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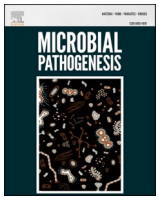

Susceptibility of the cultured Amazonian fish, *Colossoma macropomum*, to experimental infection with *Aeromonas* species from ornamental fish

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ABSTRACT

The global ornamental fish trade carries important risk factors for spreading pathogens between different countries and regions, not only for ornamental fish but also for cultured fish and even other animal species. In the current study, we reported the capacity of *Aeromonas veronii* and *A. hydrophila* isolated from ornamental fish to experimentally infect the reared Amazonian fish *Colossoma macropomum*. For this, those bacteria were identified, and a primary characterization was performed. Fish were inoculated with 0.1 mL of increasing concentrations of *A. hydrophila* or *A. veronii* (C1 = 1×10^2; C2 = 1.8×10^4; C3 = 2.1×10^6; C4 = 2.4×10^8 bacterial cells per mL) in the coelomic cavity. In the control group, fish received the same volume of sterile saline solution (0.9 %). Fish presented petechiae, skin suffusions, and mortality rates up to 100 % according to the inoculum concentration. Histopathologically, fish presented necrosis with karyolysis, loss of the cytoplasmic delimitation of cells of the renal tubules and hepatocytes, hemorrhage, cellular edema, and the presence of bacterial cells. The LD_{50-96h} of *A. veronii* on *C. macropomum* was estimated at 2.4×10^6 CFU mL^{-1} and of *A. hydrophila* at 1.408×10^5 CFU mL^{-1}. The results demonstrated that it is possible that *Aeromonas* species isolated from ornamental fish affect *C. macropomum,* causing similar clinical signs and lesions. This shows the importance of promoting risk control measures worldwide regarding the trade of ornamental fish.

1. Introduction

The market for ornamental fish is worth $6 billion worldwide. Annually, millions of fish travel thousands of kilometers across the globe in a matter of days, typically without being subject to many limitations from governmental agencies for fish health [[1–3]]. This is an important risk factor for spreading pathogens, not only for ornamental fish; these pathogens might affect cultured fish and even other animal species [4–6].

Several studies have demonstrated that bacteria from the *Aeromonas* complex can negatively affect a variety of fish species [[7–10]]. There is little information available regarding the species implicated and the susceptibility of Amazonian fish, despite the fact that *Aeromonas* species are among the most prevalent bacterial infections in tropical fish and are likely the principal bacterial agent found in those species. These bacteria cause important economic losses to producers [6].

Colossoma macropomum (gamitana) is the main cultured native fish species consumed in the Amazon of Peru and Brazil [11]. Government measures in both nations encourage the development of this species in aquaculture. However, the lack of a complete technology package for the production of gamitanas and the outbreaks of *Aeromonas* sp hinder the sustained growth of the production of this species. Considering the

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importance of this resource in the Amazon, herein we show the capacity of *Aeromonas veronii* and *A. hydrophila* isolated from ornamental fish outbreaks to experimentally infect the reared Amazonian fish *C. macropomum*. These bacteria are reported for the first time in Peru in this species.

2. Material and methods

2.1. Ornamental fish and farm conditions

An outbreak at two ornamental fish farms was reported by the farmers. Five specimens of *Astyanax* sp. (6 cm) and *Trachelyopterus galeatus* (4 cm) were analyzed. These samples were collected during the wet season of 2021 in the Amazon of Peru (Iquitos). In both cases, the fish presented hemorrhages and ulcers in the skin (Fig. 1A and B). There were also some dead fish found in the tanks where the samples were taken. Farmers reported that mortality reached up to 60 % in both cases. Temperature, dissolved oxygen, and pH of water from fish farms were recorded using a multiparameter probe (SevenGo Duo pro SG98, Mettler Toledo, Switzerland). The ethical protocol for this study was approved by the Department of Animal Ethics and Welfare under protocol number 097-S/2022 in accordance with guidelines for the care and use of laboratory animals under Peruvian law for aquaculture species.

2.2. Isolation of bacteria

Samples of spleen, kidney, and liver from diseased fish were taken, homogenized, and streaked on Trypticase soybean agar (TSA, Merck, Germany), Tryptone Yeast Extract Salts (TYES, Merck, Germany), and *Pseudomonas-Aeromonas* Selective Agar Base (GSP, Merck, Germany). Plates were incubated at 28 °C for 48 h. Bacterial colonies were selected according to their morphological characteristics and stored in 20 % TSB-glycerol at −80 °C. A clone of each bacterium isolated was stored for further phenotypic and molecular analysis.

