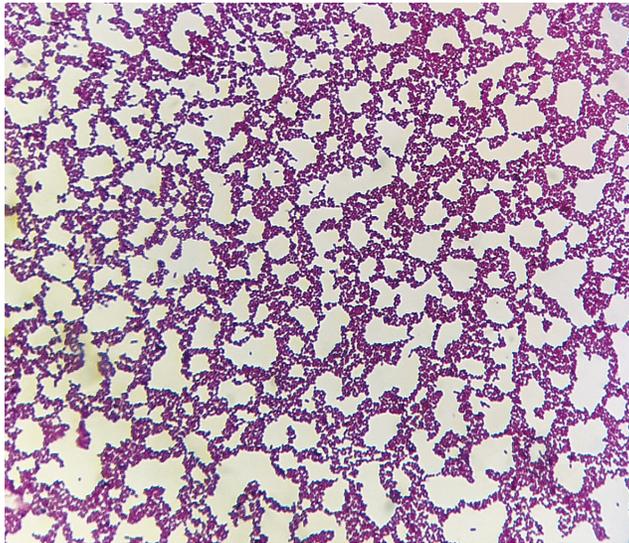


Figure 4. Gram stain of *L. garvieae* SRB NIVS-1 strain with clusters and short chains of gram-positive cocci.

Based on colony morphology, phenotypic and biochemical characteristics, the isolated bacterium was presumably identified as *L. garvieae* (Table 1).

Table 1. Phenotypic characteristic of *L. garvieae* SRB NIVS-1 strain isolated from diseased rainbow trout.

Phenotypic characteristics	<i>L. garvieae</i> SRB NIVS-1	<i>L. garvieae</i> ATCC 43921
Colony morphology	smooth	smooth
Cell morphology	ovoid-cocci	ovoid-cocci
Gram staining	+	+
Motility	-	-
Production of oxidase	-	-
Production of catalase	-	-
Production of indole	-	-
Production of H <sub>2</sub> S	-	-
Citrate utilization	-	-
Methyl red	+	+
Voges Proskauer	+	+
Nitrate reduction	-	-
Starch hydrolysis	-	-
Growth on nutrient agar	+	+
Growth on trypticase soy agar	+	+
Growth on brain heart infusion agar	+	+
Growth on Mueller-Hinton agar	+	+
Growth on MacConkey agar	-	-
Hemolysis in blood agar	α	α

Using the BBL CRYSTAL GP ID system, the isolate was identified as *L. garvieae* ID: 3440571723 (Table 2).

Table 2. The BBL Crystal profile for *L. garvieae* isolated from diseased rainbow trout

L-phenylalanine-AMC	+	p-nitrophenyl-β-D-cellobioside	+
L-tryptophan-AMC	+	p-nitrophenyl-α-D-maltoside	+
Trehalose	+	Esculin	+
Sucrose	+	L-valine-AMC	+-
Arabinose	-	pyroglutamic acid-AMC	+-

p-nitrophenyl- $\beta$ -D-glucoside	+	4MU-N-acetyl- $\beta$ -D-glucosaminide	+/-
p-nitrophenyl-phosphate	-	L-isoleucine-AMC	+
Urea	-	Methyl- $\alpha$ & $\beta$ -glucoside	+
4MU- $\beta$ -D-glucoside	+	Maltotriose	+
4MU- $\alpha$ -D-glucoside	+	Fructose	+
L-arginine-AMC	+/-	Proline & Leucine-p-nitroanilide	+
Lactose	-	o-nitrophenyl- $\beta$ -D-galactoside (ONPG) & p-nitrophenyl- $\alpha$ -D-galactoside	-
Mannitol	+	ID: 3440571723 <i>Lactococcus garvieae</i>	
Glycerol	-		

The *L. garvieae* isolate was tested in the present study with 15 different antimicrobials in terms of antimicrobial sensitivity and results are shown in Table 3.

