

CENTRO DE AQUICULTURA DA UNESP

**POTENCIAL DE FITOTERÁPICOS,
PEPTÍDEOS ANTIMICROBIANOS E
COMBINAÇÃO DE ANTIBIÓTICOS NO
TRATAMENTO DE DOENÇAS BACTERIANAS
DE PEIXES**

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CENTRO DE AQUICULTURA DA UNESP

**POTENCIAL DE FITOTERÁPICOS,
PEPTÍDEOS ANTIMICROBIANOS SINTÉTICOS
E TERAPIA COMBINADA NO TRATAMENTO
DE DOENÇAS BACTERIANAS DE PEIXES**

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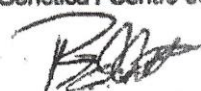
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Resumo

Esta Tese aborda a problemática da emergência de doenças bacterianas na piscicultura, num cenário de limitada disponibilidade de antimicrobianos eficazes. Os resultados são apresentados em cinco capítulos (manuscritos) que correspondem a seus objetivos. No primeiro manuscrito, são descritos surtos de doença bacteriana emergente, em piscicultura e instalações laboratoriais, respectivamente; evidenciando a ineficácia terapêutica dos antimicrobianos aprovados para uso na piscicultura brasileira. O segundo manuscrito, descreve a diversidade genética, a virulência e o perfil de resistência a antimicrobianos de uma das principais bactérias patogênicas (estreptococo do grupo B) da principal espécie de peixe cultivada no Brasil (tilápia-do-Nilo, *Oreochromis niloticus*). O terceiro e o quarto manuscrito, demonstram o potencial de peptídeos antimicrobianos sintéticos e óleos essenciais derivados de plantas, respectivamente; como uma alternativa aos antimicrobianos convencionais no controle das principais doenças bacterianas de peixes cultivados em regiões tropicais; destacando sua síntese, composição química, citotoxicidade, atividade antimicrobiana e interação com antimicrobianos convencionais. No quinto capítulo, é descrita a farmacocinética da combinação de dois antimicrobianos convencionais (tianfenicol e florfenicol) em tilápia-do-Nilo. Os resultados obtidos indicam que novas opções terapêuticas eficazes contra doenças bacterianas são necessárias no Brasil e peptídeos antimicrobianos sintéticos, óleos essenciais derivados de plantas e terapia combinada têm potencial de contribuir para minimizar a problemática.

Palavras-chave: doenças bacterianas, resistência antimicrobiana, peptídeos antimicrobianos sintéticos, óleos essenciais derivados de plantas, sinergismo, cromatografia líquida de alta eficiência, teste de sensibilidade aos antimicrobianos.

Abstract

This thesis addresses the issue of the emergence of bacterial diseases in fish farming, in a scenario of limited availability of effective antimicrobials. The results are presented in five chapters (manuscripts) that correspond to the objectives. In the first manuscript, outbreaks of emerging bacterial disease are described in a fish farm and laboratory facility, respectively; denoting the ineffectiveness of antimicrobials approved for use in Brazilian fish farming. The second manuscript describes the genetic diversity, virulence, and antimicrobial resistance profile of one of the main pathogenic bacteria (group B *Streptococcus*, GBS) of the main fish species cultivated in Brazil (Nile tilapia, *Oreochromis niloticus*). The third and fourth manuscripts describe the synthesis, chemical composition, cytotoxicity, and antimicrobial activity of synthetic antimicrobial peptides (AMPs), plant-derived essential oils (EOs) and their combinations with conventional antimicrobials, respectively; highlighting their potential as an alternative to conventional antimicrobials against the main bacterial diseases of fish cultured in tropical regions. In the fifth manuscript, the pharmacokinetics of the combination of two conventional antimicrobials (thiamphenicol and florfenicol) in Nile tilapia is described. Our findings indicate that new effective therapeutic options against bacterial diseases are needed in Brazil and AMPs, EOs and combined therapy are good treatment options for bacterial diseases in aquaculture.

Keywords: bacterial diseases, antimicrobial resistance, synthetic antimicrobial peptides, plant-derived essential oils, synergism, high-performance liquid chromatography, antimicrobial susceptibility test.

General Introduction

Aquaculture accounts for 50 per cent of the world's fish that is used for food and is probably the fastest-growing food-production sector (FAO, 2021a). Currently, 387 species and/or species groups of finfishes are being farmed commercially around the world (FAO, 2021a), and several species not farmed are known to be suitable for aquaculture, mainly due to their zootechnical characteristics such as fast growth, large size or low requirement for fishmeal and oil (Mylonas et al., 2019). Moreover, selective breeding for genetic improvement of production traits has great potential to increase efficiency and reduce the environmental impacts of most species currently being farmed (Houston et al., 2020). Nevertheless, despite this diversity, the main aquaculture production is dominated by a small number of species or species groups at national, regional, and global levels (FAO, 2020). According to the Food and Agriculture Organization of the United Nations (FAO, 2020), in 2018, carp and Nile tilapia were the major species produced in the world aquaculture, corresponding to ~ 51.8 % of finfish production.

Due to their zootechnical characteristics, such as strong environmental adaptability, ease of breeding, disease resistance, high protein content, large size, rapid growth, and palatability, Nile tilapia, *Oreochromis niloticus* L., is probably the most widely introduced species and economically important as food fish in many countries, being introduced in 114 countries or regions (FAO, 2021b), including Brazil, where it represented, in 2020, 62.3% of fish production (IBGE, 2020). Brazil has a good tilapia sector, which grows fast and represents over 74% of the production of Nile tilapia in the Americas. In the last decade, tilapia production increased from 150,000 to over 400,000 tonnes, making Brazil the 4th major Nile tilapia producer in the world, behind China, Indonesia, and Egypt (FAO, 2021b; IBGE, 2020; Peixe BR, 2021). However, this sector is being negatively impacted by bacterial diseases (Chideroli et al., 2017; Delphino et al., 2019; Junior et al., 2020; Sebastião, Furlan, Hashimoto, & Pilarski, 2015; Sebastião, Pilarski, Kearney, & Soto, 2017) and the lack of effective therapies (de Oliveira, Queiroz, Teixeira, Figueiredo, & Leal, 2018). According to Tavares-dias & Martins (2017), in Brazil, the annual loss to the inland aquaculture sector due to parasitic and bacterial diseases is estimated to be USD 84 million.

Currently, *Streptococcus agalactiae*, group B *Streptococcus* (GBS), is the main bacterial pathogen of Nile tilapia, causing massive deaths in all stages of the farming cycle throughout the year (Chideroli et al., 2017), with higher frequency when the water temperature is $> 28^{\circ}\text{C}$ (Delphino et al., 2019). On the other hand, only two antimicrobials, florfenicol (FFC) and oxytetracycline (OTC) are permitted for use in Brazilian aquaculture (SINDAN, 2021), despite the reports of vaccine failure and antimicrobial ineffectiveness after a streptococcosis outbreak (Chideroli et al., 2017; de Oliveira et al., 2018).

Due to the high economic impacts of GBS infections in Nile tilapia worldwide, several strategies are being evaluated and/or used to prevent GBS outbreaks and reduce the economic impacts when they occur. Breeding Nile tilapia that are resistant to *S. agalactiae* infection (Shoemaker et al., 2017; Suebsong et al., 2019), the use of commercial and autogenous vaccines (Barnes et al., 2021), dietary supplementation with prebiotics (Pilarski, Ferreira de Oliveira, Darpossolo de Souza, & Zanuzzo, 2017) and probiotics (Xia, Wang, Gao, Lu, & Chen, 2020; Yamashita et al., 2017), and the use of new effective and environmentally friendly treatments (de Sousa et al., 2021; Luo et al., 2018) are among the main strategies being evaluated. However, as with other bacterial infections, the use of antimicrobials remains the main strategy for disease outbreak control. Thus, the approval of new effective antimicrobials for use in fish farming is relevant.

The use of antimicrobials for prophylactic and therapeutic purposes has become a common practice in aquaculture in the last two decades. Only between 2008 and 2018, about 67 antimicrobials or their combinations were used among the main producing countries of aquatic organisms (Lulijwa, Rupia, & Alfaro, 2019). The annual consumption, in 2017, was estimated at 10,259 tons (Schar, Klein, Laxminarayan, Gilbert, & Boeckel, 2020). Nevertheless, most of the available antimicrobials are at risk of losing effectiveness due to misuse and consequent development of bacterial resistance. For example, *S. agalactiae* has been reported to be resistant to multiple antimicrobials, including gentamycin, kanamycin, trimethoprim, nitrofurantoin, ampicillin, spiramycin, oleandomycin, sulphamethoxazole, oxolinic acid, penicillin, erythromycin, OTC, and FFC (Assane, Gozi, Valladão, & Pilarski, 2019; de Oliveira et al., 2018; Faria, Leal, Carvalho-Castro, Leite, & Figueiredo, 2014; Soto et al., 2015).

Despite the lack of uniformity in antimicrobials permitted for aquaculture in many producing countries, OTC and FFC are among the few antimicrobials permitted for disease control in food fish in many countries (Yuting, Aiping, Fei, & Lan, 2020). Florfenicol is an antimicrobial of the phenicol drug family, derived from thiamphenicol (TAP), and considered a critically important antimicrobial for fish disease control (OIE, 2015). It inhibits bacterial protein synthesis and has a broad spectrum of action against bacteria (Gram-negative and positive, aerobic, and anaerobic) (Fukui, Fujihara, & Kano, 1987; Syriopoulou, Harding, Goldmann, & Smith, 1981), no prolonged post-antimicrobial effect (Rairat, Yu, Wipavee, Chi, & Chou, 2019), low toxicity to Nile tilapia, and low environmental risk (Mattioli et al., 2020). Due to its short half-life (Kogiannou, Nikoloudaki, Katharios, Triga, & Rigos, 2021), it is considered primarily time-dependent bacteriostatic. Its lipophilic characteristic allows rapid absorption (J. Feng & Jia, 2009; Kogiannou et al., 2021) and wide tissue distribution (J. B. Feng, Huang, Zhong, Liu, & Dong, 2016), ensuring that concentrations are high enough to treat intracellular pathogens and cross some anatomical barriers, such as the barrier blood-brain (Papich, 2016). Its main metabolite is florfenicol amine (FFCA), which is considered a marker residue to determine the withdrawal period in food fish.