2.3. Phenotypic and biochemical characterization

Gram reaction (Gram Color, Merck, Germany), catalase activity (Catalase reagent Liofilchem, Italy), oxidase activity (Oxidase reactive strips, Merck, Germany), hemolysis (TSB + 5 % blood), VP Voges Proskauer test, motility, sugar utilization, H_2S production, citrate utilization, esculin hydrolysis, salt, and temperature tolerance were analyzed. Commercial kits API 20E (BioMerieux, Marcy l'Etoile, France) were used to determine the biochemical profiles of isolated strains.

2.4. Molecular identification

The two isolates from diseased fish (strains 14 and 17) were evaluated using 16S rRNA and gyrase subunit B (*gyrB*) genes, for species identification. The genomic DNA from each isolate was extracted as described by Wilson [12]. PCR was performed in a Proflex PCR System (Applied Biosystems) thermal cycler, using the kit HotStartTaq Plus Master Mix (QIAGEN), with 2 ng of DNA template, and 0.1 μM of each primer, as final concentrations, in a total volume of 10 μL. The 16S rRNA gene was amplified using the universal primers 27F and 1492R [13], considering an initial denaturation of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30s and extension at 72 °C for 1.5 min with a final extension at 72 °C for 10 min. The *gyrB* gene was amplified with the primers gyrB3F and gyrB14R described in Yañez et al. [14], with an initial denaturation of 95 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplified products were sequenced for both strands, on an ABI 3500 Genetic Analyzer (Applied Biosystems), using the same primers as in the previous PCR. The obtained sequences were compared using the BLASTn tool from NCBI. The nucleotide sequences of both genes were aligned independently and analyzed, including the sequences registered in GenBank. Kimura-2 parameter (K2P) pairwise distance was calculated, and phylogenetic trees were constructed using Neighbor-Joining (NJ) method, considering 1000 replicates, in the MEGA-X program [15]. *Pseudomonas aeruginosa* (CP072783) was included as an out-group.

2.5. Experimental infection in Colossoma macropomum and LD_{50-96h}

Experimental infections were performed considering the two isolates from diseased fish (*A. veronii* and *A. hydrophila*). Strains were seeded into TSB and incubated at 28 °C overnight. Afterward, the bacterial suspension was centrifuged (3000 g for 10 min at 4 °C), washed three times, and resuspended in sterile phosphate buffered saline (PBS).

Prior to infection, fish were anesthetized in 90 mg/L of ethyl 3-aminobenzoate methanesulfonate salt (MS-222, Sigma Aldrich). Three hundred and sixty healthy Gamitanas (*C. macropomum*) (~9 cm) were stocked in 90L tanks at 28 ± 0.4 °C (n = 10) in a completely randomized design. Fish were inoculated with 0.1 mL of increasing concentrations of *A. hydrophila* or *A. veronii* ($C1 = 1 \times 10^2$; $C2 = 1.8 \times 10^4$; $C3 = 2.1 \times 10^6$; $C4 = 2.4 \times 10^8$ bacterial cells per mL approximately) in the coelomic cavity.

Fish injected with 0.1 ml of PBS were considered the control group. An experimental infection was performed with four replicates (n = 10) for 10 days. Results were presented as an average of the data from each replicate. Moribund fish were used for bacteriological and molecular analysis. At the end of the experiments, surviving fish were euthanized by MS-222 overdose [16] and the same analyses were performed.

2.6. Histopathology

Samples of the brain, gills, heart, liver, kidney, and spleen were fixed in 10 % neutral buffered formalin for histopathology analysis. Tissues were processed routinely, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E) [17].

2.7. Statistical analysis

The LD_{50-96h} result was estimated using Trimmed Spearman-Karber [18]. The survival rate followed the Kaplan-Meier method, correlation, and linear regression. The experimental statistical program used for the calculations was R software, version 3.4 (GNU GPL v2) [19].

