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THIAGO DUARTE MIELKE

The strategic use of water additives for tambaqui *Colossoma macropomum* transport: New insights of bacteriosis and productivity approach

COLORADO DO OESTE
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Artigo Científico apresentado ao curso Bacharelado em Zootecnia do Instituto Federal de Educação, Ciência e Tecnologia de Rondônia (IFRO) - *Campus* Colorado do Oeste, como requisito parcial para obtenção do Título de Bacharel em Zootecnia.

Orientador: Prof. Sílvia Umeda Gallani.
Presidente da banca avaliadora:
Prof. Thainara Camila Fernandes de Quadros

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The strategic use of water additives for tambaqui *Colossoma macropomum* transport: New insights of bacteriosis and productivity approach

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ABSTRACT

The transport of live fish is a major sanitary issue for aquaculture. This study addresses two critical steps regarding natural recurrent mortality outbreaks after transport of tambaqui *Colossoma macropomum*. At first, five post-transport mortality outbreaks of juveniles showing clinical signs suggestive of columnaris were investigated. *Flavobacterium columnare* was ascertained as the etiological agent in all outbreaks, causing massive mortality up to 72 h after transport. Based on these findings, the next step of consisted of a trial to investigate the use of three additives (8 g/L sodium chloride, 11.12 mg/L *Melaleuca alternifolia* essential oil and 60 mg/L oxytetracycline) in the water of transport, simulating fish farmers' practices to prevent disease outbreak. Juveniles (ÿ 8 cm) were transported for 3 h under trade-mimicking conditions, then transferred to 20 tanks (5 groups, 4 replicates) and monitored for 30 days for analysis of health status. Zootechnical parameters (weight gain, total feed intake, feed conversion, specific growth rate and growth analysis) were evaluated on days 1, 15 and 30. The best health status was attributed to the group transported with 0.8% NaCl, which showed 100% survival , in addition to being the only group that did not show behavioral changes and clinical signs, indicating the absence of any infectious biological source after transport. A poly-infection *(Aeromonas jandaei* and *F. columnare)* was detected in fish from all other groups *(M. alternifolia,* oxytetracycline and control). Furthermore, fish exposed to sodium chloride showed better weight gain and feed conversion compared to those transported with oxytetracycline *(p* ÿ 0.05). Then, the pathogenicity of the two isolated strains *(A. jandaei* and *F. columnare)* was ascertained by Koch's Postulate assay, which established two emerging pathogens for *C. macropomum.* This study addresses fundamental information for the development of fish farming and suggests the addition of salt at 0.8% in the water of transport to prevent columnaris and motile *Aeromonas* septicemia (MAS) associated disease.

1. Introduction

In fish farming, consistent management practices are important indicators of a successful industry. The intensive procedures must be carefully monitored and performed in a controlled environment, to not compromise the health and survival of fish (Martos-Sitcha et al., 2020), due to subsequent disease outbreaks (Baldissera et al., 2020a). Transport and intensive handling are the most common causes of stress in juvenile fish; therefore, the conditions imposed on organisms at this critical stage of production raise great concerns (Sampaio and Freire, 2016). In an attempt to improve welfare and reduce mortalities, transport practices should be the target for industry-academic efforts.

Tambaqui *Colossoma macropomum* is a Neotropical fish of major economic importance in artisanal fisheries and aquaculture. Recently, this fish has reached the milestone of the most produced Amazonian native species in continental aquaculture of South America (Vallad˜ao et al., 2018; FAO, 2020); due to its unique features, tambaqui was also introduced in regions outside its native range and, currently, the production of tambaqui has spread to most countries in South and Central America, some Caribbean countries, and various countries in Asia: particularly China, Indonesia , Malaysia, Myanmar and Vietnam (Woyn´arovich and Van Anrooy, 2019). Since aquaculture production is much higher than inland capture fisheries, the development of sanitary protocols is necessary. Recent investigations carried out by Barbas et al. (2020), Costa et al. (2020), Santos et al. (2020) and Silva et al. (2020) have been highlighting the importance of using additives in the

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water of transport, as a strategic measure to improve production rates. Most of the time, fish farmers transport a thousand tambaqui juveniles in 60 L plastic bags with the addition of pure oxygen. However, during the process, there is a common lack of standardization in the density and period for transport, as well as a limited control of water quality and the health status of the batch.

Among the pathogens associated with fish handling and transport, *Flavobacterium columnare,* which is the etiological agent of columnaris, stands out as the bacterium that causes one of the greatest impacts on fish productivity (Lange et al., 2017), causing mortalities of up to 100% within 24 h (Pradhan et al., 2019). It is suggested that larvae and ju veniles are highly susceptible to columnaris (Seghouani et al., 2017) and other opportunistic pathogens, like *Aeromonas* spp. (Hayakijkosol et al., 2017; Shirajum Monir et al., 2020), which overlaps the period when handling and transportation conditions are intensively imposed, from larviculture to fattening farms (Chockmangmeepisan et al., 2020).

Considering the negative effects of stress on transported fish, the search for practical-effective additives in the water of transport is one of the best choices for fish farmers to combine feasibility, efficiency and safety. Akinola and Olakunbi (2019) demonstrated that 1% of salt in the water for 30 min was effective to control columnaris of African catfish *Clarias gariepinus* juveniles (11 weeks old), without causing environmental mental or public health damage. Santos et al. (2020) found that the addition of tea tree and clove essential oils (EO) at 10.4 mg/ L in the water of transport (up to 36 h) of *C. macropomum* juveniles (65.2 ± 1.20 g) reduces fish stress and ensures water quality. However, in the fish farm, many producers make empirical or inadequate use of antimicrobials. Leal et al. (2019) highlight the disseminated use of oxytetracycline (OTC) in aquaculture because they are water-soluble, with a broad spectrum against pathogens and low toxic to fish. These features please its inappropriate use as prophylactic measure by fish farmers in the fish transport. The authors emphasize that the incorrect use of OTC raises concerns due to effects on human and animal health, the environmental contamination and the consequences arising therefrom, such as the risk of selection of resistant pathogens.

Disease outbreaks after transport are a threat to fish farming. Thus, applied studies are encouraged for aquaculture development. In view of the need and urgency of fundamental knowledge for growth of *C. macropomum* production, the aim of this study was to investigate the etiological agent responsible for several mortality outbreaks in Ama zona, Brazil, after the transport of *C. macropomum* juveniles and also to investigate the use of additives in the water of transport, providing a practical strategic measure that enhances production parameters and prevent disease outbreaks caused by emergent pathogens after transport.

2. Material and methods

This study addresses two critical steps regarding natural recurrent mortality outbreaks after fish transport.

2.1. Step 1: Investigation of mortality in five batches after transport

2.1.1. History of transport and clinical signs

Four natural mortality outbreaks were monitored after transport of 4000 juveniles of *C. macropomum* from different fish farms in Amazonas, located in the cities of Presidente Figueiredo (n of fish farms = 1; average travel time: 108 min) , Iranduba (*n* = 1; 52 min) and Rio Preto da Eva (*n* = 2; 76 min), to Manaus.

Fish were sold in batches of 1000 fingerlings (± 0.5 to 10 g). Fish from each batch were placed in 1 to 2 plastic bags of 60 L, according to lot size (batches with larger fish were divided into 2 plastic bags for transport, while batches with small fish were transported in a single plastic bag). The bags were filled with 1/3 of pond water and 2/3 of 100% O2 and then transported directly to Manaus. Immediately after transport, fish were acclimatized in quarantine tanks (150 L of water, 310 L of capacity – D xdxh = 0.97 × 0.68 × 0.60 m), where infectious disease was manifested.

Fish were assessed with regard to clinical signs and disease progression (daily monitoring, twice a day) and non-specific bacteria were identified through routine microbiological examination (item 2.1.2.). The experimental procedures of the following step were approved by the Animal Use Ethics Committee (CEUA) of Nilton Lins University under protocol 003/2019 and were carried out in accordance with ethical principles of animal use adopted by the Brazilian College of Animal Experimentation (COBEA, Brazil) and the Federal Council of Veterinary Medicine (CFMV, Brazil).

