

Brief Report

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Brief Report

Susceptibility and Clinicopathological Findings of three Amazonian Fishes Experimentally Infected with *Lactococcus* spp.

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Abstract

This study evaluated the susceptibility of three Amazonian fish species, *Arapaima gigas*, *Brycon amazonicus* and *Colossoma macropomum* to *Lactococcus* spp., through experimental infection, and performed a detailed examination of the pathological damage. While lactococcosis-causing bacteria have already been isolated from these fish species, their pathogenic role remains unconfirmed. *Lactococcus formosensis*, *L. garvieae* and *L. petauri* strains were used to intracoelomically infect juvenile Amazonian fish. Following infection, the fish were monitored for 15 days to evaluate clinical signs and mortality. Fish that died or survived until the end of the experiment underwent bacteriological and histopathological examinations. Clinical signs were observed in the *L. garvieae*-challenged *A. gigas*, with one fish dying at five days post-infection. The remaining experimental animals, regardless of fish species, did not die. Bacteriological examination confirmed re-isolation of asymptomatic animals in the *L. garvieae*-challenged *A. gigas* and *L. formosensis*-challenged *C. macropomum* groups. No bacterial growth or histological alterations were observed in the control groups nor in the groups infected with *L. petauri*. Microscopic examination revealed *L. garvieae*-induced fibrinoid-necrotic hepatitis, lymphohistiocytic myocarditis and myositis in *A. gigas*, while *L. formosensis* caused lymphohistiocytic pericarditis in *C. macropomum*. These findings demonstrate that *A. gigas* and *C. macropomum* are susceptible to *L. garvieae* and *L. formosensis*, respectively, and that they can become bacterial carriers. The distinct histopathological profiles—particularly myositis (*L. garvieae*) and pericarditis (*L. formosensis*)—highlight species-specific manifestations of piscine lactococcosis in Amazonian fish species.

Keywords: lactococcosis; experimental infection; native fish species; Brazil

1. Introduction

Piscine lactococcosis is an emerging bacterial disease that affects both wild and farmed fish species worldwide in freshwater and marine environments. It is caused by three *Lactococcus* species:

L. formosensis, *L. garvieae* and *L. petauri* [1–4]. The disease leads to high mortality rates, particularly in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*) farms, and results in significant economic losses for farmers [2,5]. In addition to fish, lactococcosis-causing bacteria (LCB) have been isolated from terrestrial animals, humans, and other aquatic organisms, such as prawns, octopuses, and dolphins, as well as various meat, dairy and cereal products and vegetables [6–10]. This broad distribution highlights their remarkable adaptability to diverse environmental conditions. Moreover, these pathogens have zoonotic potential, which is primarily linked to the handling and consumption of raw fish or seafood, with endocarditis being the most frequently reported clinical manifestation in infected humans [8]. Given their impact on both animal and human health, these pathogens are of major importance in the One Health framework.

Bacteriological examination of fish (both diseased and healthy) from Brazilian fish farms, including ornamental fish farms, has led to the isolation of LCB [2,11–13]. Among the species associated with piscine lactococcosis in Brazil, *L. petauri* has been the most frequently identified pathogen in disease outbreaks, particularly in *O. niloticus* farms in different regions of the country [2]. However, this bacterium, along with other LCBs, has also been detected in native Brazilian fish species [12]. The production of native fish species has significant economic and social importance, especially in northern Brazil, though it is also relevant nationwide. Species such as *Arapaima gigas*, *Brycon amazonicus* and *Colossoma macropomum* are crucial for the growth of Amazonian aquaculture, serving both domestic and international markets. These fish play a vital role in local food security and cultural practices, underscoring their broader societal value [14].

Previous studies in Brazil have evaluated the pathogenicity of LCBs in *O. niloticus* and *Pseudoplatystoma* spp. under controlled laboratory conditions following experimental infection, including histopathological assessments of affected organs [2,11,15]. However, due to the lack of comprehensive and detailed studies, there is still no conclusive evidence linking LCBs to disease etiology in Amazonian fish species. Consequently, the potential impact of *Lactococcus* spp. on the production chains of key species such as *A. gigas*, *B. amazonicus* and *C. macropomum* remains unknown.