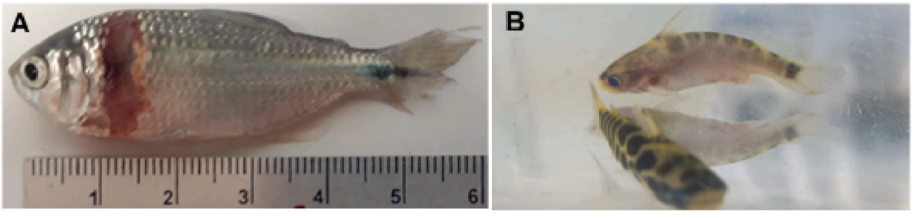

Fig. 1. A. *Astyanax* sp with hemorrhages and skin ulcers. B. *Trachelyopterus galeatus* with hemorrhages, erratic swimming, and abdominal distension.

3. Results

Both bacterial infections presented the same morphological characteristics. Colonies presented a regular border, a smooth, shiny appearance, a convex surface, a whitish cream color, and mucous (Fig. 2). Microscopically, both isolates presented the typical morphology of *Aeromonas* sp, short Gram-negative rods. Biochemical analysis showed some differences between both isolates, mainly in citrate utilization, esculin hydrolysis, salt tolerance, and API20E profile number (Table 1).

Gene sequences of strains 14 and 17 were registered in GenBank with accession numbers OP801421 and OP801422 for 16S rRNA (1300 bp), OP795658 and OP795659 for gyrB (830 bp), respectively. Strain 14 was similar to *A. veronii* (99.92 % with the 16S gene, and 100 % with the gyrB sequence gene); while strain 17 showed 100 % identity with several *Aeromonas* species (100 % identity) compared with the 16S gene, and to *A. hydrophila* with gyrB gene.

The phylogenetic analysis derived from the 16S gene showed that strain 14 was closely related to *A. veronii* and *A. veronii* bv. *veronii* (0.002), but also clustered with 10 other species (group a) such as *A. jandaei* (0.006), *A. lacus* (0.007), *A. diversa* (0.009), *A. finlandiensis* (0.01) and *A. schubertii* (0.011). The strain 17 was related to *A. hydrophila* (0.002–0.012) and grouped with *A. caviae* (0.011), *A. rivipollensis* (0.006), *A. dhakensis* (0.012), *A. taiwanensis* (0.014), *A. enteropelogenes*, *A. sanarellii* (0.012), *A. rivuli* (0.013) and *A. sobria* (0.014). K2P pairwise distance between strains 14 and 17 was 0.0017, while the distance between sequences obtained in this study and *P. aurigonosa* was >0.15.

On the other hand, NJ analysis based on the gyrB gene showed a clear discrimination between strains 14 and 17 in different clades related to *A. veronii* and *A. hydrophila*, respectively. The genetic distance between the two strains was 0.045. The strain 14 clustered with *A. veronii*, *A. veronii* bv. *veronii* and *Aeromonas* sp (CP077209) showing a genetic distance between 0 and 0.021, while strain 17 grouped with sequences from *A. hydrophila* with a K2P distance between 0 and 0.023. All *Aeromonas* species discriminated from *P. aeruginosa* with distance >0.28 (Fig. 3).

Similar histopathological lesions were observed in both ornamental fish and in *Colossoma macropomum*. Mainly, necrosis with karyolysis, loss of the cytoplasmic delimitation of cells of the renal tubules and hepatocytes, hemorrhage, cellular edema, and the presence of bacterial cells in the spleen, liver, and kidney with leukocyte infiltrates (Figs. 4–6).

Fig. 2. Colonies of *Aeromonas veronii* isolated from *Trachelyopterus galeatus*.

Table 1
Biochemical characterization of isolate 14 (*Trachelyopterus galeatus*), isolate 17 (*Astyanax* sp) and ATCC 35654.

Strain	ATCC 35654	14	17
GRAM	Short rods -	Short rods -	Short rods -
CATALASE	+	+	+
OXIDASE	+	+	+
HEMOLYSIS (BLOOD AGAR)	+ β	+ β	+ β
VP VOGES PROSKAUER TEST	+	+	+
SIM (INDOLE)	+	+	+
MOTILITY	+	+	+
H_2S PRODUCTION	–	–	–
CITRATE UTILIZATION	–	+	–
ESCULIN HYDROLYSIS	+	–	+
SODIUM CHLORIDE 1 %	+	+	+
SODIUM CHLORIDE 2 %	+	–	+
SODIUM CHLORIDE 3 %	+	–	+
SODIUM CHLORIDE 4 %	+	–	+
SODIUM CHLORIDE 5 %	–	–	–
GROW 24 °C	+	+	+
GROW 28 °C	+	+	+
GROW 37 °C	+	+	+
GROW 42 °C	+	+	+
API 20E CODE	–	7246124	7046127

API20E were performed at 28 °C. Isolate 14 showed 89.3 % of similarity with *Aeromonas hydrophila/caviae/sobria*. Isolate 17 showed 98.4 % of similarity with *Aeromonas hydrophila/caviae/sobria*.