The bioavailability of FFC is high in several fish species, with reports of 91% and 99% of bioavailability after oral administration of 10 mg/kg.bw in cod (Samuelson, Bergh, & Ervik, 2003) and in Atlantic salmon (Horsberg, Hoff, & Nordmo, 1996), respectively. The recommended dose and administration regimen vary between countries and fish species (i.e., the recommended dose in Brazil is 10 - 20 mg/kg/day for 10 days, depending on the fish species, while in the USA is 10 - 15 mg/kg/day for 10 days, and in Taiwan is 10 mg/kg/day for 3-5 days) (FDA, 2007; Rairat, Hsieh, Thongpian, Sung, & Chou, 2019; SINDAN, 2022).

Despite its wide use in aquaculture and strong antimicrobial activity against the main fish bacterial pathogens, such as *Aeromonas hydrophila* (Assane et al., 2019; G. Bandeira Junior et al., 2018; Daniela T. Godoy et al., 2008), *A. jandei* (Assane et al., 2019), *A. veronii* (Assane et al., 2019; G. Bandeira Junior et al., 2018), *Vibrio fluvialis* (Assane et al., 2019), and *S. agalactiae* (Chideroli et al., 2017; de Oliveira et al., 2018), there are reports of resistant strains (Assane et al., 2019; G. Bandeira Junior et al., 2018; Gallani, Sebastião, Valladão, Boaratti, & Pilarski, 2016), treatment failure, and recurrent infection in Nile tilapia after treatment using the recommended dose and

regimen (de Oliveira et al., 2018). Therefore, new antimicrobials or therapies that are both environmentally friendly and effective must be conceived for the treatment of the increasing number of bacterial diseases that affect cultured fish.

The use of antimicrobial peptides (AMPs), plant-derived essential oils (EOs) and combination therapy (CT) are among the promising strategies to reduce the use of antimicrobials in aquaculture while reducing the economic impacts of bacterial disease outbreaks. AMPs are important components of the innate and adaptive immune system of fish and help to protect the fish from microbial infection. The mechanisms of action of AMPs vary from one to another. However, most AMPs act by disrupting the cytoplasmic membranes of both Gram-positive and Gram-negative bacteria, resulting in depolarization and cell death. Moreover, bacteria are less likely to develop resistance against AMPs due to their action as an immune system modulator (Scheenstra et al., 2019) and direct action (bind and neutralization) on bacterial lipopolysaccharides (LPS) (Sigurdardottir et al., 2006). On the other hand, it is known that some EOs have broad-spectrum antimicrobial activity against fish pathogens, and several potential benefits for fish farming, such as the improvement of fish welfare, health and zootechnical performance indices (Acar, Kesbiç, İnanan, & Yılmaz, 2019; de Souza et al., 2019; Sutili, Gatlin, Heinzmann, & Baldisserotto, 2018), and the reduction of bacterial resistance phenomenon (Dugo & di Giacomo, 2002).

Combination therapy is a therapy that consists of using more than one antimicrobial to treat the same condition or disease. This form of therapy has been shown to be promising for solving the aforementioned problem; as it reduces the emergence of bacterial resistance (Milatovic & Braveny, 1987; Xu et al., 2018), it is effective against multiresistant pathogens (Allen, Cha, & Rybak, 2002; Guner, Hasanoglu, Keske, Kalem, & Tasyaran, 2011; Lin et al., 2015; Xu et al., 2018) and can give satisfactory therapeutic results using lower doses of antimicrobials (Assane et al., 2019; Guerino Bandeira Junior et al., 2021). Combination therapy is often used to improve clinical outcomes of infections by pathogens susceptible to one or more individual antimicrobials (Petrosillo, Ioannidou, & Falagas, 2008; Rahal, 2006). Currently, there are at least four combinations of antimicrobials approved for use in aquaculture in different countries (FDA, 2007; Felleskatalogen, 2018; Health Canada, 2010) and new promising combinations are being studied (BANDEIRA JUNIOR et al., 2018; ASSANE; ASSANE et al., 2019).

The combination of TAF with FFC stands out among the promising combinations for use in Brazilian aquaculture. There are reports of a synergistic effect of this combination against both Gram-negative and Gram-positive pathogenic bacteria recovered from diseased cattle, chickens (C. F. Wei, Shien, Chang, & Chou, 2016), swine (C. Wei et al., 2016) and fish (Assane et al., 2019). Moreover, the differences in the mechanisms of bacterial resistance to these antimicrobials of the same class usually make bacteria resistant to one of them susceptible to the other (van Hoek et al., 2011), allowing the occurrence of a synergistic interaction when CT is used. However, there is no information about its effect against *S. agalactiae* isolated from Nile tilapia.

The cost of antimicrobial research and development is high, so all drug candidates selected for clinical trials in animals must be evaluated as efficiently and cost-effectively as possible. It is proven that the application of *in vitro* antimicrobial susceptibility testing, and pharmacokinetic-pharmacodynamic principles to this process improves the selection of appropriate doses and clinical trial design (CLSI, 2013, 2020; Kazakevich & LoBrutto, 2006; Rosenbaum, 2017) necessary for antimicrobial registration (MAPA, 2004).

Considering this scenario, and the Brazilian potential for an increase in aquaculture production (FAO, 2020), screening non-cytotoxic compounds for antimicrobial activity against fish pathogens, evaluating different therapeutic strategies, and understanding pharmacokinetic and pharmacodynamic proprieties of promising antimicrobials are cost-effective and sustainable solutions to address, in the long term, the issue of the emergence of bacterial diseases in a scenario of limited availability of effective antimicrobials. Thus, this thesis aims to evaluate these approaches as a solution for disease control in Brazilian aquaculture. Pieces of evidence of an emerging pathogen in the Brazilian Nile tilapia industry and genetic diversity, virulence, and antimicrobial resistance profiles of the main bacterial pathogen are provided. Additionally, the antimicrobial activity, therapeutic effectiveness, pharmacokinetics, toxicity and effects on the immunity and gut microbiota of Nile tilapia, of different antimicrobials were determined.

MANUSCRITO I

ORIGINAL ARTICLE

Phenotypic and genotypic characterization of *Aeromonas jandaei* involved in mass mortalities of cultured Nile tilapia, *Oreochromis niloticus* (L.) in Brazil

[Available online since 30 April 2021 \(Aquaculture, ISSN: 0044-8486\)](#)

Abstract

Aeromonas jandaei is an emerging fish pathogen associated with massive mortalities in cultured freshwater fish. This study investigated the morphological, biochemical, molecular, virulence, pathogenicity, and antimicrobial susceptibility characteristics of four strains of *A. jandaei* involved in the occurrence of mass mortalities of cultured Nile tilapia in an earthen pond farm and laboratory fibreglass tanks in Brazil. Isolates were identified by morphological, biochemical, and molecular analyses. Bacteria morphology was assessed under light and scanning electron microscopes, biochemical profile by conventional biochemical tests, and molecular identification by nucleotide sequencing and phylogenetic analysis of the highly conserved 16S rRNA gene, as well as the housekeeping genes, *gyrB*, and *rpoB*. The virulence and pathogenicity were confirmed by screening for 12 virulence genes and induced experimental infection. Broth microdilution method was used to assess susceptibility to enrofloxacin, florfenicol, oxytetracycline, and thiamphenicol. All isolates were confirmed as *A. jandaei*. Haemolysin, temperature-sensitive protease, haemolysin-aerolysin, and nuclease were the most predominant virulence genes. Lateral flagella B and cytolytic enterotoxin/cytotoxic enterotoxin were only detected in strains isolated from fish from fibreglass tanks. In the experimental challenge, doses above 4.3×10^7 CFU mL⁻¹ resulted in mass mortality (100%) in a short period (less than 12 h) without remarkable external clinical signs. Typical clinical signs of disease, including lethargy, inappetence, surface swimming, exophthalmia, cloudy eyes, haemorrhagic patches, and redness of the skin, below the opercula, and at the base of all fins, fin rot, and pale body surface were observed in fish challenged with doses below 4.0×10^7 CFU mL⁻¹ (10–100% mortality). The most predominant histopathological changes in the internal organs of diseased fish were melano-macrophage centres, vascular congestion with thrombus, haemorrhage, and necrosis. Strains isolated from fish from the earthen pond farm were resistant to oxytetracycline, while the strains from

fibreglass tanks were sensitive to all antimicrobials. This study provides pertinent data on *A. jandaei* host-pathogen interactions, susceptibility to antimicrobials, morphological, biochemical, and molecular characteristics, and geographical distribution, which may serve as a guideline for *A. jandaei* isolation and identification in aquaculture.

Keywords: Bacterial infection, mass mortality, virulence, antimicrobial resistance, housekeeping genes, scanning electron microscopy.

Highlights

- The multilocus phylogenetic analysis allowed to clarify the phylogenetic relationships of the clinical isolates.
- This is the first detailed report of mass mortality caused by *Aeromonas jandaei* in Brazil.
- Florfenicol (10 mg kg⁻¹ bw) and Oxytetracycline (120 mg kg⁻¹ bw) were ineffective to control the outbreak in the fish farm.
- *A. jandaei* killed 100% of fish in less than 12 h without remarkable clinical signs.
- Haemolysin, temperature-sensitive protease, haemolysin-aerolysin and nuclease were detected in all clinical isolates.

1. Introduction

Brazil stands out as one of the countries with the greatest potential for the expansion of aquaculture in the world (Gentry et al., 2017), in a time of growing demand for fish and fish products due to the rapid world population growth and the increasing preference for healthier foods (FAO, 2020). However, the intensification of aquaculture is prone to problems of bacterial diseases owing to improper husbandry practices, high stocking density, inappropriate feeding rates, exposure to poor water quality, and a lack of biosecurity measures. Disease outbreaks are unpredictable and usually followed by significant economic losses (Tavares-dias and Martins, 2017), which contribute to an increase in the risks and uncertainties about the future of the activity and a reduction of investment in the sector.

Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), is the second most cultured fish species in the world (after carps) (FAO, 2020), and it represents 61.1% of all the cultured fish in Brazil (IBGE, 2019). Mass mortality caused by bacterial diseases in Nile tilapia aquaculture is very common and has been reported in many countries (Abdel-Latif et al., 2020; Abdel-Latif and Khafaga, 2020; Amal et al., 2018; Oh et al., 2020; Raj et al., 2019). Nevertheless, no one bacterial infectious agent is specific for Nile tilapia, it being a well-adapted fish species with good resistance to bacterial infection as long as it is kept under good water quality conditions, in the proper temperature range, and using the proper husbandry practices (Plumb and Hanson, 2011).