2.1.2. Bacteria identification

At least 20 diseased fish from each mortality outbreak were sampled (brain, kidney and muscle) for bacterial isolation. The diseased fish showed lethargy, erratic swimming, anorexia, fins corrosion and saddle shaped lesion in focal areas, skin melanosis, depigmentation, and extensive ulcerative lesions, mainly in fins and muscle of the dorsal region. Moribund fish were stablished as predetermined humane endpoint for euthanizing the animals (overdose in 0.3 g/L of benzocaine; E1501 Sigma-Aldrich, China). Recently dead fish (natural mortality) were also included in this analysis. First, fish were subject to antisepsis (peeling, then washed with neutral detergent, 0.1% iodinated alcohol and 70% alcohol, respectively). In a microbiological safety cabinet, the brain and kidney were streaked on tryptone soy agar (TSA) and incubated at 28 ÿC for up to three days. Meanwhile, muscle fragments were streaked on Hsu-Shotts (HSM) or tryptone soy agar with yeast extract (TYES) and incubated at 25 ÿC for up to five days. Pure stock isolates were stored at ÿ 20 ÿC in sterile HSM broth, supplemented with 15% glycerol for further analysis. For bacterial species identification, each strain obtained was analyzed using Matrix Assisted Laser Desorption-Ionization - Time of Flight (MALDI-ToF) mass spectrometry with a Bruker Microflex MALDI Biotyper 2.0 (Bruker Daltonics, USA), according to Assis et al . (2017). The criteria of real-time identification (RT) were based on the score recommended by the manufacturer: score ÿ 2000 indicates identification at the species level, score ÿ 1700 and < 2000 indicates identity at the genus level, and *score < 1700* indicates that there is no reliable identification.

2.2. Step 2: Search for practical-effective additives in the water of transport: Influence on production performance

2.2.1. Animal use ethics statement

The experimental procedures of the following step were approved by the Animal Use Ethics Committee (CEUA) of Nilton Lins University under protocol 009/2021 and were carried out in accordance with ethical principles of animal use adopted by the Brazilian College of Animal Experimentation (COBEA, Brazil) and the Federal Council of Veterinary Medicine (CFMV, Brazil).

2.2.2. fish

A total of 480 apparently healthy tambaqui juveniles (8 cm; 6.89 ± 0.94 g), previously monitored for the presence of parasites and bacteria, were obtained from the same pond of a commercial fish farm in Ama zona. Parasite disease was ascertained as negative through routine analysis of gills and integument scrap in optical microscopy (100× and 400× magnification). Bacterial disease was ascertained as negative through culturing of brain, kidney and muscle fragments in TSA and HSM, incubated at 28 ÿC for up to three days and 25 ÿC for up to five days, respectively.

2.2.3. Transport and experimental conditions

For transport, live fish were placed in 20 plastic bags of 5 L (1 L of water from the pond, supplemented with 100% O2). In each bag, 25 fish (6.89 ± 0.94 g) were transported, simulating the density of 175 kg/m3 adopted by fish farmers, from which the four batches of fish were

purchased in Amazon. The bags were divided into five treatments, with four replicates: control group of the water of transport (C1, transported with the fish farming water); control of alcohol, indicated as solvent for EO of *M. alternifolia* (C2, 100.08 mg of absolute alcohol, corresponding to nine parts of EO); group transported with commercial EO of *M. alternifolia* (EO, added at the dose of 11.12 mg/L, diluted in absolute alcohol at 1:9 ratio); group transported with non-iodized salt (S, with NaCl added at a dose of 8 g/L or 0.8%); and group transported with oxytetracycline (OTC, at the dose of 85.71 mg of the commercial product TM700®, equivalent to 60 mg/L of the active principle). The chosen doses of EO, S and OTC were based on doses recommended by Santos et al. (2020), Gomes et al. (2003a) and Pavanelli et al. (2008), respectively.

The additives had the following composition: sodium chloride (NaCl) with purity ÿ99.0% (Sigma-Aldrich, S3014), oxytetracycline TM700® with 70% purity (700 g of terramycin for each 1000 g of the product). The composition of the commercial EO of *M. alternifolia* was verified by high-performance gas chromatography, with ÿ-terpinene and terpinen 4-ol as the main constituents (supplementary Table 1).

After three hours of transport, the fish from each bag were transferred to a corresponding tank (24 fish per 60 L of water; tanks with 84 L of capacity - wxlxh = 0.60 × 0.40 × 0.35 m), totaling 20 tanks, without using additives in the water). The fish were kept for 30 days in this static system, supplied with continuous aeration and daily partial water changes (30% of volume) for two weeks. Afterwards, the water change was carried out every three days, until the end of the experiment. The water quality parameters (oxygen, temperature, pH, conductivity) were measured with a multiparameter probe (AKSO AK87) in fish farms, in the transport water and every 15 days in the experimental tanks. The water supply for the experimental tanks was from artesian well. The quality parameters (pH, O2 and temperature) were ascertained within ideal ranges for the fish species according to Correa et al. (2018) (5–7; 4–8 mg/L; 28–30 ÿC, respectively).

2.2.4. Production performance: Zootechnical parameters and survival

After transport, the fish from the five treatments (with four replicates each) were fed for 30 days with commercial feed (2.5 mm, specific for omnivores, 42% crude protein, AcquaLine®), previously analyzed (in triplicate) regarding constituents by methodology described in Brazilian compendium of animal feed (BCAF, 2017) in moisture (drying in an oven at 105 ÿC), crude protein (bp, Kjeldahl method), ether extract (Soxhlet method) and mineral matter (determination of ash in a muffle furnace at 550 ÿC), showing values (in %) of 6.22 ± 0.29, 44.8 ± 0.08, 7.15 ± 0.29 and 9.37 ± 0.67, respectively (supplementary Table 2).

Fish were fed once a day, until apparent satiety. The leftover of each experimental unit was collected after 20 min of the offer for weighing after drying in an oven at 50 ÿC/24 h. Fish from each experimental unit were individually weighted and measured (total and standard lengths) for biometry analysis on days 1, 15 and 30. With biometry and feed intake data, weight gain (WG), total feed intake (TFI), feed conversion (FC) and specific growth rate (SGR) and growth (G) were calculated as follows: WG (g) = final weight (g) – initial weight (g); TFI (g) = this measure was ascertained for each tank (g) by previous weighing (daily) of feed supplied and withdrawn (after drying in oven at 50 ÿC /24 h); FC (g) = total feed consumption per fish/weight gain; SGR (%/day) = 100 x (ln average final weight – ln average initial weight)/experimental period; G (cm) = final length – initial length. Fish mortality was twice a day. Dead fish were discounted for the calculations of production performance, and the data for survival estimation were attributed as follows: survival (%) = 100 x (surviving fish/ total).

2.2.5. Bacteriological exam: Natural mortality outbreak of fish after transport

Fish were monitored continuously throughout the experimental period for description of the following natural infection. In order to identify potential bacterial pathogens during disease outbreak after transport, each recently dead fish (natural mortality) was subjected to the same procedures described above for bacteria isolation (brain, kid ney, liver and muscle), followed by identification using MALDI-ToF mass spectrometer and storage.

2.2.5.1. Molecular detection of F. columnare from tissue and mucus. A PCR was implemented as an additional tool to diagnose *F. columnare* in natural diseased fish after transport. First, mucus from integument were sampled for Gram staining to allow a fast detection of long and thin rods, stained by Gram-negative, suggestive of *F. columnare.* Then, an aliquot of mucus was scraped from the integument of diseased fish, and then the sample was stored in sterile Eppendorf® (with and without absolute alcohol). For recently dead fish (natural mortality), this procedure was repeated, adding muscle (aseptic sampling of tissue fragment from the dorsal region). At the end of the experimental trial, one fish from each tank was euthanized in benzocaine overdose (0.3 g/L E1501, Sigma Aldrich, China) for the same tissue sampling. All collected samples were stored in ultra-freezer (ÿ 80 ÿC), until DNA extraction.

The DNA was extracted with ZR genomic DNA II kit, according to the manufacturer's recommendations, despite the following modifications: 50 ÿL of proteinase K, activated in dry bath at 37 ÿC/5 min. The extracted DNA was analyzed in a Nanodrop spectrophotometer (approximately 10 ng) and then multiplex PCR was performed, according to LaFrentz et al. (2019) with some modifications. Briefly, PCR reaction was performed with Platinum Taq Kit (Invitrogen), corresponding to a reaction volume of 25 ÿL composed of: 2.5 ÿL of PCR Buffer (10×); 2.5 µL of MgCl2 (25 µM); 1.0 µL dNTP (10 µM); 1.5 ÿL of Taq polymerase (5 U / ÿL); 11 ÿL of ultrapure water and 2 ÿL of DNA template (50 ng). The reaction used 5 primers, as shown in Table 1.

The reaction was amplified in the Veriti AB thermal cycler, following the cycle conditions of: 1 × 95 ÿC for 15 min; 40 × 94 ÿC for 30 s, 56 ÿC for 20 s and 72 ÿC for 60 s; 1 × 72 ÿC for 10 min. A volume of 9 ÿL of each amplicon plus 1 ÿL of dye (100 ÿL of the loading dye 10× + 10 L of Nancy 520, Sigma Aldrich) were analyzed by electrophoresis on agarose gel (1%, without additive dye). The electrophoresis voltage, amperage, power and time factors were: 100v, 200 mA, 70 W and 35 min, respectively. The bands were visualized in Alpha Imager Mini trans illuminator (Biolab Tec). A 100 bp marker was used as a molecular weight standard. AM-FC08 strain (Baldissera et al., 2020b) and ultrapure water were used as a positive and a negative control, respectively.