Survival percentage in *Colossoma macropomum* presented some similarities between isolates in most bacterial concentrations; in both cases, fish mortality was observed since concentration 10^2 (Figs. 6–8). In concentration 10^6, it was observed that in the group infected with *A. hydrophila*, the survival rate was only 20 %, and in the group infected with *A. veronii,* more than 70 %.

The $LD_{50–96h}$ of *Aeromonas veronii* on *Colossoma macropomum* was estimated in 24×10^6 CFU mL^{-1} and with its inferior and higher limits at 0.7965×10^6 and 73.38×10^6 CFU mL^{-1}. *Aeromonas hydrophila* was estimated in 1.408×10^5 CFU mL^{-1} and with its inferior and higher limits at 0.4905×10^5 and 4.041×10^5 CFU mL^{-1}.

4. Discussion

The traits of the global ornamental fish trade make this industry a significant risk factor for the development of fish illnesses. A fish that leaves the Amazon today would arrive in a couple of hours in Lima, Tampa in the same day, Frankfurt or Seoul the following day. These cities are all important hubs for the ornamental fish trade. The majority of the time, no exploratory study or disease control is carried out to ascertain the animals' health status. Some of these fish might be harboring an illness, and spreading it to other fish, even without apparent clinical signs. Whittington et al. [20] highlighted the poorly defined epidemiological and pathogenetic data for the majority of bacteria and parasites in ornamental fish and why it is crucial to improve the current political trade worldwide. Some studies have identified the dangers of this situation. According to Go et al. [21], the ornamental fish trade raises the risk of an ornamental fish illness entering a nation and spreading into aquatic ecosystems. The primary risk factors are transboundary biosecurity and biodiversity of fish and pathogens in both scenarios.

In the current study, we report the presence of two pathogenic bacteria (*Aeromonas hydrophila* and *A. veronii*) that were isolated from ornamental fish (*Astyanax* sp. and *Trachelyopterus galeatus*) and are capable of infecting the tropical fish *Colossoma macropomum*, the primary native fish species cultivated in the Amazon. Both bacteria are representative of the *Aeromonas* complex and have been reported in several fish species [[22,23]] and even mammals [[24,25]]. Ghatak