Given this knowledge gap, this study aimed to evaluate the susceptibility of three Amazonian fishes (*A. gigas*, *B. amazonicus* and *C. macropomum*) to infection by *Lactococcus* spp. (*L. formosensis*, *L. garvieae* and *L. petauri*) using Koch's postulates and characterize the gross and histologic lesions induced by infection in experimentally challenged fish.

2. Material and Methods

2.1. Bacterial Strain Selection and Culture Conditions

A total of three *Lactococcus petauri* (AM-LG02, AM-LG07 and CRBP89) strains, one *L. formosensis* (AM-LG05) strain, and one *L. garvieae* (PA-LG01) strain were used in this study. The isolates were obtained from Amazonian fish species (*Arapaima gigas*, *Brycon amazonicus* and *Colossoma macropomum*), which were obtained from commercial farms in the states of Amazonas and Pará, Brazil, and were confirmed at the species level using *gyrB* sequencing, as previously reported [12].

Cryopreserved strains were cultured by streaking on tryptic soy agar (HiMedia, India) supplemented with 5% sheep blood and incubation at 28 °C for 48 hours. Bacterial colonies were collected from the plates, inoculated into brain heart infusion (BHI) broth (Kasvi, Brazil) and incubated at 28 °C under agitation (100 rpm) until they reached an optical density of 0.200–0.500 at OD₆₀₀. The bacterial pellet was collected and washed three times with phosphate-buffered saline (PBS). Then, 10-fold serial dilution was performed for each strain, and 100 µL of each dilution was plated on BHI agar plates and incubated at 28 °C for 48 hours, followed by colony counting to determine the bacterial concentration of the inoculum.

2.2. Fish and Experimental Infection

A. gigas (180.93 ± 36.32 g; *n* = 23), *B. amazonicus* (97.36 ± 29.49 g; *n* = 16) and *C. macropomum* (51.58 ± 16.81 g; *n* = 30) were acquired from the animal facility of the Federal University of Minas Gerais and Nilton Lins University, and kept under laboratory conditions for 15 days for acclimation before the experiment. The fish were housed in 310-L tanks or 57-L glass aquaria, with a 50% renewal with dechlorinated water every 24 hours. Water temperature was maintained at 28 °C, and aeration was provided using air stones to maintain adequate dissolved oxygen levels. The fish were fed twice daily with a commercial fish feed containing 42% crude protein (NutriPiscis, Brazil), at a rate of 2% of their body weight per day.

Before infection, 10% of the fish from each species were randomly sampled to confirm the absence of *Lactococcus* spp. For this purpose, the brain, kidney, spleen and liver were collected aseptically and streaked onto BHI and de Man, Rogosa and Sharp (MRS, Kasvi, Brazil) agar, followed by incubation at 28 °C for 48 hours. After the acclimation period, feeding was suspended for 24 hours before the experimental trials. Prior to handling, the fish were anesthetized by immersion in a 100-mg/L benzocaine solution (Sigma-Aldrich, USA).

The study was conducted in compliance with the guidelines of the Ethics Committee for Animal Use (CEUA) of Nilton Lins University (Protocol No. 02/2022) and the Federal University of Minas Gerais (Protocol No. 152/2020).

Nine experimental groups (challenge groups, *n* = 5; control groups, *n* = 4) were used in the infection assays (Table 1). Each experimental group consisted of 6-7 fish. For the intracoelomic challenge (G1-G5), fish were inoculated with 0.1 mL of bacterial inoculum containing 10⁶-10⁸ colony-forming units (CFU)/mL. The control groups (G6-G9) received 0.1 mL of PBS. Challenged fish were monitored three times daily for 15 days. At the end of the experiment, all surviving fish were euthanized via overdose with benzocaine (300 mg/L). Fish that died during the experiment or survived until the endpoint were subjected to bacterial re-isolation and histopathological analysis.

Table 1. Challenge dose, mortality and bacterial recovery in dead and surviving Amazonian fish species infected with lactococcosis-causing bacteria.