2.2.5.2. Pathogenicity ascertainment of isolated strains. To identify the causative agent of both natural diseases (Steps 1 and 2), a Koch's Postulate assay was carried out. Bacteria were considered the etiological agents of the disease when the same pathological manifestations were observed, along with re-isolation and identification of the microorganism, as described in item 2.1.2.

2.2.5.2.1. Fish and experimental conditions. Healthy *C. macropomum*

Table 1
Primer sequences used in this study.

Primer (10 ÿM)	Sequencing (5' ÿ 3')	bacterial target
GG-F	ACRGGRGATAAAGCAGAASA*	*Flavobacterium columnare*
GG1-R	GACTTTTGTGTTGAAACGG	*F. columnare*
GG2-R	AAGAAAATAGGGGGAGAGG	*F. columnare*
GG3-R	CAAGTTTCGTTATGATGAGG	*F. columnare*
GG4- R	TCCAAAAAAGTCCGMAATC*	*F. columnare*
gyrB- F	GGGGTCTACTGCTTCACCAA	*Aeromonas* spp.
gyrB- R	CTTGTCCGGGTTGTACTCGT	*Aeromonas* spp.
recA- F	AGAACAAACAGAAGGCACTGG	*Aeromonas* spp.
recA- R	AACTTGAGCGCGTGTACCAC	*Aeromonas* spp.
rpoD- F	GAAGGCGAAATCGACATCGC	*Aeromonas* spp.
rpoD- R	ATGCCTCATGCGRCGGTTGAT*	*Aeromonas* spp.

* Degenerate primers. GG: genetic group of *F. columnare.* GG primers were designed according to LaFrentz et al. (2019); *gyrB:* DNA gyrase, subunit b; recA: recombinase A; *rpoD:* RNA polymerase sigma factor; F: forward; A: Reverse.

(juveniles, approximately 1–3 g) were acquired from commercial fish farms. Fish were acclimated in tanks (150 L of water, 310 L of capacity – D xdxh = 0.97 × 0.68 × 0.60 m) with continuous aeration and water flow. In order to ascertain the absence of bacterial disease in experimental fish, 7 fish from the same batch were analyzed according to routine microbiological procedures, as described in item 2.1.2. After verification, fish were distributed randomly in 30 L glass aquariums (7 fish/aquarium, wxlxh = 0.30 × 0.30 × 0.30 m). Each aquarium contains a target-bacteria (2 groups of potential pathogens, besides control group, challenged with the sterile inoculum suspension).

The aquariums were provided with constant aeration (air compressor-60 Hz, until complete saturation) and static water system. Partial water changes were performed (30% of the volume) every day. Water parameters (pH, oxygen and temperature) were measured weekly and showed average values within the normal range for the species. During the experimental period, fish were fed once a day until apparent satiety. The commercial diet was specific for omnivores, with 45% crude protein content.

2.2.5.2.2. Bacterial inoculum. Based on the pathogenic potential, two strains were selected for Koch's Postulate: *F. columnare* AM-FC30 and *Aeromonas jandaei* AM-AE70. The isolates were selected because they represent the main pathogens detected in natural mortality outbreak of fish after transport in step 1 (100% of analyzed fish diagnosed with F. *columnare)* and step 2 (100% of molecular-analyzed fish diagnosed with *F. columnare* and 43% diagnosed with *Aeromonas* spp. in Maldi-ToF), respectively.

The inoculum of *F. columnare* was identified at species level by MALDI-ToF mass spectrometer, as described in 2.1.2. However, ac cording to P ´erez-Sancho et al. (2018), the technique of identification of *Aeromonas* by MALDI-ToF may not be effective at species level. There fore, additional analysis of the isolates with molecular amplification techniques of multiple genes such as *rpoD, gyrB* and *recA,* indicated by Beaz-Hidalgo et al. (2013) was performed to ensure a reliable diagnosis.

By that means, *A. jandaei* DNA was extracted using a Maxwell 16 Tissue DNA purification kit (Promega, USA) according to the manufacturer's instructions, quantified using a Nanodrop spectrophotometer (Thermo Scientific, USA) and stored at ÿ 20 ÿC until use . PCR reactions were performed using primers (Table 1) for amplification of housekeeping genes *(gyrB, recA* and *rpoD)* according to Martino et al. (2011) and Martinez-Murcia et al. (2011), with some modifications. These primers were synthesized and purified at Integrated DNA Technologies (IDT, USA). PCR for each gene was performed with a HotStart Taq polymerase kit (Qiagen, USA) in a final reaction of 25 ÿL. The reaction mixture was composed of 2.5 ÿL 1× PCR buffer, 1 ÿL dNTPs, 1.5 ÿL mM MgCl2, 1 ÿL primer forward, 1 ÿL primer reverse, 1 ÿL taq DNA polymerase, and 50 ng template DNA. The PCR conditions were performed according to manufacturer's instructions (HotStart Taq polymerase kit; Qiagen, USA) as follows: an initial step of 94 ÿC for 15 min, followed by 35 cycles of 94 ÿC for 15 s, 55 ÿC for 30s , 72 ÿC for 45 s; final elongation was carried out at 72 ÿC for 3 min. Amplification was performed in a Veriti 96-well Thermal Cycler, and PCR amplicons were separated in the QIAxcell Advanced using QX DNA Screening Kit (Qiagen). The PCR amplicons were purified using Agencourt AMPure XP (Beckman Coulter, Pasadena, USA) according to the manufacturer's instructions. Sequencing re actions were performed using a BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, Carlsbad, USA), with the same primers of the *gyrB, recA* and *rpoD* PCR protocols, and evaluated with an ABI 3500 Genetic Analyzer (Life Technologies, Carlsbad, USA). Forward and reverse sequencing products were used to generate contigs using BioEdit Software (Ibis BioSciences, Carlsbad, USA) version 7.2. The contigs were then compared to existing sequences from the NCBI database using the BLAST webserver (http://www.ncbi.nlm.nih.gov/BLAST).

The strains (stored at ÿ 20 ÿC in TSB and HSM enriched with 15% glycerol) were reactivated on the respective agar *(A. jandaei* in TSA and *F. columnare* in HSM) and incubated according to suitable conditions (28 ÿC for 24 h and 25 ÿC for 72 h, respectively).

To prepare *F. columnare* inoculum, the colonies were added into sterile TYES broth medium and incubated under shaking (100 rpm at 28 ÿC/24 h). Then, TYES broth was washed by centrifugation (ÿ 4 ÿC/ 3000G/ 10 min) with sterile saline solution (PBS). The bacterial pellet was resuspended in PBS until reaching 0.500 at OD600. For *A. jandaei,* the colonies were inoculated into sterile PBS, until reaching the same optical density conditions.

2.2.5.2.3. bacterial challenge. For experimental infection, the fish were previously anesthetized by immersion in benzocaine (0.1 g/L, Sigma-Aldrich E1501, China). After weighing, each individual was intraperitoneally inoculated with 0.1 mL of the respective inoculum for each 10 g of live weight (109 CFU/fish).

The challenged fish were monitored (twice a day) for 15 days for analysis of behavioral changes, clinical signs and mortality. The bacteria were re-isolated from recently dead fish (natural mortality due to bacterial infection), following procedures mentioned above for bacterial identification.

2.3. statistical analysis

The groups were compared at the end of the experimental period with One-way ANOVA, followed by the Tukey test. The values of the zootechnical parameters and survival were expressed as mean ± stan dard deviation. All data were normal and homoscedastic and did not require transformation for statistical analysis. Values of p ÿ 0.05 were considered significant.

3. Results

3.1. Step 1: Investigation of mortality in five batches after transport

3.1.1. History of transport and clinical signs

In less than 24 h after the transport, it became evident it was an infectious disease of easy and rapid transmission. Within 72 h, almost 100% mortality was ascertained, which resulted in the loss of five thousand fingerlings. Just a few fish from each batch survived and due to disease weakening, they were euthanized (humane endpoints according to described in item 2.1.2).

All infection outbreaks first produce massive lethargy, frayed and ragged fins and characteristic saddle-shaped lesion in the fish's back. Anorexia and erratic swimming were also common batch behavior, besides integument melanosis. Grayish wounds on the fins and focal skin discoloration on fish's back were noted, followed by scale loss, fin corrosion and deep wounds in the integument caused by *F. columnare* (Fig. 1).

3.1.2. Bacteria identification

In HSM agar, there was growth of rhizoid yellowish colonies, suggestive of *F. columnare.* In TSA, there was no significant bacterial growth besides the colonies like those ascertained in HSM. Gram-negative rods with long and thin morphology were visualized under microscope (1000 x magnification) (Fig. 2). MALDI-ToF analysis confirmed *F. columnare* as involved pathogen in the outbreaks.