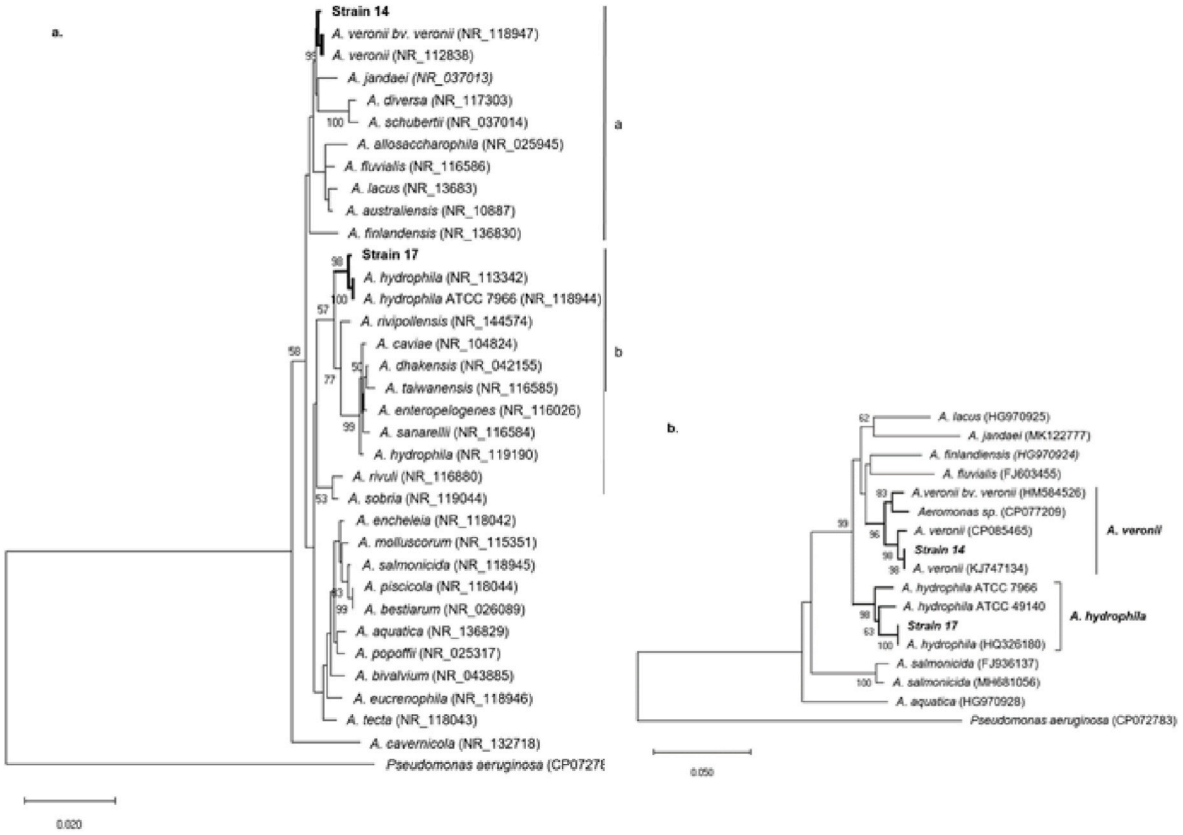

Fig. 3. Neighbor-Joining phylogenetic trees derived from 16S rRNA (a) and gyrB (b) sequence genes, showing relationship between strain 14 and 17 (obtained in this study) and others from genus *Aeromonas* (98 % identity). Numbers at branches indicate bootstrap values.

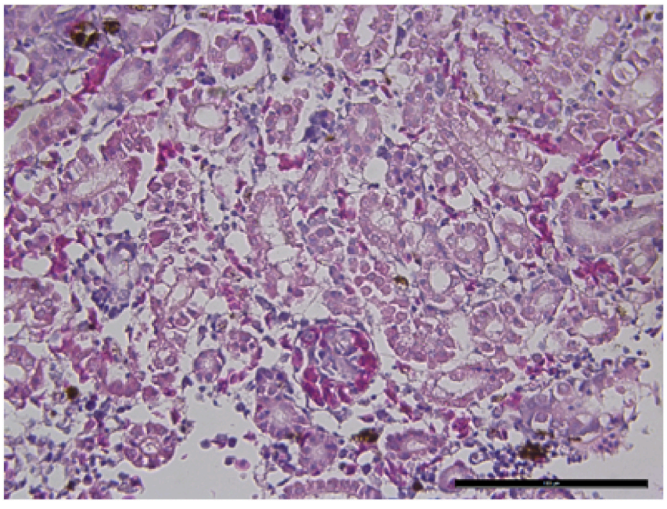

Fig. 4. Histopathological findings in *Colossoma macropomum*. Necrosis of the kidney and hydropic degeneration. Hematoxylin and Eosin stain.

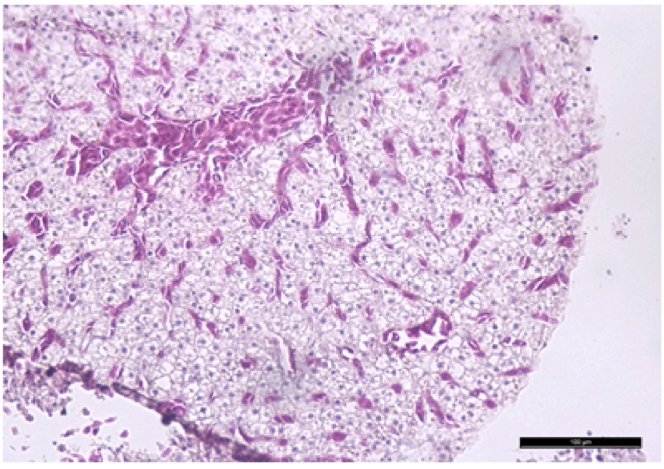

Fig. 5. Histopathological findings in *Colossoma macropomum*. Hemorrhage and necrosis in the liver. Hematoxylin and Eosin stain.

et al. [26] even suggested after a phylogenomic network analysis, the possibility of a homologous recombination and lateral gene transfer between these bacterial species, which would improve their abilities to infect.