Group	Fish species	Environment ¹	Bacteria	Challenge dose (CFU/fish)	No. fish death/total fish	Bacterial recovery from dead fish	Bacterial recovery from surviving fish
G1	<i>Arapaima gigas</i>	Tank	<i>L. garvieae</i> (PA-LG01)	10 ⁵	1/7	1/1	2/6
G2	<i>Arapaima gigas</i>	Tank	<i>L. petauri</i> (CRBP89)	10 ⁷	0/7	0	0/7
G3	<i>Brycon amazonicus</i>	Tank	<i>L. petauri</i> (AM-LG07)	10 ⁵	0/7	0	0/7
G4*	<i>Colossoma macropomum</i>	Glass aquaria	<i>L. petauri</i> (AM-LG03)	10 ⁶	0/6	0	0/6
G5	<i>Colossoma macropomum</i>	Tank	<i>L. formosensis</i> (AM-LG05)	10 ⁵	0/7	0	3/7
G6	<i>Arapaima gigas</i>	Tank	-	PBS	0/7	0	0/7
G7	<i>Brycon amazonicus</i>	Tank	-	PBS	0/7	0	0/7
G8*	<i>Colossoma macropomum</i>	Glass aquaria	-	PBS	0/6	0	0/6
G9	<i>Colossoma macropomum</i>	Tank	-	PBS	0/7	0	0/7

* The experiment conducted with groups G4 and G8 was carried out at the Veterinary School, Federal University of Minas Gerais, while the other groups were conducted at Nilton Lins University, Amazonas. ¹ The fish were housed in 310 L tanks or 57 L glass aquaria.

2.3. Bacterial Analysis

For the bacteriological analysis, brain, kidney, liver and spleen samples were aseptically collected from the dead or surviving fish, streaked on BHI and MRS agar plates, and incubated at 28 °C for 48 hours. Bacterial isolates were identified to the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), following previously established protocols [16].

2.4. Histological Analysis

For the histological analysis, samples of brain, kidney, liver, spleen, heart, intestine and stomach, along with gills, eyes and skin were collected from both dead and euthanized fish. Tissues were fixed in 10% neutral buffered formalin for 24 hours and then preserved in 70% ethanol until processing. Gills, skin, muscles, bone and eye samples underwent decalcification in 24% formic acid for 24-72 hours, as needed.

All the tissues were processed using standard protocols in a vacuum infiltration processor (ASP300S, Leica Biosystems, Germany). Following embedding in paraffin, 6 µm sections were obtained with a manual rotary microtome (MRP 2015, Lupetec, Brazil). The sections were routinely stained with hematoxylin and eosin (H&E) according to conventional histological methods [17] or, when required, with Gram staining (Goodpasture method) to highlight bacterial organisms in tissues sections.

Histopathological evaluation was conducted using light microscopy (O500R, Opticam, Brazil), with digital image acquisition performed using OPTHD software (Opticam, Brazil).

3. Results

3.1. Mortality and Gross Pathological Observations

Hyporexia and lethargy were the main clinical signs observed in all the challenge groups, especially up to the fourth day post-infection (dpi). After that, feeding and swimming behavior returned to normal in all fish species. In the control groups (G6-G9), no clinical signs were observed.

In group G1, one *A. gigas* died on the fifth dpi, presenting corneal opacity and reddened areas in the ventral region and at the base of the caudal fin (Figure 1A) as clinical signs. Other infected fish in this group developed dark grey areas on the skin by the sixth dpi and exhibited abnormal swimming behavior (opposite direction to the shoal) by the eighth dpi. No other fish died during the experiment, regardless of group (challenge or control).

At the end of the experimental period, examinations were performed on the surviving fish. Only the G1 challenge group presented macroscopic pathological changes at necropsy, including flat light red areas and pinpoint yellow foci (Figure 1B) in the liver of the *A. gigas*.

Figure 1. Lesions observed during gross pathological examination of *Arapaima gigas* infected with *Lactococcus garvieae*. A) Hemorrhagic areas in the ventral region and at the base of the caudal fin (red arrow). B) Liver with yellow spots (white arrow).

3.2. Histopathology

No histological findings were observed in groups challenged with *L. petauri* (G2-G4) nor in the control groups. The following lesions were observed in the *A. gigas* infected with *L. garvieae* (G1): acute mild focally lymphohistiocytic myocarditis (1/7 fish); multifocal lymphohistiocytic and neutrophilic myositis with moderate muscle degeneration and necrosis (3/7) (Figure 2A); moderate hyperplasia and hypertrophy of renal tissue (1/7); and fibrinoid-necrotic hepatitis (1/7) (Figure 2B).