3.2. Step 2: Search for practical-effective additives in the water of transport: Influence on production performance

3.2.1. Production performance: Zootechnical parameters and survival

The results of production performance (weight gain (g), total feed intake (g), specific growth rate (%/day), feed conversion (g), growth (cm) and survival (%)) obtained at the end of the experimental trial (30 days) are shown in Table 2.

3.2.2. Bacteriological exam: Natural mortality outbreak of fish after transport

Fish transported with oxytetracycline, *M. alternifolia* EO and both

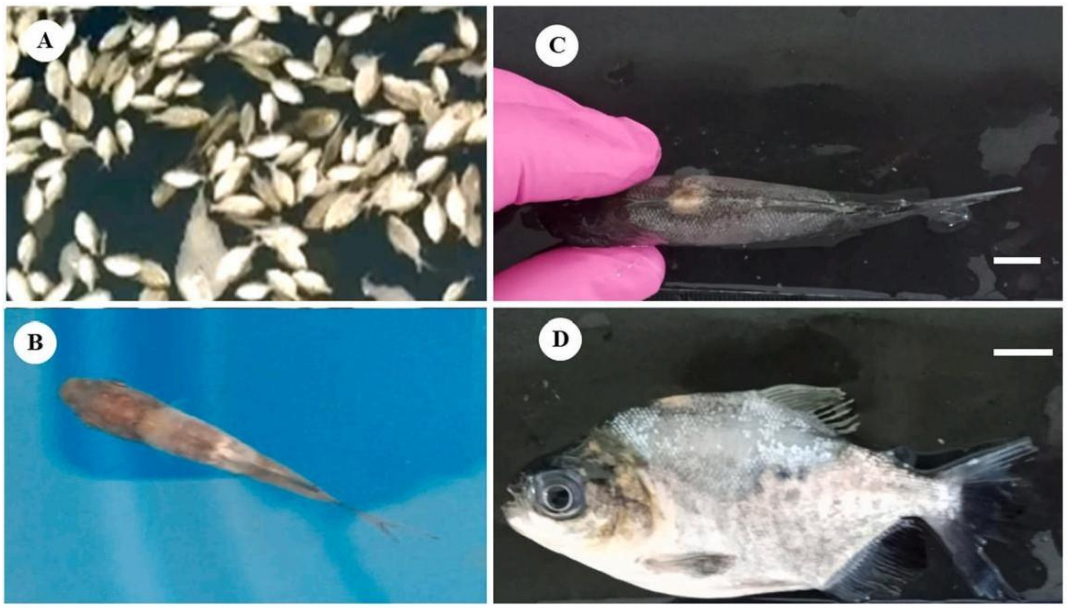

Fig. 1. *Colossoma macropomum* juvenile batch showing mortality outbreak by *Flavobacterium columnare* within 24 h after transport (A) Saddle-shaped lesion, showing focal areas of skin melanosis and depigmentation with extensive ulcerative lesions, mainly in fins and muscle of the dorsal region of *C. macropomum* (B, C and D). Bar = 1 cm.

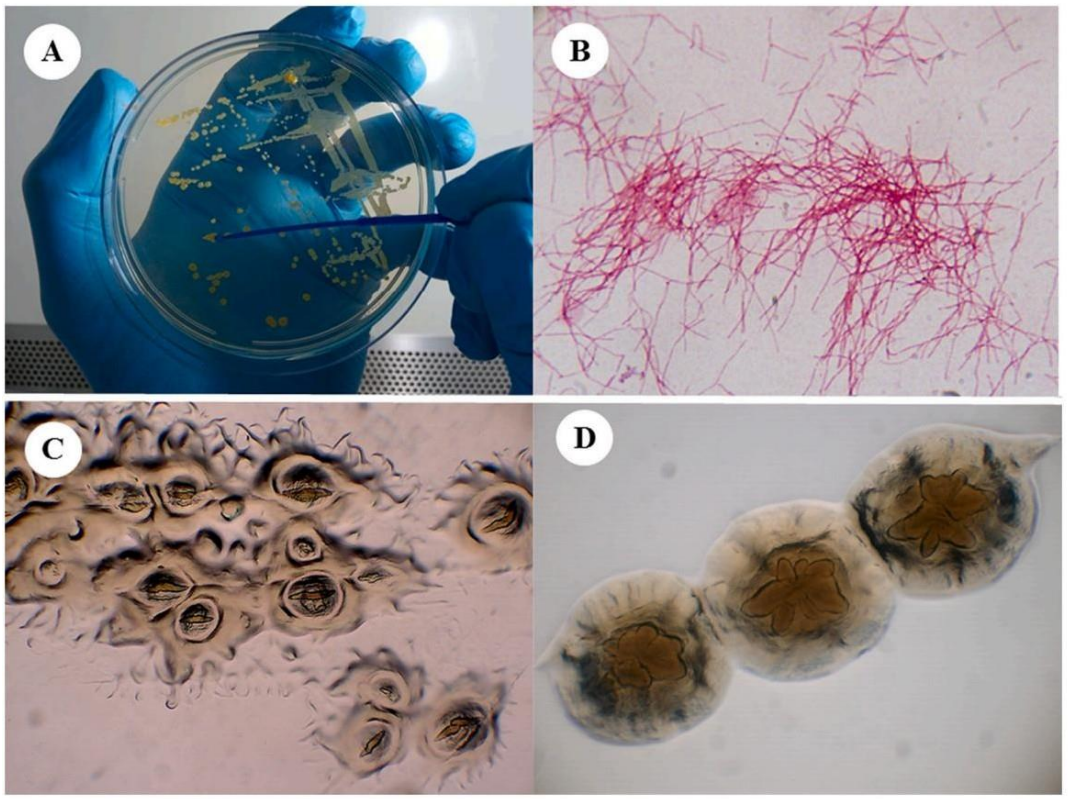

Fig. 2. Morphological characteristics of *Flavobacterium columnare* (strain AM-FC30) isolated from disease outbreak in *Colossoma macropomum* cultured in Brazil. Representative yellowish and adherent colonies on Hsu-Shotts medium (A). Long and thin rods, Gram-negative stained, under a light microscope 1000 X (B); isolated colonies achieved by streak plate procedure, showing the rhizoid morphology (C) and the yellow star-shaped center (D), under a light microscope 40× and 100×, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

control groups showed behavioral changes such as anorexia, hyporexia, lethargy, erratic swimming, dyspnea and clustering on the water surface in the first three days after transport. Additionally, moribund fish showed prominent yellowish-white lesions and fin erosions, followed by extensive ulcerative lesions, mainly in the dorsal and caudal regions, which started in the ends and progressed to extensive areas of musculature. These changes were not observed in fish transported without (Fig. 3).

Table

2 Comparison of survival, total feed intake per experimental unit (TFI); feed conversion (FC); growth (G); weight gain (WG) and specific growth rate (SGR) between both control groups transported with water of transport (C1) and diluent alcohol (C2) and with the additives of *Melaleuca alternifolia* essential oil (EO), oxytetracycline (OTC) and salt (S).

Treatment	Survival (%)	TFI (g)	FC (g)	G (cm)	WG (g)	SGR (%/ day)
C1 (water)	90.63 ± 18.75	99.10 ± 5.58	1.04 ± 0.22 ab	0.92 ± 0.37	4.59 ± 0.60 ab	5.06 ± 0.42
C2 (alcohol)	88.54 ± 2.08	98.20 ± 11.63	1.20 ± 0.25 ab	1.22 ± 0.23	4.00 ± 1.05 ab	4.54 ± 0.90
EO	94.79 ± 5.24	105.46 ± 17.61	1.35 ± ab 0.70	1.25 ± 0.55	3.52 ± 1.77 ab	3.83 ± 1.88
OTC	89.58 ± 8.68	116.95 ± 6.71	1.81 ± 0.45 a	1.27 ± 0.27	3.14 ± b 0.72	3.75 ± 0.71
S	100 ± 0	118.68 ± 6.23	0.89 ± b 0.10	1.66 ± 0.22	5.62 ± 0.89 a	5.72 ± 0.54

Values are presented as mean ± standard deviation. Different superscript letters indicate statistical difference between treatments (p ÿ 0.05). The absence of letters indicates no statistical difference between treatments.

Fig. 3. Recently dead (natural mortality) of *Colossoma macropomum* juveniles, at day 4 post-transport, showing a diffuse ulcerative lesion, mainly in the caudal region. Grayish wounds are prominent and the fin was almost completely corroded. The muscle showed focal skin discoloration with yellowish fungus like patches at the edges and above the deep ulcerative wounds, suggestive of *Flavobacterium columnare* colonies (A). Recently dead (natural mortality) *C. macropomum* juveniles from the group with *Melaleuca alternifolia* essential oil added in the water of transport, showing diffuse hemorrhage, suggestive of the coinfection by *Aeromonas* spp. (B). Bar = 1 cm.