Despite the two different bacteria being isolated in this study, all three of these species displayed similar clinical signs and lesions, suggesting that most *Aeromonas* spp can cause the same clinical picture regardless of the fish species impacted, primarily hemorrhages and, skin ulcers, as were seen in earlier studies [[7,27,28]]. This is the first report of a bacterial pathogen in *Trachelyopterus galeatus*.

Histopathologically, the lesions observed in the current study corresponded to the clinical picture, mainly hemorrhage, congestion, and necrosis, primarily in the spleen, liver, and kidney. These lesions were also reported in *Piaractus mesopotamicus* by Marinho-Neto et al. [29] and in Nile tilapia by El Latif et al. [30]). The majority of these clinical signs and lesions are linked to the virulence components found in the species *Aeromonas*, particularly aerolysins and hemolysins [[31–33]]. However, the heterogeneity seen in this group is not just restricted to virulence factors; this genus is known for its variance in biochemical profiles [34]. The bacterium's capacity to endure various conditions and hostile settings may be related to the variations seen in citrate utilization, esculin

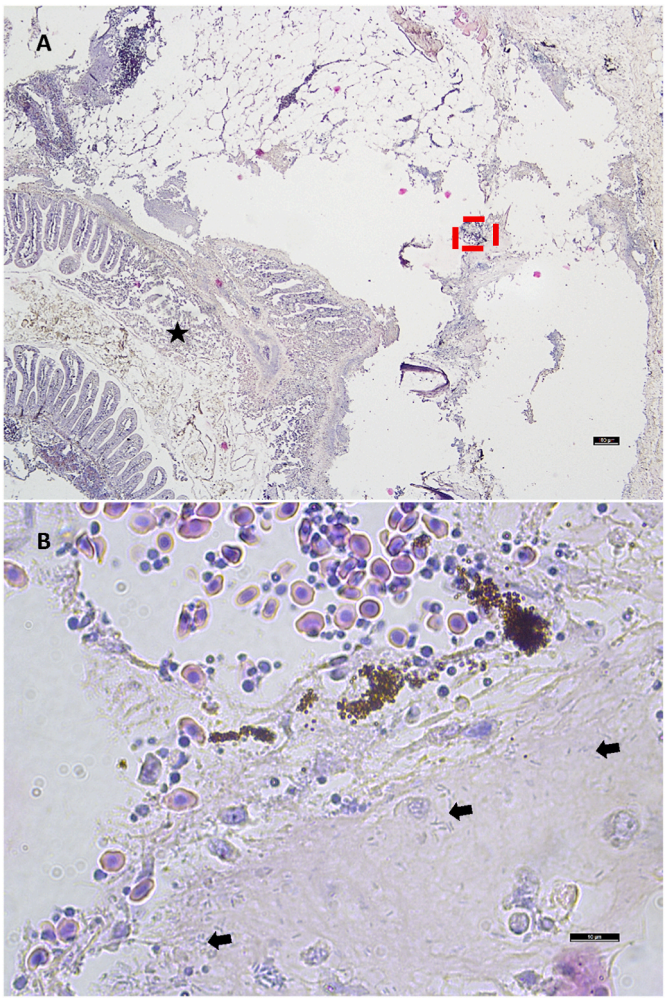

Fig. 6. A). Panoramic view of the intestine showing detachment of enterocytes from the lamina propria followed by necrosis (star). Cellular remnants of the peritoneal layer accompanied by lymphocytes and red blood cells (discontinuous lines amplified in B) are observed in the peritoneal cavity. Inoculated bacterial cells invade the tissue (arrow). Hematoxylin and Eosin stain.