In the *Colossoma macropomum* infected with *L. formosensis* (G5), moderate acute multifocal lymphohistiocytic and fibrinous pericarditis (6/7) was observed, accompanied by multifocal thrombosis in pericardial vessels, marked edema (Figure 2C) and the presence of aggregates of Gram-positive cocci bacteria (Figure 2D). For the remaining tissues evaluated in the fish from groups G1 and G5, no histopathological alterations were observed.

Figure 2. Histopathological lesions in organs of *Arapaima gigas* (A, B) and *Colossoma macropomum* (C, D) experimentally infected with *Lactococcus garvieae* and *Lactococcus formosensis*, respectively. A) Skeletal muscle showing multifocal areas of marked lymphohistiocytic inflammatory infiltrate (white arrow) and muscle necrosis (black arrow). Hematoxylin and eosin (H&E), 400×. B) Liver displaying extensive focal lytic necrosis (arrow) with lymphohistiocytic inflammatory infiltrate and fibrin deposition. H&E, 400×. C) Pericardium with marked thickening due to lymphohistiocytic inflammatory infiltrate (arrow). H&E, 400×. D) Pericardium containing lymphohistiocytic inflammatory infiltrate with numerous intralesional Gram-positive coccoid bacteria (arrows). Goodpasture stain (Gram histology), 1000×.

3.3. Bacteriology

Positive bacterial recovery was confirmed in 3 out of 7 of the *Arapaima gigas* challenged with *L. garvieae* (one dead and two surviving fish) and in 3 out of 7 of the *Colossoma macropomum* infected with *L. formosensis* (all survivors). No bacterial growth was observed in groups challenged with *L. petauri* (G2, G3 and G4) nor in the control groups.

4. Discussion

The present study investigated the pathogenicity and pathological findings in the organs of three Amazonian fish species challenged with *Lactococcus* spp. Lactococcosis-causing bacteria produce the same clinical signs in fish [1]. In our experimental infection assay, only G1 (*A. gigas*) infected with *L. garvieae* (PA-LG01 strain) exhibited clinical manifestations of the disease, with clinical signs consistent with piscine lactococcosis observed in natural outbreaks and experimental infections in other fish species [2,11,15,18–20].

In an outbreak of bacterial disease in farmed *A. gigas* in the state of Pará, Brazil—the origin of the strain used in our experimental infection—the fish displayed exophthalmia, corneal opacity, cachexia, swimming abnormalities and cutaneous hyperemia [21]. Thus, the experimental assay conducted here fulfilled Koch's postulates, since the same clinical signs were observed, mortality occurred, and the pathogen was re-isolated. In addition to PA-LG01, the *L. petauri* AM-LG07 strain was also isolated from a mortality event in *B. amazonicus* in the state of Amazonas, Brazil [12]. However, the experimentally infected animals did not exhibit clinical signs nor mortality. The same was observed for the other tested strains, which were obtained from studies that aimed to isolate potential probiotic candidates from intestinal tissue.

In Amazonian fish species, the significance of *Lactococcus* spp. as a pathogenic organism remains unclear, as *L. garvieae* appears to be part of the microbiota in some fish, such as *A. gigas* [22]—a finding consistent with our previous isolation in this species, as well as in *C. macropomum* [12]. Since LCBs are considered pathogens in aquatic and terrestrial animals, as well as humans, they are excluded from selection criteria for potential probiotics in fish applications [23]. Thus, it is necessary, through experimental challenge assays, to evaluate whether these bacteria act as direct or opportunistic pathogens in their host species. Among the strains isolated from the intestinal tract, only AM-LG05 induced histological damage in challenged individuals.

Although the mortality rate in the experimental groups was low or nonexistent, some fish became carriers of *L. garvieae* (G1) and *L. formosensis* (G5), as evidenced by the re-isolation of these pathogens from asymptomatic fish after the experimental period. The mechanisms underlying the natural resistance of Amazonian fish species to bacterial pathogens remain unclear, and it is uncertain whether this contributed to low or no mortality in the challenged animals. Previous studies involving another Brazilian native species, *Pseudoplatystoma* spp., inoculated with two different strains—BR-

LG3 (*L. petauri*) and 31MS (*L. garvieae*)—reported mortality rates of 66% and 0%, respectively. Bacterial re-isolation was also achieved from asymptomatic animals in both studies [11,15]. In contrast, exotic fish species exhibited higher susceptibility: *L. petauri* isolates caused mortality rates of approximately 80% and 90% in *O. niloticus* and *O. mykiss*, respectively. While no carrier fish were detected in *O. niloticus*, bacterial persistence was observed in 50-67% of the surviving *O. mykiss* [2,20]. In our study, no bacterial re-isolation occurred in any fish species infected with *L. petauri*, aligning with findings from the studies on *O. niloticus*.