It is worth noting that hemorrhagic lesions in the fins and tegument could be noted during microbiological analysis (Fig. 3). Also, from day 4 after transport, it was possible to observe the acclimatization of most of the fish in the system.

The group transported with salt was the only one to show voracious eating behavior, in which the individuals ingested the food immediately, requiring additional amount of food until satiety. This behavior was not observed in the other groups.

Sporadic mortality (Fig. 4) remained until the end of the second week after transport. Then, stability was observed. From this period, the fish remain without signs of illness.

A set of 39 diseased fish (9, 11, 9 and 10 recently-dead individuals (natural mortality) from groups C1, C2, EO and OTC, respectively) were subjected to bacteriological examination and the majority of isolates obtained from both HSM and TSA showed bright cream colony. Those isolates were subsequently identified as *Aeromonas* spp. Besides this pathogen, *Chryseobacterium* spp., *Citrobacter freundii* and *Plesiomonas shigelloides* were also ascertained by MALDI-ToF. There was no growth of *Flavobacterium columnare* in HSM agar, up to 5 days of incubation at 25 ÿC.

3.2.2.1. Identification of F. columnare from tissue and mucus by PCR. All fish that showed clinical signs suggestive of columnaris were ascertained with long Gram-negative bacilli and were diagnosed by PCR using DNA samples extracted from mucus and/or tissue. In addition, fish analyzed at the end of the experimental period (n = 10) also showed positive results for *F. columnare.*

3.2.2.2. Pathogenicity assessment of isolated strains

3.2.2.2.1. bacterial challenge. Healthy fish inoculated with *Aeromonas jandaei* (species confirmed by sequencing of housekeeping genes) and *F. columnare* developed the same clinical signs and behavioral changes as diseased fish from both natural outbreaks after transport (Steps 1 and 2).

Fish infected with *F. columnare* displayed the highest mortality rates (100% of the challenged group) in a short period (up to the fourth day post-infection). Meanwhile, infection of fish with *A. jandaei* resulted in the loss of 86% of juveniles in less than 48 h (Fig. 5).

Colossoma macropomum infected with *F. columnare* showed mainly lethargy, erratic swimming in the water surface and anorexia. Some infected fish showed the saddle-shaped lesion on the back; however, most individuals had focal ulcerative and depigmented lesions, which were related to the region of inoculum application at the ventral region (A). Hemorrhage was the major clinical sign of *C. macropomum* infected with *Aeromonas jandaei,* which also show the same behavioral changes of columnarosis, associated with septicemia (Fig. 6).

The validation of reisolated bacterial identities using MALDI-ToF and combined technique with gene sequencing allowed the confirmation of *F. columnare* and *A. jandaei,* respectively, fulfilling the Koch's Postulate.

4. Discussion

The transport of fish in aquaculture trade is a neglected critical step in fish farming, which can cause mass mortalities and economic loss due to exposure of fish to multiple stressors. Based on recurrent disease outbreaks after transport, we found that *F. columnare* must be recognized by epizootic mortality in *Colossoma macropomum,* especially in finger lings, affecting a very large proportion of the batch with dramatic decline within a few hours after stress induction.

Seghouani et al. (2017) highlight columnaris under non-standard conditions during transport. Since the ideal density for transport of *C. macropomum* is 78 kg/m3 (Gomes et al., 2003b), the high density adopted by producers in Amazonas (175 kg/m3), simulated in our study, was probably the main factor that favors columnaris outbreak. Whereas fish bacteria are opportunistic pathogens, stress is a major factor in columnaris disease, causing infection in hosts that face atypical environmental conditions or impaired immune function (Derome et al., 2016). So, transport practices such as excessive handling and thermal variations between locations are also associated with risk factors for this disease (Sebastiao et al., 2013). It is noteworthy that, according to Gallani et al. (2021), when *C. macropomum* are infected by bacteria, they exhibit lower expression of the HSP70 heat shock genes, which makes them even more susceptible to environmental stress and infections. So, it seems that *C. macropomum* is highly sensitive to environmental conditions and, thereby, highly predisposed to columnaris infection after stress of transport.

Although *F. columnare* has already been isolated from several fish species in Brazil (Figueiredo et al., 2005; Pilarski et al., 2008; Sebasti˜ao et al., 2013; Barony et al., 2015), little is known regarding its virulence (Li et al., 2017); however, due to outbreaks of mortality of 100% of

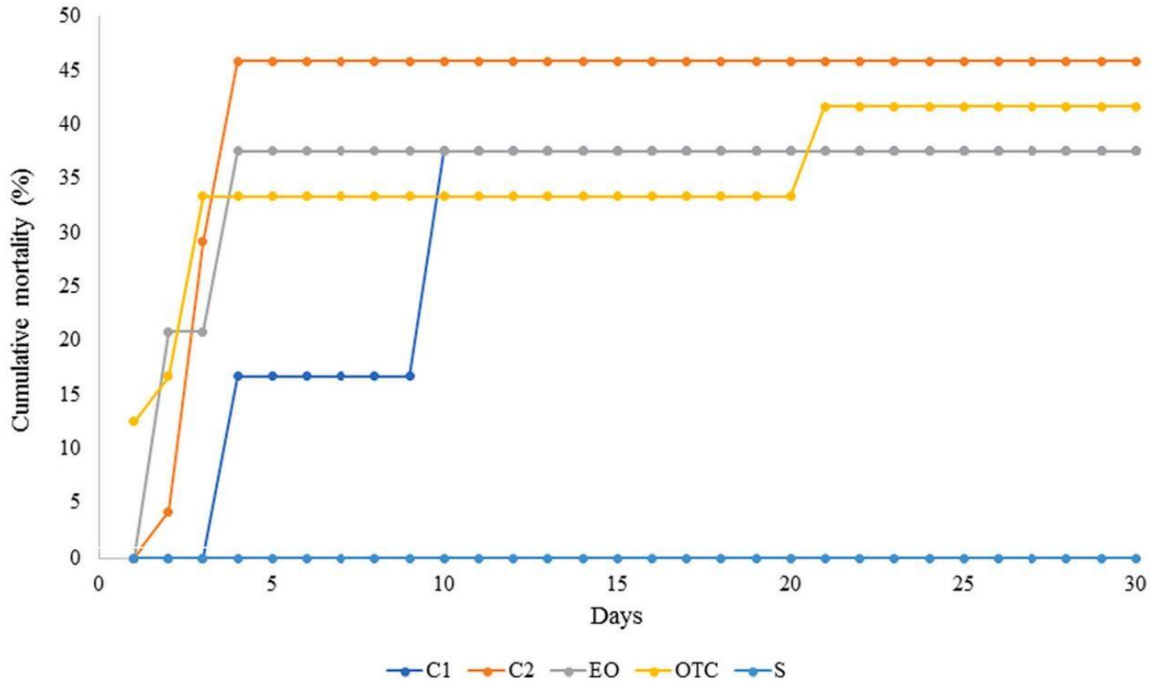

Fig. 4. Data of 30-day cumulative mortality (%) of fish transported with the additives in the water of fish farming. The groups were compared as follows: control groups corresponding to fish transported with the water of fish farming (C1) and with 100.08 mg/L of absolute alcohol used as diluent (C2); and three groups of fish transported with additive in the water of transport, as follows: 11.12 mg/L of *Melaleuca alternifolia* essential oil, diluted in absolute alcohol 1:9 (EO); non-iodized sodium chloride at 8 g/L or 0.8% (S); and 60 mg/L of oxytetracycline, the equivalent to 85.71 mg/L of TM700®.