Although the role of aeromonads as primary or secondary pathogens of fish has been known for decades (Austin and Austin, 2016; Hoai et al., 2019; Janda and Abbott, 2010; Rasmussen-Ivey et al., 2016), and at least 14 taxa (out of 36 recognized taxa) of *Aeromonas* are known to be pathogenic in fish (Carriero et al., 2016; Dong et al., 2017; Fernández-Bravo and Figueras, 2020; Ren et al., 2019; Yi et al., 2013), only *A. hydrophila* and *A. veronii* are commonly reported species associated with mass mortality in Nile tilapia in most countries (Delphino et

al., 2019; Hassan et al., 2017; Sudheesh et al., 2012). Thus, their clinical signs, diagnosis, bacterial characteristics, epizootiology, pathology, and significance in Nile tilapia aquaculture is well described in the literature compared to other pathogenic aeromonads. In contrast, there is little information about *A. jandaei* as a causative agent of disease in Nile tilapia, despite it being capable of causing mass mortality (Dong et al., 2017).

A. jandaei was first isolated and associated with disease in humans (Carnahan et al., 1991b), and has been recently isolated from diseased freshwater crocodiles (Pu et al., 2019) and fish (Dong et al., 2017; Guo et al., 2016). Due to the challenging process of identifying *Aeromonas* to the species level, mainly when only routine methods are used, such as conventional biochemical tests, commercial phenotypic tests and systems, or 16S rRNA gene sequencing (Fernández-Bravo and Figueras, 2020), *A. jandaei* can be misidentified as *A. caviae*, *A. hydrophila* or *A. veronii* (Carnahan et al., 1991a; Martinez-Murcia et al., 1992). In fact, *A. jandaei* shows 99.5%, 99.0%, and 98.3% similarity with *A. veronii*, *A. caviae*, and *A. hydrophila* in the complete 1,502 bp 16S rRNA sequence (Martinez-Murcia et al., 1992), respectively. The correct identification relies on DNA-DNA hybridization methods (Esteve et al., 2003) and nucleotide sequencing of housekeeping genes (Guo et al., 2016), which are not routine methods for disease diagnosis.

This study aimed to report mass mortality caused by *A. jandaei* in Brazil along with its pathogenicity against Nile tilapia, morphological, molecular, and biochemical characteristics, and antimicrobial susceptibility. We identified and characterized isolates of *A. jandaei* recovered from fish exhibiting disease and mass mortality after exposure to stress by classification and transport in a commercial fish farm and laboratory facility, respectively. The identity of bacteria was confirmed by biochemical tests, morphological evaluation, nucleotide sequencing and phylogenetic analysis of the highly conserved 16S ribosomal RNA (rRNA) gene, as well as the housekeeping genes, *gyrB*, and *rpoB*. The virulence and pathogenicity were

confirmed by screening for 12 virulence genes and artificial infection. The antimicrobial activity of four commercial antimicrobials was determined.

2. Materials and methods

2.1. Outbreak description

The first outbreak of haemorrhagic septicaemia and mass mortality ($\geq 90\%$) occurred between August and September 2017 (winter season) in the Laboratory of Microbiology and Parasitology of Aquatic Organisms of São Paulo State University (Unesp), at the Aquaculture Center of Unesp (Caunesp), Jaboticabal, São Paulo, Brazil. In June 2017, a batch of healthy Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) fry (approximately 5 g body weight (bwt)) was obtained from a commercial fish farm in Sales Oliveira, State of São Paulo (Fig. 1), and reared for three months in an earth pond located inside Caunesp (Fig. 1A). In the last week of August, 340 healthy Nile tilapia juveniles (approximately 50 g bwt) were transported from the earth pond to 17 fibreglass tanks (500 L) with constant water flow and aeration, in the Laboratory of Microbiology and Parasitology of Aquatic Organisms facilities. After exposure to transport-induced stress, fish started dying from day 1 and mortality increased gradually and reached 45.4% on day 3 post-stocking. Cumulative mortality reached approximately 90% at day 10 post-stocking. Clinical signs were recorded and freshly dead fish were collected for parasitological and microbiological analyses.

During the outbreak, 20 fish exhibiting typical clinical signs of bacterial infection, including lethargy, inappetence, surface swimming, exophthalmia, cloudy eyes, haemorrhagic patches and redness of the skin, below the opercula, and at the base of all fins, fin rot, and pale body surface (Fig. 2A–C) were separated into two groups (G1 and G2) of 10 fish each and administered 10 mg kg⁻¹ bwt florfenicol (FFC) (G1) and 10 mg kg⁻¹ bwt thiamphenicol (TAP) (G2) through gavage. The typical clinical signs of the disease started to disappear 3 days post-

medication with a single oral dose of antimicrobial, and at day 10 post-medication all fish were healthy.

The second outbreak of disease and mass mortality occurred between April and May 2019 (autumn season) on a commercial fish farm in Panorama, São Paulo State, Brazil (Fig. 1B). In April 2019, a batch of apparently healthy Nile tilapia fry (approximately 5 g bwt) was transported from one commercial fish farm located in Panorama, São Paulo to another fish farm in the same city (3 km apart). One week after acclimation in earthen ponds, heavy mortality with no significant external clinical signs of disease or fish behaviour alteration was recorded. Two other episodes of heavy mortality with similar characteristics were recorded 10 and 20 days after the first outbreak, immediately after fish grading. Upon visual inspection of freshly dead fish, a swollen abdomen was the most evident pathological sign (Fig. 2D–F). FFC (10 mg kg⁻¹) and oxytetracycline (OTC, 120 mg kg⁻¹ bwt) were administered following the manufacturer's instructions during the outbreaks, but still, the deaths continued.

Cumulative mortality reached approximately 50% at day 30 post-stocking. A sample of fingerlings (15 g bwt) showing a swollen abdomen and/or small haemorrhages in the operculum, and the pelvic fin (Fig. 2D–F) was collected for parasitological and microbiological analyses at the Laboratory of Microbiology and Parasitology of Aquatic Organisms.

Water physicochemical parameters were appropriate for Nile tilapia maintenance during both outbreaks (data not shown).

2.2. Parasitological analyses

Samples from the gills, body mucus, stomach, and intestine were collected separately through scraping for the parasitological surveys. The material collected was deposited on glass slides and observed while fresh under an optical microscope. After observing a relatively high

prevalence of parasites in smears from the stomach during the first outbreak, the stomach was collected and preserved for scanning electron microscopy (SEM) analysis and histopathology.

2.3. Bacterial isolation and identification

For bacterial isolation, swabs from the kidneys and brain of each collected fish were inoculated on Tryptic Soya broth (TSB, KASVI, Italy), Brain Heart Infusion broth (BHIB, KASVI, Italy), and Cation-adjusted Mueller–Hinton broth (CAMHB, BD, EUA) then incubated at 28 °C for 24 and 48 h. A loopful from each broth tube was streaked onto Tryptic Soy agar (TSA, KASVI, Italy), Brain Heart Infusion agar with Sheep blood (BHIASB, KASVI, Italy), and Cation-adjusted Mueller-Hinton agar (CAMHA, KASVI, Italy) then incubated at 28 °C for 24 and 48h. Purified isolates were labelled and used as stocks for further studies. Stocks were made with 15% glycerol and kept at -80 °C until use.

The identity of pure colonies was determined and confirmed by morphological, biochemical, and molecular (16S rRNA, *gyrB*, and *rpoB* gene sequencing) tests.

2.4. Morphological and biochemical characterization

Colony morphology and arrangement of each isolate were described after 24 and 48h incubation at 24, 28 and 35 °C in Brain Heart Infusion agar (BHIA), BHIASB, CAMHA, TSA, and MacConkey agar (MAC, Acumedia, USA). BHIASB and MAC were also used to evaluate the haemolysin production and lactose fermentation of each strain, respectively. Strain motility and ability to ferment sugars and produce hydrogen sulfide were evaluated using Motility Test Agar (TTC, Sigma-Aldrich, Brazil), and Triple Sugar Iron Agar (TSI, KASVI, Italy), respectively. Catalase, coagulase, and oxidase activity were determined following standard laboratory procedures. Growth in TSB with 6% NaCl after 24 h and 48 h incubation at 24, 28,

and 35 °C was also evaluated. *Escherichia coli* ATCC 25922 and non-inoculated medium were used as the positive and negative control, respectively.

Bacterial films were prepared from each 24 and 48 h pure culture of purified isolate and stained with Gram stain, then examined under an optical microscope (1000 ×) to describe the bacterial morphology (shape and arrangement of bacterial cells). Additionally, a morphological study on cells grown overnight in Mueller–Hinton agar (MHA, Sigma-Aldrich, Brazil) was performed through SEM.

2.5. Molecular characterization and phylogenetic analysis

For the identification of bacteria, genomic DNA was extracted from the bacteria of freshly grown culture plates using a DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's instructions (Qiagen, 2020). The extracted DNA was quantified using a NanoDropTM One/One^C Microvolume UV-Vis Spectrophotometer (ThermoFisher ScientificTM, Brazil) and used to perform polymerase chain reaction (PCR) to amplify the 16S rRNA, *gyrB*, and *rpoB* genes, using specific primers.

The 25-μL reaction consisted of 2.5 μL 10X PCR Buffer (-Mg), 0.8 μL MgCl₂ (50mM), 0.5 μL dNTP mix (10 mM), 1.0 μL of each primer (10 μM), 17 μL of UltraPure DEPC-treated water, 2.0 μL of extracted DNA (20–50 ng μL⁻¹ at a ratio of 260/280⁻¹ between 1.8 and 2.0), and 0.2 μL of PlatinumTM Taq DNA polymerase (ThermoFisher ScientificTM, Brazil). The specific diagnostic primers and PCR conditions are described in Table 1.