Table 3. Antibiotic sensitivity of *L. garvieae* isolate SRB NIVS-1 isolated from diseased rainbow trout

Antibiotic	Sensitivity
Flumequine (15 $\mu$ g)	R
Oxytetracycline (30 $\mu$ g)	S
Sulfamethoxazole/trimethoprim (25 $\mu$ g)	R
Enrofloxacin (5 $\mu$ g)	R
Florfenicol (30 $\mu$ g)	S
Penicillin G (10 IU)	S
Ampicillin (10 $\mu$ g)	S
Amoxicillin (25 $\mu$ g)	S
Tetracycline (30 $\mu$ g)	S
Gentamycin(10 $\mu$ g)	R
Neomycin (10 $\mu$ g )	R
Streptomycin (10 $\mu$ g )	R
Trimethoprim (5 $\mu$ g)	R
Erythromycin (15 $\mu$ g)	S
Oxacillin (1 $\mu$ g)	R

To further confirm our result, the *L. garvieae* strain SRB NIVS-1 was identified with MALDI-TOF, which also confirmed the identity of our isolate. To

determine the genotype identity, we have extracted and sequenced the 16S rRNA (1419 bp, NCBI Genbank accession number MT000099) of strain SRB NIVS-1 and blasted using BLAST search program in the GenBank of NCBI, which revealed 99.6% sequence identity with *L. garvieae*. The sequence was compared with the sequences of reference species in Genebank data library by the BLAST program. Blast analysis of 16S rRNA sequence of our isolate presented a 99.4 to 99.6% similarity in sequences to the *L. garvieae* strains previously isolated from diseased fish. The isolate showed high percentage sequence similarity to other strains of *L. garvieae*: 99.6 % sequence similarity to MG016446 from China, 99.5 % sequence similarity to KT428591 from Turkey and 99.4 % sequence similarity to KU360715 from Iran. The relationship between the strain SRB NIVS-1 and the closest taxonomic species based on 16S rDNA sequences is described in the phylogenetic tree (Figure 5).

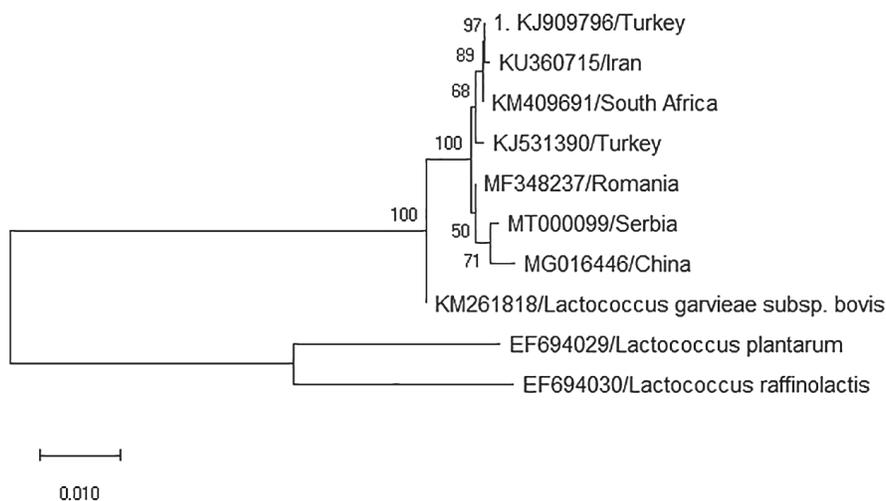


Figure 5. Phylogenetic tree based on 16S rRNA gene sequences showing the position of *L. garvieae* SRB NIVS-1 strain

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.15577609 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

## DISCUSSION

The outbreak of the lactococcosis affecting rainbow trout occurred at a trout aquaculture facility in Zaovine Lake, an artificial lake in the Central-West Serbia, on the Tara Mountain, created on the Beli Rzav River as a reservoir for the Bajina Bašta II reversible hydro power plant. This outbreak with a total mortality of around 40% lasted for three weeks. Rainbow trout mortality due to *L. garvieae* infection can be up to 60%, depending on the water temperature, stress for fish and strain type of the bacterium (Shahi et al, 2018). The water temperature during the outbreak was consistently higher than 14°C (with highest temperature of 20°C). Such high temperature was a critical environmental condition for the appearance of the disease with a high mortality (Pereira et al., 2004, Castro et al. 2017). Clinical findings during the course of the disease, including characteristic symptoms as hyperpigmentation and bilateral exophthalmia, were similar to those previously described in other studies (Evans et al. 2009, Algöet et al.2009, Austin and Austin, 2016).