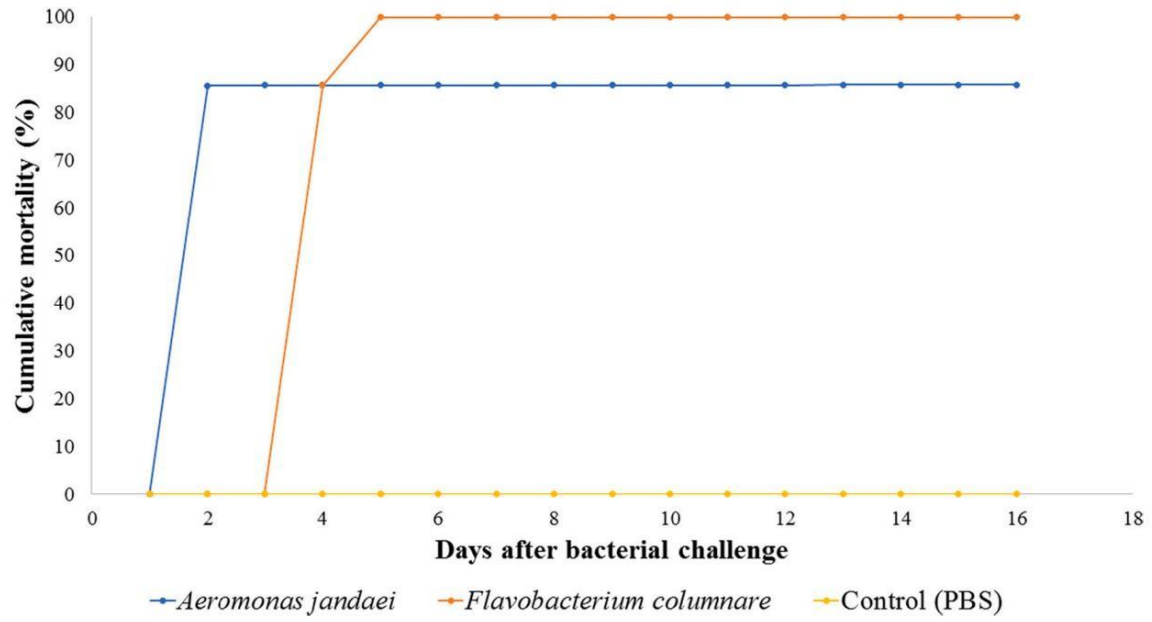

Fig. 5. Cumulative mortality (%) of fish infected with *Aeomonas jandaei* and *Flavobacterium columnare.* After intraperitoneal infection (0.1 mL/ 10 g live weight), fish were monitored for 15 days. Control group was inoculated with sterile PBS (same dose) and did not show mortality or clinical signs and behavioral changes until the end of the experimental trial.

batches in less than 72 h, our study suggests that the bacterium is highly virulent and pathogenic.

Due to the high pathogenicity and virulence of *F. columnare* (Chockmangmeepisan et al., 2020), the prevention of disease seems to be the best choice to avoid massive mortalities. According to Suoma Lainen et al. (2005), the use of salt in the water is not effective when the aim is to treat the host. The authors assert that this occurs because, after infection, the bacteria are located below the mucus surface. even though it is known that *F. columnare* is highly sensitive to salt and inhibits bacteria growth (Akinola and Olakunbi, 2019), if the bacteria penetrate the host, the substance does not exert the desired microbicidal effect. Therefore, disinfection of water containing *F. columnare* cells eliminated by infected fish is the best alternative for disease control (Suomalainen et al., 2005), which corroborates the use of additives in the water of transport. Furthermore, the transmission of columnaris occurs through the water column, without the need for contact between individuals

Fig. 6. Fish challenged with *Flavobacterium columnare* showing grayish focal wound. The muscle shows focal skin discoloration with scale loss. Hemorrhagic yellowish area can be noted at the ventral region (fin and muscle). The eye shows hemorrhagic lesion (A). Fish challenged with *Aeromonas jandaei* showing diffuse hemorrhagic lesions in the muscle (B). Bar = 1 cm.

(Seghouani et al., 2017). Bacteria infect the fish mainly through the gills (Chettri et al., 2018) and skin lesions (Klesius et al., 2008), therefore, the need for biosecurity practices must be highlighted, since the presence of a single infected individual is an important risk factor for columnaris, which threatens with the loss of the entire batch.

Based on these findings, the search for additives in the water of transport showed that 8 g/L of sodium chloride combines efficiency, practicality and sustainability and proves to be the best choice for fish farmers to prevent disease outbreak after transport. It is known that the stressful condition caused by transportation can increase the loss of ions in freshwater fish because of a blood flow increase in the gills and consequent changes in cell permeability (Cech Jr et al., 1996; Santos et al., 2020) . Indeed, it has been hypothesized that salinity affects the energy budget in fish (Tavares-Dias, 2022). So, fish transported with 0.8% of salt in the water of transport may spent less energy on osmotic regulation. Thus, the findings regarding the improvement of zootechnical parameters in this group would also be explained. The group transported with salt was the only one with 100% of fish survival, with sanitary integrity certified by absence of clinical signs, behavioral changes and lack of bacteria in microbiological and PCR analysis. These findings represent a relevant biological accomplishment, proving that salt prevents juveniles from being infected by *F. columnare* due to stress of transport. Meanwhile, all the other groups display fish mortality by columnaris after environmental stress, indicating that fish farmers who opt for these methods may be subject to great economic losses.

In this study, one of the groups with the greatest negative effects related to the use of the additive in the water of transport was OTC. According to Limbu et al. (2020), the side effects of the inappropriate use of OTC impair fish performance in terms of weight gain, feed efficiency and survival rate. Since these were the same parameters that differed this group from the group transported with salt, OTC was pointed out as a substance that might negatively influence zootechnical parameters of fish. In the other groups, the energy from the ingested food may have been diverted towards the control of infection and the side effects of the drug. Also, when the batch of fish is infected by columnaris, survival and growth may have been compromised as a result of the disease (Loch and Faisal, 2015). Furthermore, as weight gain directly interferes with feed conversion, the better conversion of individuals transported with salt, when compared to the group transported with OTC, is justified. Xuan et al. (2009) also pointed out that photolysis can be the dominant degradation pathway of OTC in environments with transparent and low amounts of water (like in plastic bags used for transport), so decrease of the drug activity during transport should be considered. The preventive use of antimicrobials can result in negative effects for teleost. The depression of the cellular (Rijkers et al., 1980) and humoral (Rijkers et al., 1981) immune response, hepatotoxicity (Limbu et al., 2020), enhancement of cortisol and pathological tissue damage, inducing oxidative stress (Nakano et al., 2020) al., 2018), are some of the side effects already described in fish, which in crease the susceptibility of fish to stressors and pathogens.

Although essential oils have been described as antibacterial, immu nostimulant and antioxidant for fish and thus used as additive in the water of transport, the EO of *M. alternifolia* recommended by Santos et al. (2020) for the transport of *C. macropomum* was not effective to mitigate *F. columnare* and associated infection by *A. jandaei.* The prop erties of EO interfere with the antimicrobial activity, but since ÿ-terpinene and terpinen-4-ol were the main active principle, the low effectiveness may be related to specific features of target-pathogens. In fact, it seems that EOs are more effective against Gram-positive than Gram-negative bacteria (Chandra et al., 2017), and that is a great concern, since most fish pathogens are Gram-negative (Van Doan et al., 2021).

With the exception of fish transported with salt, all other groups showed focal wounds suggestive of columnaris, besides hemorrhagic lesions. In fact, *F. columnare* can release proteolytic enzymes and degrade the fish tissue, causing the extensive lesions (Chockmangmee pisan et al., 2020), corroborating our findings. However, *F. columnare* bacteria were not isolated from these fish by routine microbiological examinations. We assumed that columnaris lesions potentially play a key role for pathogenicity of opportunistic bacteria, predisposing the juveniles to co-infection by *Aeromonas* spp. after transport. Due to the competitive ness with this bacterium, the growth of *F. columnare* is compromised (Tekedar, 2020), which may have inhibited its growth in HSM agar and resulted in false-negative diagnoses.

In this study, *F. columnare* was presumed to be a major issue for *C. macropomum* juvenile transport, as well as *A. jandaei.* The motile *Aeromonas* septicemia (MAS) is a hemorrhagic septicemia disease, caused by a group of bacteria of great relevance in aquaculture. Although MAS is considered one of the main diseases of tropical fish (Valladao ˜ et al., 2018; Gallani et al., 2020; Gallani et al., 2021), only recently Gallani et al. (2020) determined the pathogenicity of the first bacteriosis for *C. macropomum,* caused by *A. hydrophila.* Dong et al. (2017) highlight that diseases caused by MAS in freshwater fish have been generally assumed to be linked mainly to *A. hydrophila,* while other species were probably overlooked. In this study, we certify *A. jandaei* as an emerging bacterial pathogen of *C. macropomum* in Brazil that causes acute infection associated with MAS. Since larvae and juveniles are highly susceptible to columnaris (Seghouani et al., 2017) and MAS (Abdelhamed et al., 2019), the use of salt as additives in the water of transport is highlighted as an important strategy measure to reduce the risk of these emerging diseases.

5. Conclusion

Recurrent mortality outbreaks by columnaris were identified as a major threat for *C. macropomum* farming and this is the first report of two key-pathogens for this fish species: *F. columnare* (columnaris) and *A. jandaei* (MAS - columnaris associated disease). Also, the use of salt at 8 g/L (0.8%) in the water of transport is highlighted as the best choice for fish farmers as an effective input for biosecurity practices during the critical stage of fish transport, since it prevents the introduction and spread of emerging transport-related pathogens and results in 100% of fish survival, besides improvement of the zootechnical parameters in relation to OTC-transported group.