The PCR product was analyzed by electrophoresis in a 1.5% agarose gel stained with 1 μL fluorescent stain for dsDNA (Nancy 520®, Sigma-Aldrich, Brazil), visualized under UV light, and sent to the Centro de Recursos Biológicos e Biologia Genômica (CREBIO, Faculty of Agricultural and Veterinary Sciences – Unesp) for gene sequence analysis (Sebastião et al., 2015). Briefly, the 10 μL sequencing reaction consisted of 50 ng of the purified PCR product,

3.0 µL of sequencing buffer (Save money 2.5X), 1.0 µL of BigDye v3.1 (Applied Biosystems, USA), 10 pmol of primer, and double distilled water to make a 10 µL reaction. The specific diagnostic primers and sequencing conditions are described in Table 1. Isopropanol 75% (80 µL) was added to each reaction. After 15 min at room temperature, the reactions were centrifuged at 3,040 *g*, in a Rotanta 46R plate centrifuge (Hettich, Germany) with microplate rotor, for 30 min at 20 °C. After DNA precipitation, the supernatant was discarded and the samples were washed twice with 200 µL of 70% ethanol followed by centrifugation at 3,040 *g* for 10 min at 20 °C. The samples were vacuum dried, resuspended in 10 µL formamide, denatured for 5 min at 95 °C, and subjected to sequencing on an ABI 3730 XL DNA Analyzer (Applied Biosystems, USA) as recommended by the manufacturer.

The initial sequencing quality was assessed in BioEdit (BioEdit version 7.0.0; Hall, 1999) and FinchTV (Geospiza) software (Gallagher and Wiley, 2008). Gene sequences were identified using the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Nucleotide sequence identity $\geq 98\%$ was used as the criterion for identification. Raw sequences of *gyrB* and *rpoB* genes were translated to protein to confirm the absence of stop codons in the middle of the sequence. All gene sequences were deposited in the NCBI GenBank database under the accession numbers: MH155229, MN134409, MN133840, MT735587, MT796422–MT796425, MT904880–MT904882, and MT937283.

The 16S rRNA (37), *gyrB* (35), and *rpoB* (35) gene sequences of clinical isolates and environmental *Aeromonas*, isolated in different locations (Brazil, China, Czech Republic, France, India, Japan, Mexico, Portugal, Sri Lanka, The United States of America, and Viet Nam) and reference strains of two related organisms, *Plesiomonas shigelloides* NCTC10360 (Enterobacterales) and *Vibrio anguillarum* NCTC12159 (Vibrionales), were obtained from the NCBI GenBank Database and aligned with the sequences from this study, using MAFFT

(Kato et al., 2019). Each gene alignment was used for phylogenetic reconstruction by Maximum Likelihood (ML, 10,000 replicates) and Bayesian Inference (BI) methods, using MEGA version X (Kumar et al., 2018) and MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003), respectively.

The best evolutionary model for ML (16S rRNA: K2 + G + I; *gyrB*: T92+G ; *rpoB*: K2 + G) and BI (16S rRNA: HKY + I; *gyrB*: SYM + G; *rpoB*: TIM2ef + G) was selected by using the Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) in MEGA version X and jModelTest 2.1.0 (Darriba et al., 2012), respectively. The AICc value (Akaike Information Criterion, corrected), ML value (lnL), and the number of parameters of each model are presented in Supplementary Tables 1 and 2.

Consensus trees were visualized using Figtree 1.4.4 (Rambaut, 2012) and refined in CorelDraw 2017 (Corel Corporation, Ottawa, Canada).

2.5.1. Multilocus sequence analysis

Sequences of 16S rRNA, *gyrB*, and *rpoB* were concatenated using Geneious 5.6.3 (Drummond et al., 2012) and used for phylogenetic reconstruction by ML (10,000 replicates) and BI methods, using MEGA version X (Kumar et al., 2018) and MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003), respectively. The best-fit partitioning schemes and models of evolution for nucleotides (Supplementary Table 2) were selected based on PartitionFinder2 on XSEDE (Lanfear et al., 2016).

2.6. Scanning electron microscopy (SEM)

For examination through SEM, the specimens (excised stomach tissues from the first outbreak and small blocks (~0.5 × 0.5 × 0.2 cm) of MHA with one of the largest surfaces covered with bacterial cells grown overnight) were fixed by immersion in 2.5% glutaraldehyde

in 0.1 M Sodium Cacodylate buffer, pH 7.4, dehydrated in a graded ethanol series of 70%, 80%, 90%, 95%, and then placed 3× in 100% ethanol for 30 min, dried with liquid CO₂ in an EMS 850 critical point dryer (Electron Microscopy Sciences, USA), placed on stubs, sputter-coated with gold (Au, 50 nm) in a Denton Vacuum Desk II sputter coater, and photographed using a ZEISS EVO 10 scanning electron microscope (ZEISS, Germany). Specimens were imaged at 15.00 kV electron high tension (EHT), with an 8.0 mm working distance.

2.7. Virulence and pathogenicity

The virulence and pathogenicity were confirmed by screening for 12 virulence factors genes, histone-like protein (*hlyA*), serine protease (*ahp*), glycerophospholipid cholesterol acyltransferase (*GCAT*), cytotoxic enterotoxin (*act*), type III secretion system (TTSS) (*ascv*), temperature-sensitive protease (*eprCAI*), lateral flagella B (*lafB*), haemolysin- aerolysin (*aeraA*), lipase (*lip*), cytolytic enterotoxin/cytotonic enterotoxin (*alt*), cytolytic enterotoxin ast (*ast*), nuclease (*nuc*), and artificial infection.

The presence of virulence genes was evaluated by PCR, using specific primers for each gene. The DNA extraction and 25 µL PCR reaction were performed as described in section 2.5, and the primers and PCR conditions are described in Table 1.

2.8. In vivo experimental challenge – Koch's Postulate

2.8.1. Study design

After an acclimation period of 14 days, 80 healthy Nile tilapia juveniles (71.07 ± 15.82 g, 15.18 ± 0.95 cm) were divided into seven groups. All groups had 10 fish, except for G7 that had 20. G1, G2, G3, G4, G5, and G6 were anaesthetized and challenged by intraperitoneal injection of 1.8×10^8 , 1.1×10^8 , 4.3×10^7 , 4.0×10^7 , 3.4×10^7 , and 2.5×10^7 CFU mL⁻¹ of IA07, respectively. G7 (negative control) was injected with phosphate-buffered-saline (PBS).

Clinical signs of disease and mortalities were recorded for 10 days post-challenge. Freshly dead animals were collected for microbiological examination, following standard microbiological procedures and histological analyses.

2.8.2. Physicochemical parameters of water

The *in vivo* experiments were conducted in 500 L fibreglass tanks with constant water flow and aeration, in the Laboratory of Microbiology and Parasitology of Aquatic Organisms facilities. Water temperature, pH, dissolved oxygen, conductivity, and salinity were measured every 3 days in all groups and were appropriate for Nile tilapia maintenance (Supplementary Table 3).

2.9. Histopathology

Samples from gills, liver, stomach and kidney of fish from the outbreaks and the pathogenicity study were fixed in 10% buffered formalin, trimmed, dehydrated using ethanol, and embedded in paraffin blocks, which were sectioned and stained with haematoxylin and eosin (H&E) for histopathological examination.

2.10. Susceptibility to antimicrobials

To evaluate the antimicrobial susceptibility of the clinical isolates, the broth microdilution method was performed according to a previous study (Assane et al., 2021a) following the guidelines described in CLSI VET04-A2 (CLSI, 2014) using 100 μL of a bacterial cell suspension of approximately $1.5 \times 10^8 \text{ CFU mL}^{-1}$ with enrofloxacin – ENRO (Fluka, $\geq 98.0\%$ purity), FFC (Sigma, $\geq 99.0\%$ purity), OTC (Supelco, $\geq 95.0\%$ purity) or TAP (Sigma, $\geq 99.0\%$ purity) and 24 h incubation at 28 °C.

The ranges of antimicrobial concentrations in the tests were 1.92–0.00375 $\mu\text{g mL}^{-1}$ ENRO, 128–0.25 $\mu\text{g mL}^{-1}$ FFC, 128–0.25 $\mu\text{g mL}^{-1}$ OTC, and 1,024–0.25 $\mu\text{g mL}^{-1}$ TAP.

2.11. Statistical analysis

Data normality and homogeneity of variance were evaluated, and rare non-normal data were normalized using square root transformation. Data were analysed using one-way ANOVA. Significant results ($p < 0.05$) from dependent variables were tested using the Holm–Sidak multiple comparison test of means, at a significance level of 0.05.

3. Results

3.1. Post-mortem findings

The freshly dead fish from the first outbreak had the peritoneal cavity filled with yellow fluid and showed a grey liver and haemorrhages in the spleen and kidney, in addition to the gross pathological signs described in section 2.1 (Fig. 2J). Upon internal organ examination of fish from the second outbreak (collected on 11 May 2019), signs of acute peritonitis, ascites, and hepatic necrosis were evident (Fig. 2K).

High prevalence and abundance of *Enterogyrus* (Monogenea: Ancyrocephalinae) parasitizing the stomach were detected in fish from the first outbreak (Assane et al., 2021b). It was possible to observe the presence of bacteria in lesions originated by the parasitism (Fig. 4I). The parasitological analysis of fish from the second outbreak showed a negative result.

3.2. Bacteria isolation and identification

Satisfactory bacterial growth was observed in all culture media (TSB, BHIB, CAMHB) 18 h after incubation at 25, 28, and 35 °C. However, the morphological and biochemical

characteristics of the clinical isolates were evaluated 24 and 48 h after incubation in each medium.

Four strains (IA17, GV1, IA04, and IA07) of motile, rod-shaped Gram-negative bacteria, 0.3 to 0.9 μm in diameter and 0.6 to 3.3 μm in length (Table 2), were recovered from the kidney (IA17 and GV1) and brain (IA04 and IA07) of sampled fish during the first and second outbreaks, respectively (Fig. 3). Notwithstanding differences in phenotype and genotype, morphological, biochemical, and molecular analysis indicated that all strains were *Aeromonas jandaei* (Table 3, Fig. 3–6).