Pure colonies of the gram positive streptococcus, non-motile, negative for oxidase and catalase, with properties typical for the *Lactococcus garvieae*, as previously described by Collins et al. (1983), were recovered from eye and kidney samples from the diseased rainbow trout. *L. garvieae* strains are phenotypically homogeneous, regardless of their geographic location or the aquatic host from which they were isolated (Buller, 2014). The biochemical properties of the isolated bacteria from rainbow trout were analogous to those described by Austin & Austin (2016). The taxonomy based on a molecular level was used to establish the phylogenesis and taxonomic position of the bacterium. Preferred methods for the identification of *L. garvieae* are MALDI-TOF MS and 16s rRNA gene PCR (Heras Cañas et al. (2015). It is important to emphasize that this pathogen causes serious economic losses due to increased mortality rates (up to 50%), decreasing the growth rates. The appearance of sick fish makes them unmarketable (Vendrell et al 2006). *L. garvieae* is a major fish pathogen of rainbow trout in Europe. To our knowledge, this is the first report of *L. garvieae* associated with trout diseases in Serbia. However, it is likely that the lactococcosis in rainbow trout will become more frequent in the future.

## CONCLUSION

This study confirmed that *L. garvieae* was the etiological agent of a hemorrhagic septicemia in farmed rainbow trout and that the lactococcosis of rainbow trout caused by *L. garvieae* emerged in Serbia. The occurrence could

be attributed to the significant increase in water temperature during summer months, since water temperature is described as the most important environmental factor in the development of the *L. garvieae* infections in trout. In addition, variations in water temperature can affect fish immune response against bacterial infection. Lactococcosis is a limiting problem for rainbow trout culture in many South European countries. After the first occurrence in Spain and Italy, the pathogen and the associated disease has spread rapidly throughout the South Europe, and further to the South-Eastern part of the continent, with disease outbreaks in Greece, Bulgaria and Turkey. In the affected countries, lactococcosis is a major threat to trout culture, especially during a warm period. The rapid spread of the pathogen is a result of the multiple routes of dissemination and transmission of this pathogen. This includes direct spread through the movement of infected fish or asymptomatic carriers and transmission via contaminated water (Savvidis et al., 2007). Since *L. garvieae* have the ability to adapt and survive in many environmental conditions including a wide range of pH, temperatures, salinity concentrations and nutrient sources (Kusuda et al. 1991), the occurrence of the disease in Serbia is a warning for the neighboring countries with trout aquaculture. It is evident that this agent spreads to the new geographic areas, causing the disease with high mortality in susceptible population. The source of outbreak is not known, but since the disease is present in the region and susceptible species are in a river basin which is shared between countries, under adequate conditions, further spread of the causative agent and concomitant disease is inevitable. Due to the ability of *L. garvieae* to colonize multiple, diverse environments, and because it causes infection in a broad range of different hosts, it is reasonable to expect further spread of the bacterium and the disease. Since vaccination is considered the best option to control lactococcosis in rainbow trout, we hope that an appropriate strategy to prevent this infection on Serbian trout farms will be available in the future.

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## Author's contributions:

VR, OR, NZ and MS made contributions to concept and design of the study, they collected data and drafted the manuscript. JMZ and BS carried out the molecular diagnostic tests and prepared the alignment of nucleotide

sequences and conducted the molecular genetic analysis. LJV carried out the data analysis. KN revised the manuscript critically and together with VR prepared the final draft of the manuscript. All the authors read and approved the final manuscript.

## Competing Interests

The authors declare that they have no competing interests.

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