CRediT authorship contribution statement

Thiago Duarte Mielke: Writing – original draft, Writing – review & editing, Conceptualization, Methodology, Formal analysis, Validation, Investigation, Project administration. **Claire Juliana Francisco:** Methodology, Formal analysis, Validation, Investigation. **Fernanda Alves Dorella:** Methodology, Formal analysis, Validation, Investigation. **Henrique Cesar Pereira Figueiredo:** Conceptualization, Visualization, Investigation, Writing – review & editing, Resources, Funding acquisition. **Guilherme Campos Tavares:** Conceptualization, Visualization, Investigation, Writing – review & editing, Resources, Funding acquisition, Supervision. **Sílvia Umeda Gallani:** Project administration, Conceptualization, Visualization, Writing – original draft, Writing – review & editing, Investigation, Resources, Funding acquisition, Supervision.

Declaration of Competing Interest

All authors declare absence of conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.aquaculture.2022.738406.

References

Abdelhamed HA, Banes M, Karsi A, Lawrence ML, 2019. Recombinant ATPase of virulent *Aeromonas hydrophila* protects channel catfish against motile *Aeromonas* septicemia. Front. Immunol. 10, 1641. https://doi.org/10.3389/fimmu.2019.01641.

Akinola, OG, Olakunbi, OO, 2019. Antibiotic sensitivity and sodium chloride susceptibility patterns of *Flavobacterium columnare* isolated from clinical columnaris in cultured *Clarias gariepinus*. J. Veterin. Med. Animal Health 11(3), 59–63. https://doi.org/10.5897/JVMAH2018.0724 .

Assis, GB, Pereira, FL, Zegarra, AU, Tavares, GC, Leal, CA, Figueiredo, HC, 2017. Use of MALDI-TOF mass spectrometry for the fast identification of gram-positive fish pathogens. Front. Microbiol. 8, 1492. https://doi.org/10.3389/fmicb.2017.01492.

Baldissera, MD, de Freitas Souza, C., Val, AL, Baldisserotto, B., 2020a. Involvement of purinergic signaling in the Amazon fish *Pterygoplichthys pardalis* subjected to handling stress: relationship with immune response. Aquaculture 514, 734481. https://doi.org/10.1016/j.aquaculture.2019.734481.

Baldissera MD, Souza CF, Tavares GC, Valladao ˜GM, Da Silva AS, Antoniazzi, A., Baldisserotto, B., 2020b. Purinergic signaling and gene expression of purinoceptors in the head kidney of the silver catfish *Rhamdia quelen* experimentally infected by *Flavobacterium columnare*. Microb. Pathhog. 142, 104070 https://doi.org/10.1016/j.micpath.2020.104070 .

Barbas, LAL, de Araujo, ERL, Torres, MF, Maltez, LC, de Oliveira Garcia, L., Heinzmann, BM, Sampaio, LA, 2020. Stress relieving potential of two plant-based sedatives in the transport of juvenile tambaqui *Colossoma macropomum*. Aquaculture 520, 734681. https://doi.org/10.1016/j.aquaculture.2019.734681.

Barony, GM, Tavares, GC, Assis, GBN, Luz, RK, Figueiredo, HCP, Leal, CAG, 2015. New hosts and genetic diversity of *Flavobacterium columnare* isolated from native Brazilian species and Nile tilapia. Dis. Aquat. Org. 117(1), 1–11. https://doi. org/10.3354/dao02931.

BCAF, 2017. Brazilian Compendium of Animal Feed, 5th. National Union of Animal Feed Industry, Sao Paulo, Brazil.

Beaz-Hidalgo, R., Martínez-Murcia, A., Figueras, MJ, 2013. Reclassification of *Aeromonas hydrophila* subsp. *dhakensis* Huys et al. 2002 and *Aeromonas aquariorum* Martinez-Murcia et al. 2008 *Aeromonas dhakensis* sp. nov. comb nov. and amendment of the species *Aeromonas hydrophila*. Syst. app. Microbiol. 36(3), 171–176. https://doi.org/10.1016/j.syapm.2012.12.007.

Cech Jr., JJ, Bartholow SD, Young PS, Hopkins TE, 1996. Striped bass exercise and handling stress in freshwater: physiological responses to recovery environment. Trans. Am. Fish. Soc. 125(2), 308–320. https://doi.org/10.1577/1548-8659(1996)125<0308:SBEAHS>2.3.CO;2.

Chandra H, Bishnoi P, Yadav A, Patni B, Mishra AP, Nautiyal AR, 2017. Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials—a review. Plants 6 (2), 16. https://doi.org/10.3390/plants6020016 .

Chettri JK, Al-Jubury A, Dalsgaard I, Heegaard PMH, Buchmann K, 2018. Experimental anal infection of rainbow trout with Flavobacterium psychrophilum: a novel challenge model. J. Fish Dis. 41 (12), 1917–1919. https://doi.org/10.1111/jfd.12888 .

Chockmangmeepisan P., Sakulworakan R., Dong HT, Kayansamruaj P., Rung ruangkijkrai T., Pirarat N., Rodkhum C., 2020. Virulence properties and pathogenicity of Flavobacterium columnare in hybrid red *tilapia* (Oreochromis sp.). *The Thai*. J. Vet. Med. 50(1), 103–108. https://he01.tci-thaijo.org/index.php/tjvm/article/view/243263.

Correa, RD-O, Sousa, ARB, Martins Junior, H., 2018. Tambaqui breeding. Embrapa amazon Oriental-Folder/Leaflet/Booklet (INFOTECA-E).

Costa, JDS, Silva, HNPD, Sousa, EMDO, Silva, RCS, Valente, ASO, Machado, S. DDS, Silva, LVFD, 2020. The use of *Myrcia sylvatica* hydrolate (G. MEY.) DC. (Myrtaceae) as a sedative under simulated conditions of transport of juvenile tambaqui *(Colossoma macropomum)*. J. Appl. Aquac. 1–13. https://doi.org/10.1080/10454438.2020.1777243.

Derome N., Gauthier J., Boutin S., Llewellyn M., 2016. Bacterial opportunistic pathogens of fish. In: The Rasputin Effect: When Commensals and Symbionts Become Parasitic. Springer, Cham, pp. 81–108. https://doi.org/10.1007/978-3-319-28170-4_4 .

Dong, HT, Techatanakitarnan, C., Jindakittikul, P., Thaiprayoon, A., Taengphu, S., Charoensapsri, W., Senapin, S., 2017. *Aeromonas jandaei* and *Aeromonas* veronii caused disease and mortality in Nile tilapia, *Oreochromis niloticus* (L.). J. Fish Dis. 40 (10), 1395–1403. https://doi.org/10.1111/jfd.12617.

FAO, 2020. Food and Agriculture Organization of the United Nations. http://www.fao. org/state-of-fisheries-aquaculture.

Figueiredo HCP, Klesius PH, Arias CR, Evans J., Shoemaker CA, Pereira Jr., DJ Peixoto MTD, 2005. Isolation and characterization of strains of Flavobacterium columnare from *Brazil* . J. Fish Dis. 28(4), 199–204. https://doi.org/10.1111/j.1365- 2761.2005.00616.x.

Gallani, SU, Valladao, ˜GMR, Assane, IM, de Oliveira Alves, L., Kotzent, S., Hashimoto, DT, Pilarski, F., 2020. Motile Aeromonas septicemia *in* tambaqui *Colossoma macropomum:* pathogenicity, lethality and new insights for control and disinfection in aquaculture. Microb. Pathhog. 149, 104512 https://doi.org/10.1016/j.micpath.2020.104512.

Gallani SU, Valladao ˜GMR, de Oliveira Alves L., Kotzent S., Hashimoto DT, Wiegertjes G., Pilarski F., 2021. Patterns of the innate immune response in tambaqui Colossoma macropomum: *modulation* of gene expression in haemorrhagic septicaemia caused by *Aeromonas hydrophila*. Microb. Pathhog. 150, 104638 https://doi.org/10.1016/j.micpath.2020.104638 .

Gomes LDC, Araujo-Lima CARM, Roubach R., Urbinati EC, 2003a. Evaluation of the effects of adding salt and density on tambaqui transport. Research Agrop. Brasileira 38, 283–290. https://doi.org/10.1590/S0100-204X2003000200016.

Gomes LC, Roubach R, Araujo-Lima CA, Chippari-Gomes AR, Lopes NP, Urbinati, EC, 2003b. Effect of fish density during transportation on stress and mortality of juvenile tambaqui *Colossoma macropomum*. J. World Aquacult. Soc. 34(1), 76–84. https://doi.org/10.1111/j.1749-7345.2003.tb00041.x.

Hayakijkosol, O., Owens, L., Picard, J., 2017. Case report of bacterial infections in a redclaw crayfish *(Cherax quadricarinatus)* hatchery. Aquaculture 475, 1–7. https://doi.org/10.1016/j.aquaculture.2017.03.038 .