The colonies were smooth, convex, rounded, tan in Brain Heart Infusion agar (BHIA), Cation-adjusted Mueller-Hinton agar (CAMHA), and Tryptic Soy agar (TSA), and buff tan in BHIA with Sheep blood (BHIASB) and MacConkey agar (MAC) (Fig. 3 and 4) 24 and 48 h after incubation. Nevertheless, the colonies were bigger and darker 48h after incubation and the haemolytic activity was more visible in plates incubated at 35 °C, as shown in Fig. 4. IA04 was β -haemolytic while IA07, IA17, and GV1 were α -haemolytic (Fig. 4). The clinical isolates were uniformly negative for oxidase, catalase, and coagulase, and fermented glucose but not lactose and sucrose. Neither gas nor H_2S was produced in the TSI agar test (Fig. 4).

The clinical isolates were uniformly positive for catalase, oxidase, and glucose fermentation (Fig. 4).

2.1. Phylogenetic analysis

The partial sequences of 16S rRNA (409–874 bp), *gyrB* (544–615 bp), and *rpoB* (453–507 bp) genes of strain IA17, GV1, IA04, and IA07 showed 100%, $\geq 95.45\%$, and $\geq 99.34\%$ similarity to several *A. jandaei* sequences deposited in GenBank, including those isolated from water treatment plants in Portugal (AF1) and the United States of America (ASH05), an aquatic plant in Mexico (CPO 4.0024), fish from different locations (strain HX201006 from China,

B10 from Bangladesh, M34 from Malaysia, and Til5 from India), and the reference strain ATCC 49568, respectively. As shown in Fig. 5, the clinical isolates from this study were included in the clade of *A. jandaei* and were differentiated from other *Aeromonas* recognized taxa in the phylogenetic tree constructed using both single (16S rRNA, *gyrB*, or *rpoB*) and concatenated (16S rRNA-*gyrB*-*rpoB*) gene sequences.

3.3. Virulence and pathogenicity

The clinical isolates from this study expressed four (IA04), five (IA17 and GV1), and six (IA07) out of 12 virulence genes analysed (Table 3). Four pathogenicity factors, haemolysin (*hlyA*), temperature-sensitive protease (*eprCAI*), haemolysin-aerolysin (*aeraA*), and nuclease (*nuc*) were detected in all strains. Lateral flagella B (*lafB*) and cytolytic enterotoxin/cytotoxic enterotoxin (*alt*) were only detected in strains isolated from the first outbreak (IA17 and GV1, respectively).

A. jandaei IA07 was pathogenic to Nile tilapia juveniles and the mortality was dose-dependent (Fig. 6). The cumulative percentage mortality of experimental challenged Nile tilapia juveniles depending on the dose of *A. jandaei* IA07 is presented in Fig. 6.

Fish challenged with the higher doses (1.8×10^8 , 1.1×10^8 , and 4.3×10^7 CFU mL⁻¹) died between 0 and 12 h post-challenge without remarkable external clinical signs (Fig. 2H). However, remarkable changes were observed internally (Fig. 2M) and pure bacterial colonies resembling *Aeromonas* (and then confirmed as *A. jandaei*) were recovered from the internal organs (brain and kidney).

Signs of disease, including changes in feeding and swimming behaviour, started to appear 3 and 21 h post-challenge in fish injected with 4.0×10^7 (3 h), 3.4×10^7 (21 h), and 2.5×10^7 (21 h) CFU mL⁻¹, respectively. The mortality in these groups was recorded 6–12 h after the emergence of the first clinical signs of disease. Like the naturally infected fish from the first

outbreak, diseased fish from these groups floated on the water surface and sank to the bottom of the tank before death (Fig. 2A). The external and internal clinical signs were also identical to those recorded in the first outbreak, as shown in Fig. 2 and Fig. 7.

3.4. Histopathological findings

No abnormal changes were observed in the fish injected with PBS (data not shown). Although there were differences between the external signs of early (naturally infected fish from the second outbreak and fish challenged by intraperitoneal injection of 1.8×10^8 , 1.1×10^8 , and 4.3×10^7 CFU mL⁻¹ of IA07) and 6 h post-challenge (fish challenged by intraperitoneal injection of 4.0×10^7 , 3.4×10^7 , and 2.5×10^7 CFU mL⁻¹ of IA07) dead fish, the histopathological findings were similar in all analysed fish (Fig. 7). However, more severe signs were found in the internal organs of fish challenged by intraperitoneal injection of 4.0×10^7 , 3.4×10^7 , and 2.5×10^7 CFU mL⁻¹ of IA07.

As shown in Fig. 7A–C, a multifocal process of interlamellar hyperplasia, lamellar fusion, epithelial cells elevation and increased mucus production were observed in the gills of diseased fish.

Melano-macrophage centres (MMCs, macrophage aggregates), vascular congestion with thrombus, haemorrhage, and necrosis were found in the spleen (Fig. 7D and E).

In the liver, swollen and diffuse hepatocytes, with cytoplasm showing pallor and weakly basophilic areas, were observed. There was also observed inflammatory cell infiltration, vacuolization, vascular congestion with thrombus, haemorrhage, haemosiderin, necrosis, and diffuse disorganization of the parenchyma, mild to moderate swelling with pale irregular cytoplasmic areas of varying size being present in most of the remaining hepatocytes, suggesting marked glycogen deposit (Fig. 7F–H).

The intestine presented inflammatory cell infiltration, congestion, and abnormal changes in the villi structure, a considerable number of inflammatory cells being observed in the intestinal mucosa (Fig. 7J and F).

MMCs, vascular congestion with thrombus, haemorrhage, necrosis, haemosiderin, tissue degradation, and interstitial nephritis were observed in the kidney of diseased fish (Fig. 4M). The first four abnormalities were the most predominant in the evaluated organs.

3.5. Antimicrobial sensitivity

The antimicrobial activity, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of enrofloxacin (ENRO), florfenicol (FFC), oxytetracycline (OTC), and thiamphenicol against *A. jandaei* strains IA17, GV1, IA04, and IA07 is displayed in Table 4. All strains were sensitive to the antimicrobials tested, except to the strains from the second outbreak, which were resistant to OTC.

4. Discussion

Due to the challenging process of identifying *Aeromonas* to the species level through routine methods, such as conventional biochemical tests, commercial phenotypic tests and systems, or 16S rRNA gene sequencing (Fernández-Bravo and Figueras, 2020; Jung-Schroers et al., 2019), *A. hydrophila* remained for decades as the only taxon, among *Aeromonas*, linked to mass mortalities in different cultured freshwater fish around the world. Thus, its epizootiology, diagnosis, bacterial morphological, biochemical, and molecular characteristics, geographical distribution, and significance in Nile tilapia aquaculture are well described in the literature compared to other pathogenic aeromonads. However, in the last five years, with the advance of diagnostic tools, a significant number of different taxa have been identified and linked to mass mortalities in cultured freshwater fish in Africa (El Latif et al., 2019), Asia

(Chen et al., 2019; Dong et al., 2017), and America (Carriero et al., 2016; Coscelli et al., 2017), but pertinent data on *A. jandaei* host-pathogen interactions, susceptibility to the available antimicrobials, morphological, biochemical, and molecular characteristics, and geographical distribution are still lacking. Hence, we believe that the outputs of this study may serve as a guideline for *A. jandaei* isolation, identification, and treatment in aquaculture.

According to the morphological, biochemical, and molecular characteristics of the clinical isolates from both outbreaks, fish were infected by *A. jandaei*. Though 16S rRNA gene sequencing is mainly used to identify *Aeromonas* to the genus level only (Küpfer et al., 2006), in this study, 16S rRNA gene sequencing results were consistent with *gyrB* and *rpoB* gene sequencing and were useful for identifying the isolates to the species level as housekeeping genes (HKGs). However, the use of 16S rRNA, *gyrB*, and *rpoB* gene sequencing and multilocus phylogenetic analysis (MLPA) allowed us to clarify the phylogenetic relationships of *A. jandaei* isolated from the two outbreaks.

The phylogenetic analysis (Fig. 5) indicated that the isolates from each outbreak were closely related to each other and *A. jandaei* strains isolated from both the environment (Figueira et al., 2011; Quisehuatl-Tepexicuapan et al., 2016) and disease outbreaks in cultured fish (Iqbal, Mohammed Mahbub, Tajima and Ezura, 1998; Preena et al., 2021) around the world.

A. jandaei seems to be an opportunistic pathogen with a cosmopolitan distribution like *A. hydrophila* (Dong et al., 2017; Harikrishnan and Balasundaram, 2005). According to Pakingking et al. (2020), *Aeromonas* spp. constitute a part of the normal microbiota of healthy tilapia and their production environment, and their density can vary from 10^1 to 10^3 CFU mL⁻¹ in the culture environment, 10^2 to 10^5 CFU mL⁻¹ in the intestines, and 10^4 to 10^7 CFU mL⁻¹ in the gills.

Although the mass mortalities described in both disease outbreaks reported in this study occurred after exposure to stress by classification and transport, which are known to contribute

to disease manifestations (Dong et al., 2017), the results obtained from microbiological and parasitological analysis from both outbreaks, combined with the clinical signs and cumulative mortality after experimental infection, indicate that in juvenile Nile tilapia, *A. jandaei* density above 4.0×10^7 CFU mL⁻¹ is capable of causing mass mortality in a short period (less than 24 h), without apparent clinical signs of disease, even when there is no evident stress inducer. In the experimental challenge, typical clinical signs (such as changes in feeding and swimming behaviour, the peritoneal cavity filled with yellow fluid, grey liver, haemorrhages in the spleen and the kidney) were only observed in fish challenged with doses below 4.0×10^7 CFU mL⁻¹ (Fig. 2). Similar results of Nile tilapia high mortality within 24 h without remarkable clinical signs after bacterial challenge using a relatively lower density of *A. jandaei* (3.7×10^6 CFU mL⁻¹) were recently reported by Dong et al. (2017). Based on these results, it can also be suggested that parasitism (by Monogenea) during the first outbreak played a significant role in the outbreak, and fish from the second outbreak were exposed to a higher density of *A. jandaei*.

The gross lesions and the histopathological findings observed in both naturally and experimentally infected fish could be attributed to the virulence factors detected in the clinical isolates (Table 3 and Fig. 4). At least four virulence factors, haemolysin (*hlyA*), temperature-sensitive protease (*eprCAI*), haemolysin-aerolysin (*aeraA*), and nuclease (*nuc*), were identified in all clinical isolates. In the clinical isolates from the first outbreak was also detected the presence of lateral flagella B (*lafB*) and cytolytic enterotoxin/cytotoxic enterotoxin (*alt*) genes. The production of haemolysin was confirmed on sheep blood agar (5%). The presence of these virulence factors allows *A. jandaei* to adhere, invade, colonize and destroy the fish cells, overcoming the fish immune response mechanisms, resulting in a disease outbreak (Fernández-Bravo and Figueras, 2020).