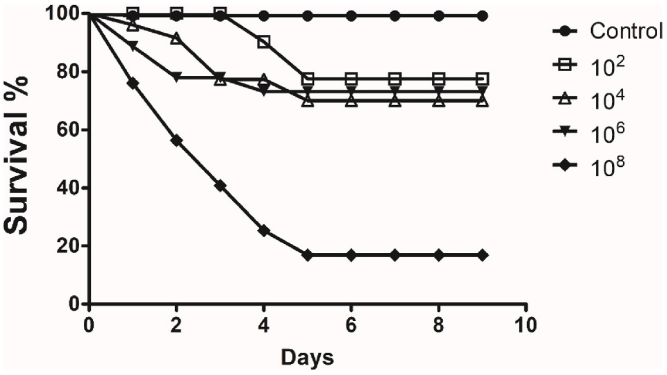

Fig. 7. Survival of *Colossoma macropomum* experimentally infected with *Aeromonas hydrophila* isolated from *Astyanax* sp (isolate 17) and the control group.

hydrolysis tests, and salt tolerance. This explains why salt treatment for bacterial diseases in Amazonian fish occasionally fails (commonly reported by farmers of Amazonian fish in Loreto).

There has been a prior report of the presence of *Aeromonas veronii* in gamitanas [35], but this is the first time that the species' infective potential has been confirmed. Sequencing analysis confirms the identity of

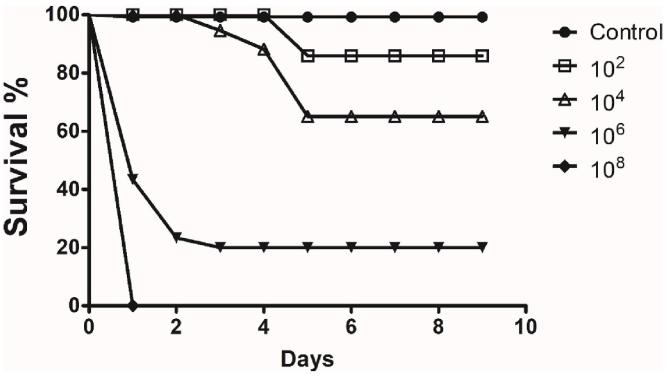

Fig. 8. Survival of *Colossoma macropomum* experimentally infected with *Aeromonas veronii* isolated from *Trachelyopterus galeatus* (isolate 14) and the control group.

the isolates. On the other side, it is well-known that this fish species is affected by the bacteria *Aeromonas hydrophila* [[36–39]]. But it is now established for the first time that an *A. hydrophila* isolated from an *Astyanax* sp, infected *C. macropomum*. It is significant to note that *Astyanax* sp. is frequently found in aquaculture facilities because it is widely used as snack, bait and food for carnivorous fish in Brazil and Peru [[7,11]].

The mortality and LD_{50} observed in the experimental infection revealed how these bacteria have a high potential to infect *C. macropomum* and cause elevated mortality. Similar levels of fish mortality have been caused by other *Aeromonas* species in tropical fish. Infected tilapia with 8.9 x 10^5 CFU/mL of *A. veronii* caused 50 % of mortality in a couple of days [40], and Crucian carp with 13.1 x 10^6 CFU/mL of *A. veronii* caused similar mortality [41]. However, other researchers found higher LD50 values for *Aeromonas* species in *Brycon amazonicus* [42], *Piaractus mesopotamicus* [8], and *Arapaima gigas* [43], indicating that bacterial strain and species susceptibilities vary.

5. Conclusion

Aeromonas veronii and *A. hydrophila* can have a negative impact on native farmed fish like *C. macropomum* and cause high mortality, as demonstrated by experimental challenges and field observations. Ornamental fish can act as reservoirs for these bacteria, posing a significant risk for the spread of fish diseases due to the variety of ornamental fish and pathogens, their proximity to farmed fish, and the global transportation of these species. The absence of regulation by governmental animal health organizations must be added to this. The danger of disease transmission will persist if the risk management strategies are not widely supported, and it is probable that it will continue to have an economic impact on producers of ornamental fish as well as aquaculture workers and even the environment.

CRediT authorship contribution statement

Jefferson Yunis-Aguinaga: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Giovanna Sotil:** Writing – review & editing, Methodology, Formal analysis. **German Augusto Murrieta Morey:** Project administration, Methodology, Investigation, Conceptualization. **Carla Fernandez-Espinel:** Writing – original draft, Validation, Formal analysis, Data curation. **Violeta Flores-Dominick:** Resources, Project administration, Formal analysis. **Gino Rengifo-Marin:** Methodology, Formal analysis, Data curation. **Gustavo da Silva Claudiano:** Methodology, Investigation, Funding acquisition. **Marco Medina-Morillo:** Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare as no conflict of interests.

Data availability

Data will be made available on request.

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