Klesius PH, Shoemaker CA, Evans JJ, 2008. *Flavobacterium columnare* chemotaxis to channel catfish mucus. FEMS Microbiol. Lett. 288(2), 216–220. https://doi.org/10.1111/j.1574-6968.2008.01348.x .

LaFrentz BR, Garcia JC, Shelley JP, 2019. Multiplex PCR for genotyping *Flavobacterium columnare*. J. Fish Dis. 42 (11), 1531–1542. https://doi.org/10.1111/jfd.13068 .

Lange MD, Farmer BD, Declercq AM, Peatman E, Decostere A, Beck BH, 2017. Sickeningly sweet: L-rhamnose stimulates *Flavobacterium columnare* biofilm formation and virulence. J. Fish Dis. 40 (11), 1613–1624. https://doi.org/10.1111/jfd.12629 .

Leal, JF, Santos, EB, Esteves, VI, 2019. Oxytetracycline in intensive aquaculture: water quality during and after its administration, environmental fate, toxicity and bacterial resistance. Rev. Aquac. 11 (4), 1176–1194. https://doi.org/10.1111/raq.12286 .

Li N, Zhu Y, LaFrentz BR, Evenhuis JP, Hunnicutt DW, Conrad RA, McBride M. J., 2017. The type IX secretion system is required for virulence of the fish pathogen *Flavobacterium columnare*. app. Environ. Microbiol. 83 (23) https://doi.org/10.1128/AEM.01769-17 e01769–17.

Limbu, SM, Zhang, H., Luo, Y., Chen, LQ, Zhang, M., Du, ZY, 2020. High carbohydrate diet partially protects Nile tilapia *(Oreochromis niloticus)* from oxytetracycline-induced side effects. Environ. Pollut. 256, 113508 https://doi.org/10.1016/j.envpol.2019.113508 .

Loch, TP, Faisal, M., 2015. Emerging flavobacterial infections in fish: a review. J. Adv. Res. 6(3), 283–300. https://doi.org/10.1016/j.jare.2014.10.009.

Martinez-Murcia AJ, Monera A., Saavedra MJ, Oncina R., Lopez-Alvarez M., Lara E., Figueras MJ, 2011. Multilocus phylogenetic analysis of the genus Aeromonas . Syst. app. Microbiol. 34(3), 189–199. https://doi.org/10.1016/j. syapm.2010.11.014.

Martino ME, Fasolato L., Montemurro F. , Rosteghin M., Manfrin A., Patarnello T., Cardazzo B., 2011. Determination of microbial diversity of *Aeromonas* strains on the basis of multilocus sequence typing , phenotype, and presence of putative virulence genes. app. Environ. Microbiol. 77 (14), 4986–5000. https://doi.org/10.1128/AEM.00708-11.

Martos-Sitcha JA, Mancera JM, Prunet P., Magnoni LJ, 2020. Welfare and stressors in fish: challenges facing aquaculture. Front. Physiol. 11, 162. https://doi.org/10.3389/fphys.2020.00162 .

Nakano, T., Hayashi, S., Nagamine, N., 2018. Effect of excessive doses of oxytetracycline on stress-related biomarker expression in coho salmon. Environ. Sci. Pollut. Res. 25 (8), 7121–7128. https://doi.org/10.1007/s11356-015-4898-4.

Pavanelli GC, Eiras J, Takemoto RM, 2008. Fish disease, prophylaxis, diagnosis and treatment. 3rd Ed. Brazil (in Portuguese).

P´erez-Sancho, M., Cerda, I., Fern´andez-Bravo, A., Domínguez, L., Figueras, MJ, Fernandez-Garayzabal, JF, Vela, AI, 2018. Limited performance of MALDI- TOF for identification of fish *Aeromonas* isolates at species level. J. Fish Dis. 41 (10), 1485–1493. https://doi.org/10.1111/jfd.12837.

Pilarski, F., Rossini, AJ, Ceccarelli, PS, 2008. Isolation and characterization of *Flavobacterium columnare* (Bernardet et al. 2002) from four tropical fish species in Brazil. Braz. J. Biol. 68, 409–414. https://doi.org/10.1590/S1519-69842008000200025.

Pradhan PK, Paria A, Pande V, Verma DK, Arya P, Rathore G, Sood N, 2019. Expression of immune genes in Indian major carp, *Catla catla* challenged with *Flavobacterium columnare*. Fish Shellfish Immunol. 94, 599–606. https://doi.org/10.1016/j.fsi.2019.09.052 .

Rijkers GT, Teunissen AG, Van Oosterom R., Van Muiswinkel WB, 1980. The immune system of cyprinid fish. The immunosuppressive effect of the antibiotic oxytetracycline in carp *(Cyprinus carpio* L.). Aquaculture 19 (2), 177–189. https://doi.org/10.1016/0044-8486(80)90018-6 .

Rijkers, GT, Van Oosterom, R., Van Muiswinkel, WB, 1981. The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp *(Cyprinus carpio)*. Vet. Immunol. Immunopathol. 2(3), 281–290. https://doi.org/10.1016/0165-2427(81)90029-5 .

Sampaio, FD, Freire, CA, 2016. An overview of stress physiology of fish transport: changes in water quality as a function of transport duration. Fish Fish. 17 (4), 1055–1072. https://doi.org/10.1111/faf.12158.

Santos, ELR, Rezende, FP, Moron, SE, 2020. Stress-related physiological and histological responses of tambaqui *(Colossoma macropomum)* to transportation in water with tea tree and clove essential oil anesthetics. Aquaculture 735164. https://doi.org/10.1016/j.aquaculture.2020.735164 .

Sebasti˜ao, FDA, Pilarski, F., Lemos, MVF, 2013. Composition of extracellular polymeric substances (EPS) produced by *Flavobacterium columnare* isolated from tropical fish in Brazil. Braz. J. Microbiol. 44(3), 861–864. https://www.scielo.br/j/bjm/a/GCq6c9vJj5TMjVgGkm6nNvq/?format=pdf&lang=en .

Seghouani, H., Garcia-Rangel, CE, Füller, J., Gauthier, J., Derome, N., 2017. Walleye autochthonous bacteria as promising probiotic candidates against *Flavobacterium columnare*. Front. Microbiol. 8, 1349. https://doi.org/10.3389/fmicb.2017.01349.

Shirajum Monir M, Yusoff SM, Mohamad A, Ina-Salwany MY, 2020. Vaccination of tilapia against motile *Aeromonas* septicemia: a review. J. Aquat. animation Health 32(2), 65–76. https://doi.org/10.1002/aah.10099.

Silva, HNP, dos Santos-Machado, SD, de Andrade Siqueira, AM, da Silva, ECC, de Oliveira Canto, MA, Jensen, L., Baldisserotto, B., 2020. Sedative and anesthetic potential of the essential oil and hydrolate from the fruit of *Protium heptaphyllum* and their isolated compounds in *Colossoma macropomum* juveniles. Aquaculture 529, 735629. https://doi.org/10.1016/j.aquaculture.2020.735629.

Suomalainen LR, Tiirola MA, Valtonen ET, 2005. Influence of rearing conditions on Flavobacterium columnare infection of rainbow trout, Oncorhynchus mykiss (Walbaum). J. Fish Dis. 28(5), 271–277. https://doi.org/10.1111/j.1365-2761.2005.00631.x.

Tavares-Dias, M., 2022. Toxicity, physiological, histopathological, handling, growth and antiparasitic effects of the sodium chloride (salt) in the freshwater fish aquaculture. Aquac. Res. 53(3), 715–734. https://doi.org/10.1111/are.15616.

Tekedar, HC, 2020. Sequencing and Analysis of the *Flavobacterium Columnare* ATCC 49512 *Genome* (Doctoral dissertation). Mississippi State University. https://ir.library.msstate.edu/handle/11668/17687 .

Vallad˜ao, GMR, Gallani, SU, Pilarski, F., 2018. South American fish for continental aquaculture. Rev. Aquac. 10(2), 351–369. https://doi.org/10.1111/raq.12164.

Van Doan, H., Soltani, M., Ringø, E., 2021. In vitro antagonistic effect and in vivo protective efficacy of gram-positive probiotics versus Gram-negative bacterial pathogens in finfish and shellfish. Aquaculture 540, 736581. https://doi.org/10.1016/j.aquaculture.2021.736581 .

Woynarovich, A., Van Anrooy, R., 2019. Field guide to the culture of tambaqui *(Colossoma macropomum,* Cuvier, 1816). *FAO Fisheries and Aquaculture Technical Paper,* (624), I-121. https://www.fao.org/3/ca2955en/ca2955en.pdf.

Xuan R, Arisi L, Wang Q, Yates SR, Biswas KC, 2009. Hydrolysis and photolysis of oxytetracycline in aqueous solution. J. Environ. Sci. Health B 45(1), 73–81. https://doi.org/10.1080/03601230903404556 .