The development of resistance to the antimicrobials used in aquaculture is an increasing concern for both the aquaculture industry and public health. Notwithstanding the fact that *A.*

jandaei is an emerging pathogenic *Aeromonas* that was never isolated from a disease outbreak in Panorama, São Paulo State, Brazil (where the second outbreak occurred), the farmer reported ineffectiveness of FFC (10 mg kg⁻¹) and OTC (120 mg kg⁻¹ bwt) during the outbreak. The strain of *A. jandaei* isolated from the outbreak also exhibited *in vitro* resistance to OTC. In our previous study (Assane et al., 2019), we also observed OTC resistance in an *A. jandaei* strain (AKJ561032) isolated in 2013 from Nile tilapia cultured in Porto Ferreira, São Paulo State, Brazil. The resistance to these antimicrobials is probably due to their empirical therapy and prophylactic use in the fish farm, considering that they are among the main antimicrobials used in aquaculture around the world (Lulijwa et al., 2019) and are the only antimicrobials approved for use in aquaculture (food species) in Brazil (SINDAN, 2021).

To our knowledge, this is the first detailed report of Nile tilapia mass mortality caused by *A. jandaei* infection in cultured Nile tilapia in Brazil. So far, *A. jandaei* has been linked to mass mortalities in tambaqui, *Colossoma macropomum* (Pessoa et al., 2020), and pirarucu, *Arapaima gigas* (Proietti-Junior et al., 2021) in Brazil, farmed Siamese crocodiles, *Crocodylus siamensis* (Pu et al., 2019) in China, farmed climbing perch, *Anabas testudineus*, in India (Mazumder et al., 2021) and Nile tilapia in Thailand (Dong et al., 2017; Guo et al., 2016). Since Nile tilapia is the main cultured fish in Brazil (representing 61.1% of all fish production in 2019) (IBGE, 2019), infection by *A. jandaei* in this fish species could represent an important obstacle in Brazilian aquaculture and generate huge economic losses, if strict biosecurity and welfare measures are not adopted in the fish farms. Furthermore, the presence of pathogenicity factors and relative resistance to oxytetracycline (OTC) could contribute to the emergence of a great health problem in the future, considering that *Aeromonas* is also pathogenic to humans and, in cases of diarrhoea, the infective dose is relatively low (Fernández-Bravo and Figueras, 2020). Therefore, there is a need for more detailed epidemiologic studies about *A. jandaei*.

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7. Tables

Table 1. Primers (P) and cycling conditions used for PCR amplification of 16S rRNA, *gyrB*, *rpoB*, *hlyA*, *ahp*, *GCAT*, *act*, *ascV*, *eprCAI*, *laf B*, *aera A*, *lip*, *alt*, *ast*, and *nuc* genes of *Aeromonas jandaei*.

Gene	P	Sequence (5'→3')	Cycling conditions (temperature, time, cycles)					Ampl icon (bp)	Reference
			Initial denaturatio n	Denaturatio n	Annealin g of primers	Extensio n	Final extensio n		
16S rRNA	F	AGAGTTTGATYMYGGCTCAG	95 °C, 5 min.	95 °C, 1 min.	54 °C, 1.5 min.	72 °C, 1 min.	72 °C, 10 min.	900	Sebastião et al. (2015)
	R	CCGTCAATTCMTTTRAGTTT	1x	30x			1x		
<i>gyrB</i>	F	TCCGGCGGTCTGCACGGCGT	95 °C, 5 min.	94 °C, 30 sec.	55 °C, 30 sec.	72 °C, 1 min.	72 °C, 10 min.	1100	Yáñez et al. (2003)
	R	TTGTCCGGGTTGTACTCGTC	1x	35x			1x		
<i>rpoB</i>	F	GCAGTGAAAGARTTCTTTGGTTC	95 °C, 5 min.	94 °C, 30 sec.	55 °C, 30 sec	72 °C, 30 sec.	72 °C, 7 min.	560	Korczak et al. (2004)
	R	GTTGCATGTTNGNACCCAT	1x	35			1x		
<i>hlyA</i>	F	CCACGCAAATTCATCACG	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	1079	Heuzenroeder et al. (1999)
	R	ATCCTTGTTACCTCGAC	1x	45x			1x		
<i>ahp</i>	F	ATTGGATCCCTGCCTA	94 °C, 2 min.	94 °C, 20 sec.	55 °C, 30 sec.	72 °C, 1 min	72 °C, 10 min	911	Hu et al. (2012)

	R	GCTAAGCTTGCATCCG	1x	32x			1x		
<i>GCAT</i>	F	CTCCTGGAATCCCAAGTATCAG	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	237	Jung-Schroers et al. (2019)
	R	AGTGACCCAGGAAGTGCACCTTG AG	1x	45x			1x		
<i>act</i>	F	GAGAAGGTGACCACCAAGAACA	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	232	Jung-Schroers et al. (2019)
	R	AACTGACATCGGCCTTGAAGCTC	1x	45x			1x		
<i>ascV</i>	F	GTAARCAGATGAGTATCGATGG	94 °C, 2 min.	94 °C, 20 sec.	60 °C, 30 sec.	72 °C, 1 min	72 °C, 7 min	331	Jung-Schroers et al. (2019)
	R	GAGACSCGGGTGACGATAAT	1x	32x			1x		
<i>eprCA I</i>	F	GCTCGACGCCCAGCTCACC	94 °C, 5 min.	94 °C, 30 sec.	49 °C, 1.5 min	72 °C, 1 min	72 °C, 10 min	387	Hu et al. (2012)
	R	GGCTCACCGCATTGGATTCTG	1x	30x			1x		
<i>laf B</i>	F	GACCAGCAAGGATAGTGGGTTGG AG	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	624	Jung-Schroers et al. (2019)
	R	AAGCACCATCGCGTTGGTATAAG G	1x	45x			1x		
<i>aera A</i>	F	TTTGGAACCCATTTCTCGTGTGGC	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	1236	Jung-Schroers et al. (2019)
	R	TCGAAGTAGTCCGGGAAGGTCTT GG	1x	45x			1x		

<i>lip</i>	F	GACCCCCTACCTGAACCTGAGCT AC	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	155	Jung-Schroers et al. (2019)
	R	AGTGACCCAGGAAGTGCACCTTG AG	1x	45x			1x		
<i>alt</i>	F	TGACCCAGTCCTGGCACGGC	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	442	Jung-Schroers et al. (2019)
	R	GGTGATCGATCACCACCAGC	1x	45x			1x		
<i>ast</i>	F	TCTCCATGCTTCCCTTCCACT	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	331	Jung-Schroers et al. (2019)
	R	GTGTAGGGATTGAAGAAGCCG	1x	45x			1x		
<i>nuc</i>	F	CAGGATCTGAACCGCCTCTATCA GG	94 °C, 2 min.	94 °C, 1 min.	64 °C, 30 sec.	72 °C, 45 sec.	72 °C, 7 min	504	Jung-Schroers et al. (2019)
	R	GTCCCAAGCTTCGAACAGTTTAC G	1x	45x			1x		

Table 2. Measurements of *Aeromonas jandaei* clinical isolates from this study. The data are presented as the arithmetic mean $\pm$ standard deviation (minimum-maximum values; the number of measurements).

Strain	Sample preparation	Electron Imaging	Diameter	Length
IA17	Sputter coating	SEM	0.59 ± 0.08 (0.35 - 0.93; 618)	1.69 ± 0.40 (0.89 - 3.29; 568)
GV1	Sputter coating	SEM	0.50 ± 0.06 (0.35 - 0.73; 637)	1.45 ± 0.37 (0.65 - 2.89; 500)
IA04	Sputter coating	SEM	0.48 ± 0.05 (0.30 - 0.73; 1160)	1.42 ± 0.31 (0.60 - 2.47; 631)
IA07	Sputter coating	SEM	0.45 ± 0.03 (0.35 - 0.56; 734)	1.24 ± 0.26 (0.57 - 2.08; 506)

Table 3. Distribution of putative virulence genes amongst *Aeromonas jandaei* clinical isolates from this study.

Strain	Gene											
	<i>hlyA</i>	<i>ahp</i>	<i>GCAT</i>	<i>act</i>	<i>ascV</i>	<i>eprCAI</i>	<i>lafB</i>	<i>aeraA</i>	<i>lip</i>	<i>alt</i>	<i>ast</i>	<i>nuc</i>
IA17	+	-	-	-	-	+	+	+	-	-	-	+
GV1	+	-	-	-	+	+	-	+	-	+	-	+
IA04	+	-	-	-	-	+	-	+	-	-	-	+
IA07	+	-	-	-	+	+	-	+	-	-	-	+

Table 4. Minimum inhibitory (MIC) and bactericidal (MBC) concentrations ($\mu\text{g/mL}$), and MBC/MIC ratio of enrofloxacin (ENRO), florfenicol (FFC), oxytetracycline (OTC), and thiamphenicol (TAP) against clinical isolates of *Aeromonas jandaei* linked to mass mortalities of cultured Nile tilapia, *Oreochromis niloticus* (L.) in Brazil.