No significant histological findings were observed in the tissues of fish experimentally infected with *L. petauri*, regardless of the host species. This finding suggests a lower pathogenicity of this bacterium in Amazonian fish, which is consistent with the absence of clinical signs or mortality in our study. These results contrast with a study of *L. petauri* infection in *O. niloticus*, in which significant pathological alterations were noted in hepatic, renal, cardiac, splenic and intestinal tissues, demonstrating the systemic nature of infection in this species [24]. The histopathological lesions observed in the Amazonian fish infected with *L. garvieae* and *L. formosensis* align with previous reports of *Lactococcus* spp. infections in other fish species regarding affected tissues. Similar findings were noted in renal, cardiac and hepatic tissues in studies on *Pseudoplatystoma* spp. [15], *Rachycentron canadum* [4], *Trachinotus blochii* [19] and *Onchorhynchus mykiss* [20]. However, these hosts exhibited splenic lesions (edema, multifocal hemorrhage, necrosis and lymphoid depletion) [4,15] and cerebral lesions (meningitis and congestion) [4,19] that were absent in our study. Notably, no previous studies have identified myositis as a histopathological feature of piscine lactococcosis, making our study the first to report this finding.

Our results demonstrate the tissue-specific distribution of inflammation and necrosis caused by these bacteria, indicating tissue tropism of the tested isolates. The hepatic necrosis and myocarditis in *Arapaima gigas* suggest that *L. garvieae* can induce significant systemic inflammatory response, likely related to sepsis, under experimental conditions. Similarly, the pericarditis and severe edema observed in *C. macropomum* highlight this species' particular susceptibility to *L. formosensis*, which may cause severe functional impairment, even without high mortality rates.

Our results suggest that clinical manifestation, mortality and histopathological changes depend on the bacterial isolate used in the experimental challenges, with potential influences from strain-specific genetic profiles, and host susceptibility. Further studies are needed to confirm these hypotheses. Additionally, research on the carrier state in Amazonian fish species is essential, as it remains unknown whether these animals can shed pathogens into the aquatic environment. Such transmission could pose a risk to more susceptible species, such as *O. niloticus*, when cohabiting with Amazonian or other native fish, potentially increasing the likelihood of outbreaks in intensive farming systems. These findings underscore the necessity for ongoing surveillance, including expanded bacteriological screening of native fish species, along with preventive measures to mitigate the impacts of bacterial infections in aquaculture production. Furthermore, comprehensive studies are needed to better characterize *Lactococcus*-Amazonian fish interactions through the identification of transmission pathways, analysis of tissue tropism, quantification of bacterial loads across different tissues, identification of predisposing factors that may trigger disease, and susceptibility to *O. niloticus*-derived *L. petauri* strains. Such research will provide the foundation for developing effective disease control and management strategies in aquaculture systems.

In conclusion, *A. gigas* and *C. macropomum* are susceptible to infection by *L. garvieae* and *L. formosensis*, respectively, establishing them as potential carriers of these pathogens. Moreover, these bacteria elicit distinct histopathological alterations, with myositis and pericarditis representing the predominant lesion patterns in *L. garvieae*- and *L. formosensis*-infected fish, respectively.

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Costa: Methodology, Writing – review & editing; **Cynthia Rafaela Monteiro da Silva Maia:** Methodology, Investigation, Writing – review & editing; **Suzana Kotzent:** Methodology, Writing – review & editing; **Sóstenes Apolo Correia Marcelino:** Investigation, Methodology, Writing – review & editing; **Felipe Pierezan:** Conceptualization, Supervision, Resources, Writing – review & editing; **Gustavo Moraes Ramos Valladão:** Conceptualization, Supervision, Resources, Writing – review & editing; **Ronald Kennedy Luz:** Funding acquisition, Resources, Writing – review & editing; **Henrique César Pereira Figueiredo:** Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Resources, writing – review & editing; **Silvia Umeda Gallani:** Conceptualization, Methodology, Supervision, Resources, writing – review & editing; **Guilherme Campos Tavares:** Conceptualization, Investigation, Methodology, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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