Strain	ENRO			FFC			OTC			TAP		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
GV1	0.015	0.015	1	1	4	4	0.125	0.125	1	4	8	2
IM01R	0.015	0.015	1	1	1	1	0.5	2	4	2	2	1
IA07	0.004	0.004	1	1	2	2	32	32	1	1	2	2
IA04	0.48	0.48	1	1	2	2	16	32	2	1	4	4
QC*	0.015	-	-	8	-	-	0.5	-	-	64	-	-

*Quality control: *Escherichia coli* ATCC 25922

8. Figures

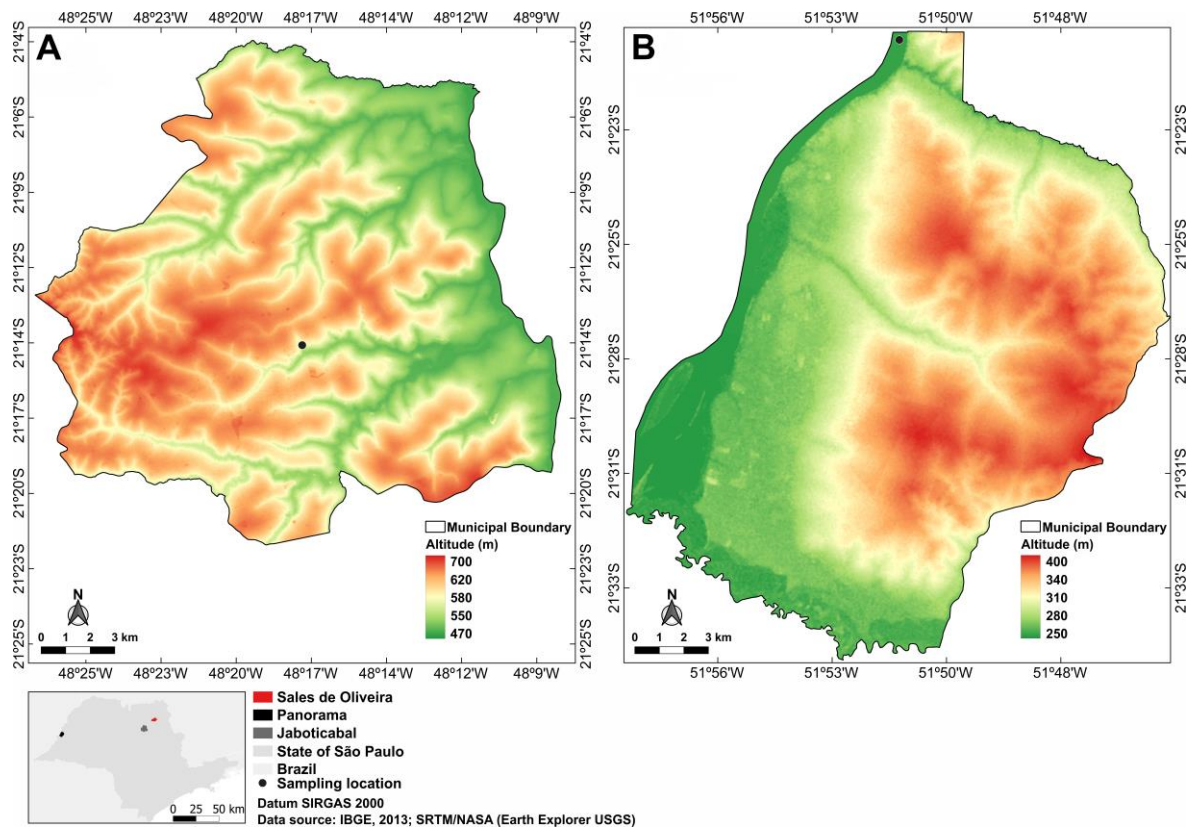


Figure 1. Map showing the geographical location of *Aeromonas jandaei* outbreaks linked to mass mortalities of cultured Nile tilapia, *Oreochromis niloticus* (L.) in Brazil.

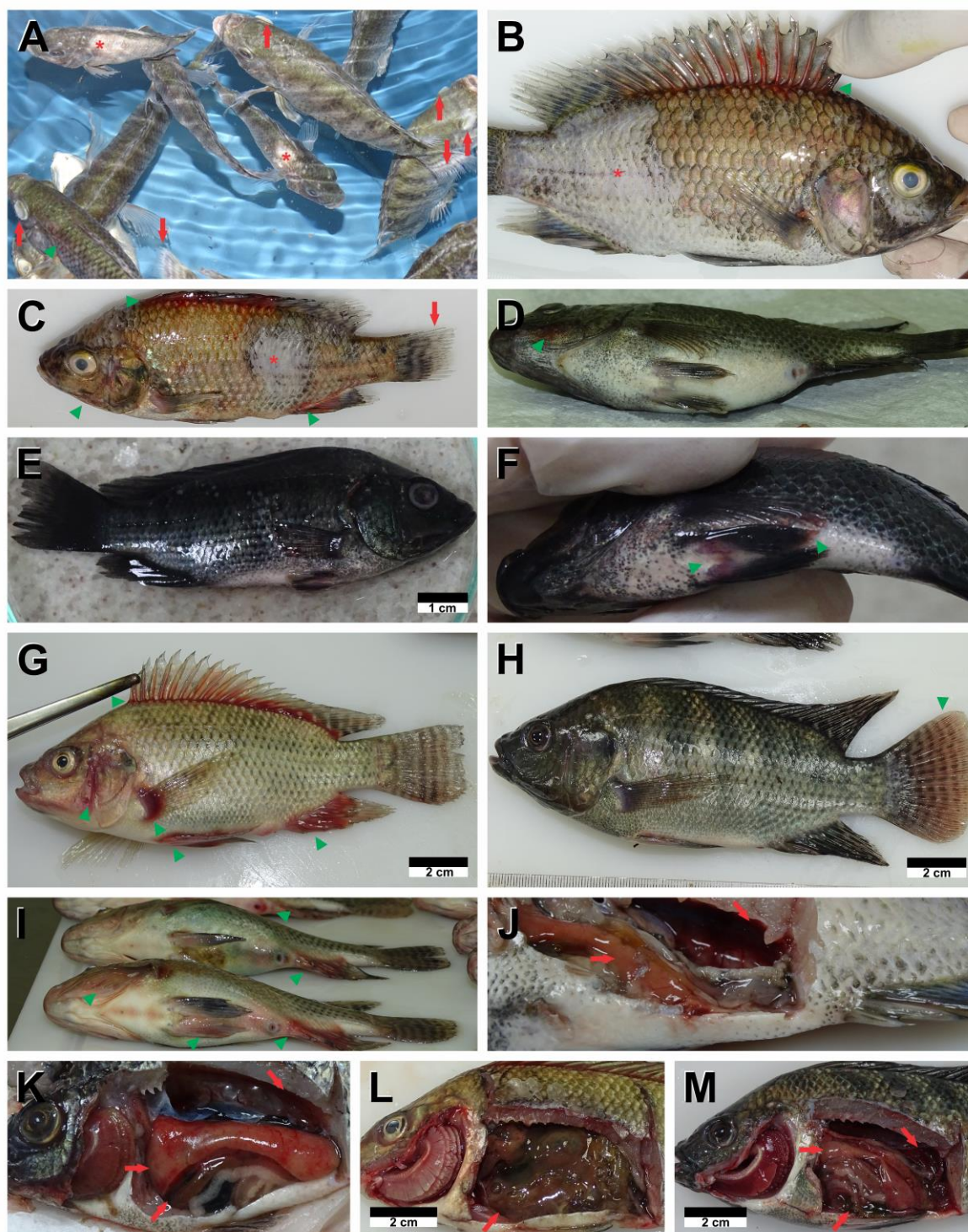


Figure 2. Clinical signs observed in Nile tilapia juveniles naturally and experimentally infected with a pathogenic strain of *Aeromonas jandaei*. A, B, and C – external signs of fish from the first outbreak; D, E, and F- external signs of fish from the second outbreak; G, I - external signs of fish injected lower doses of bacteria (4.0×10^7 , 3.4×10^7 , and 2.5×10^7

CFU/mL); H- external signs of fish injected higher doses of bacteria (1.8×10^8 , 1.1×10^8 , and 4.3×10^7 CFU/mL); internal organs of fish from the first outbreak (J), the second outbreak (K), injected lower (L) and higher (M) dose of bacteria (L). The asterisk indicates discolouration of skin along with loss of scales; upwards thick arrow, cloudy eyes; green medium triangle centred, haemorrhage and redness in the skin; downwards thick arrow, fin rot; rightwards thick arrow, necrotic liver; south-east arrow, haemorrhagic kidney; northeast thick arrow, yellow exudate. Note that both naturally and experimentally infected fish displayed similar clinical signs of disease, and there are fish suspended on the water surface before sunk to the bottom of the tank and die.

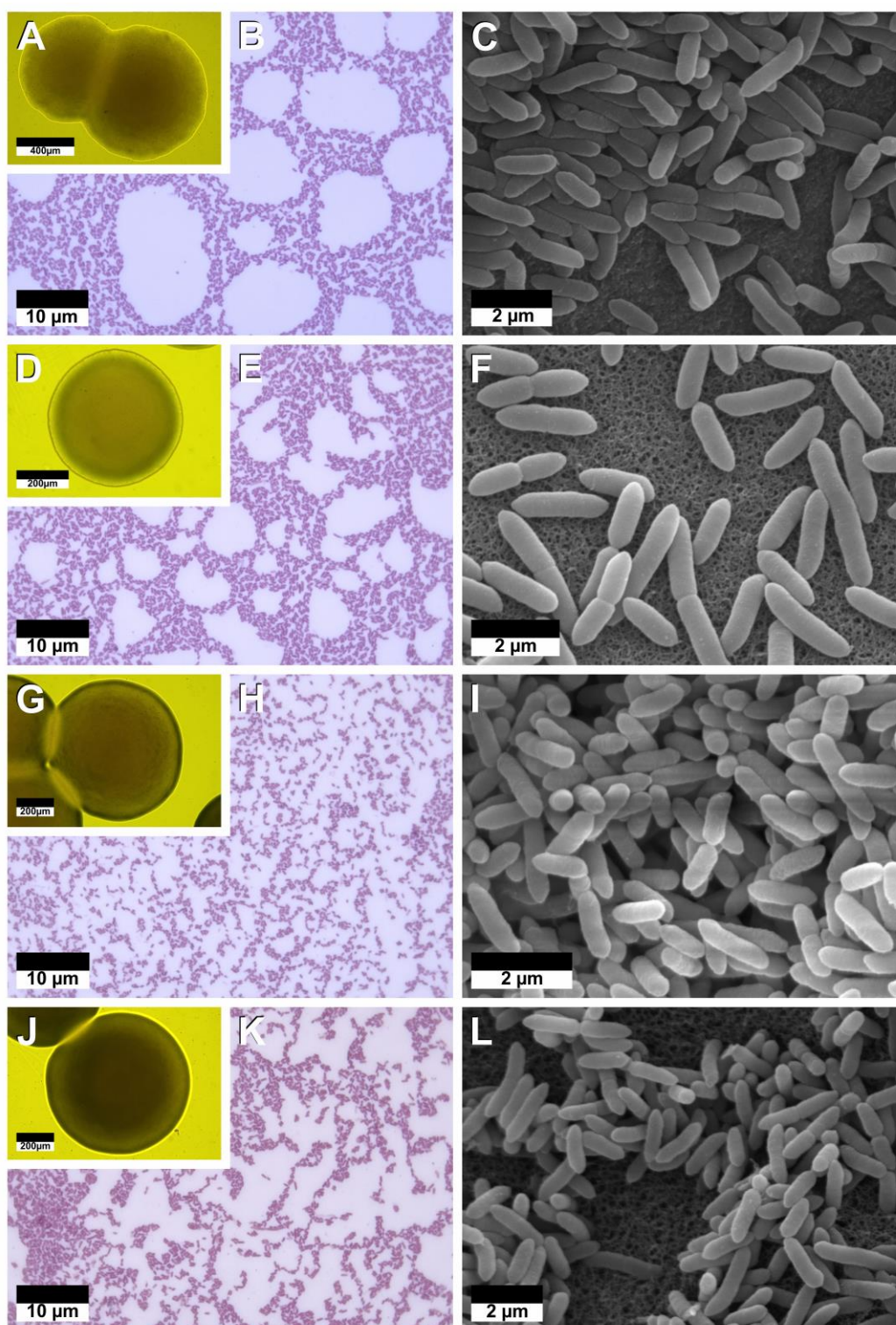


Figure 3. Morphological characteristics of *Aeromonas jandaei* isolated from disease outbreaks in Nile tilapia cultured in Brazil. Strain GV1, IA17, IA07, and IA04 single colonies (A, D, G, and J), Gram stained under a light microscope (B, E, H, and K), and individual cells under a scanning electron microscope (C, F, I, and L), respectively.

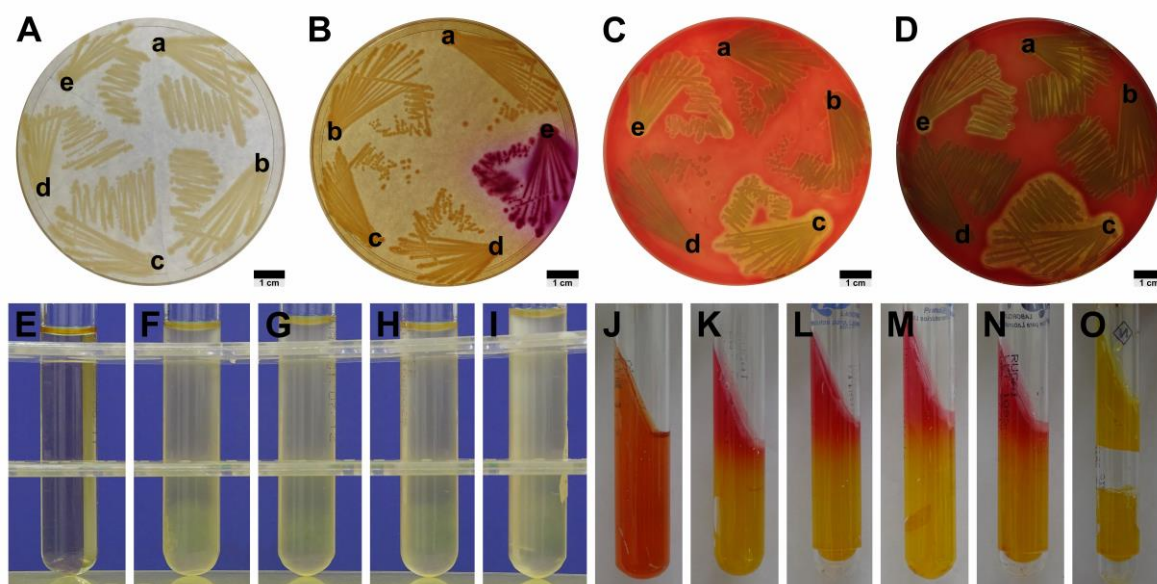


Figure 4. Biochemical characteristics of *Aeromonas jandaei* isolated from a disease outbreaks in Nile tilapia cultured in Brazil. Strain IA17 (a), GV1 (b), IA04 (c), and IA07 (d) after 24h incubation at 28 °C (A, B, C) and 35 °C (D) in trypticase soy agar (A), MacConkey agar (B), brain heart infusion agar with 5% defibrinated sheep blood (C, D), trypticase soy broth (TSB) with 6% NaCl (F, G, H, I), and triple sugar iron agar - TSI (K, L, M, N), respectively. *Escherichia coli* ATCC 25922 (e, O) and non-inoculated medium (E, J) were used as a positive and negative control in the tests, respectively. No growth differences were observed in *A. jandaei* incubated for 24h at 24 °C, 28 °C or 35 °C in TSB or TSI (pictures not shown).

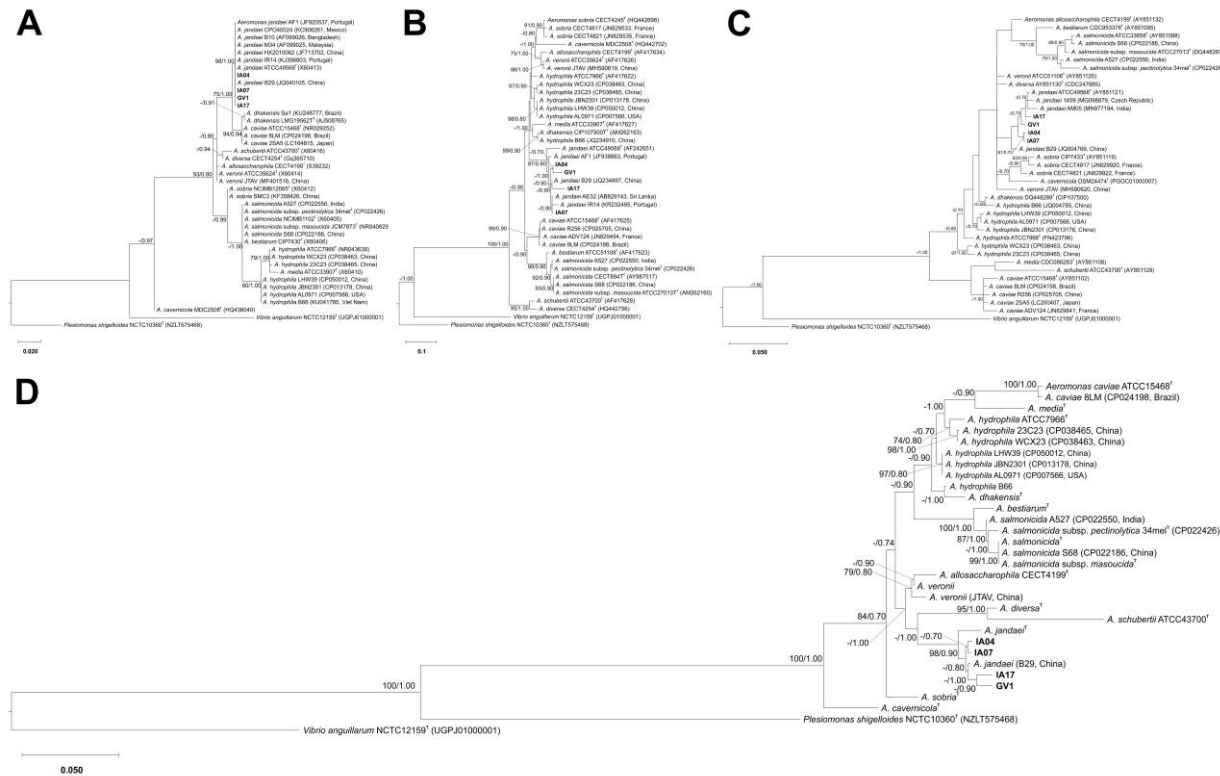


Figure 5. Rooted phylogenetic tree inferred by Maximum Likelihood (ML) and Bayesian Inference (BI) methods based on individual analyses of 16S rRNA (A), *gyrB* (B), and *rpoB* (C) gene sequences, and multilocus (16S rRNA-*gyrB*-*rpoB*) sequence analysis (D) of four clinical isolates from this study (bold) and 33-35 recognized *Aeromonas* isolated from different sources. *Plesiomonas shigelloides* NCTC10360^T and *Vibrio anguillarum* NCTC12159^T were used as outgroups. Support values at the nodes are for ML bootstrap values ($\geq 70\%$)/BI posterior probabilities (≥ 0.70) (BP/PP). Type strains of different species are indicated with ^T.

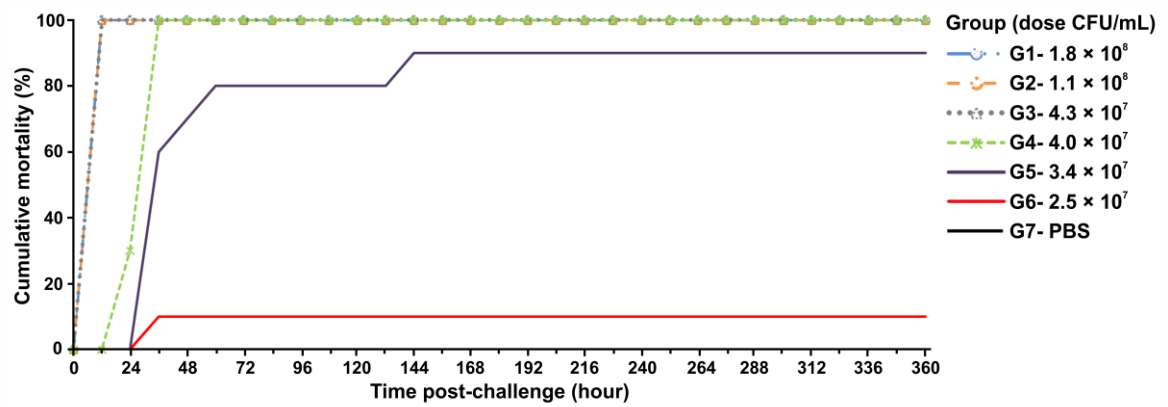


Figure 6. Cumulative mortality (%) of Nile tilapia juveniles experimentally challenged by intraperitoneal injection of six different doses of *Aeromonas jandaei* isolated from a disease outbreak in Brazil. The control group (G7) received an intraperitoneal injection of phosphate-buffered-saline (PBS).

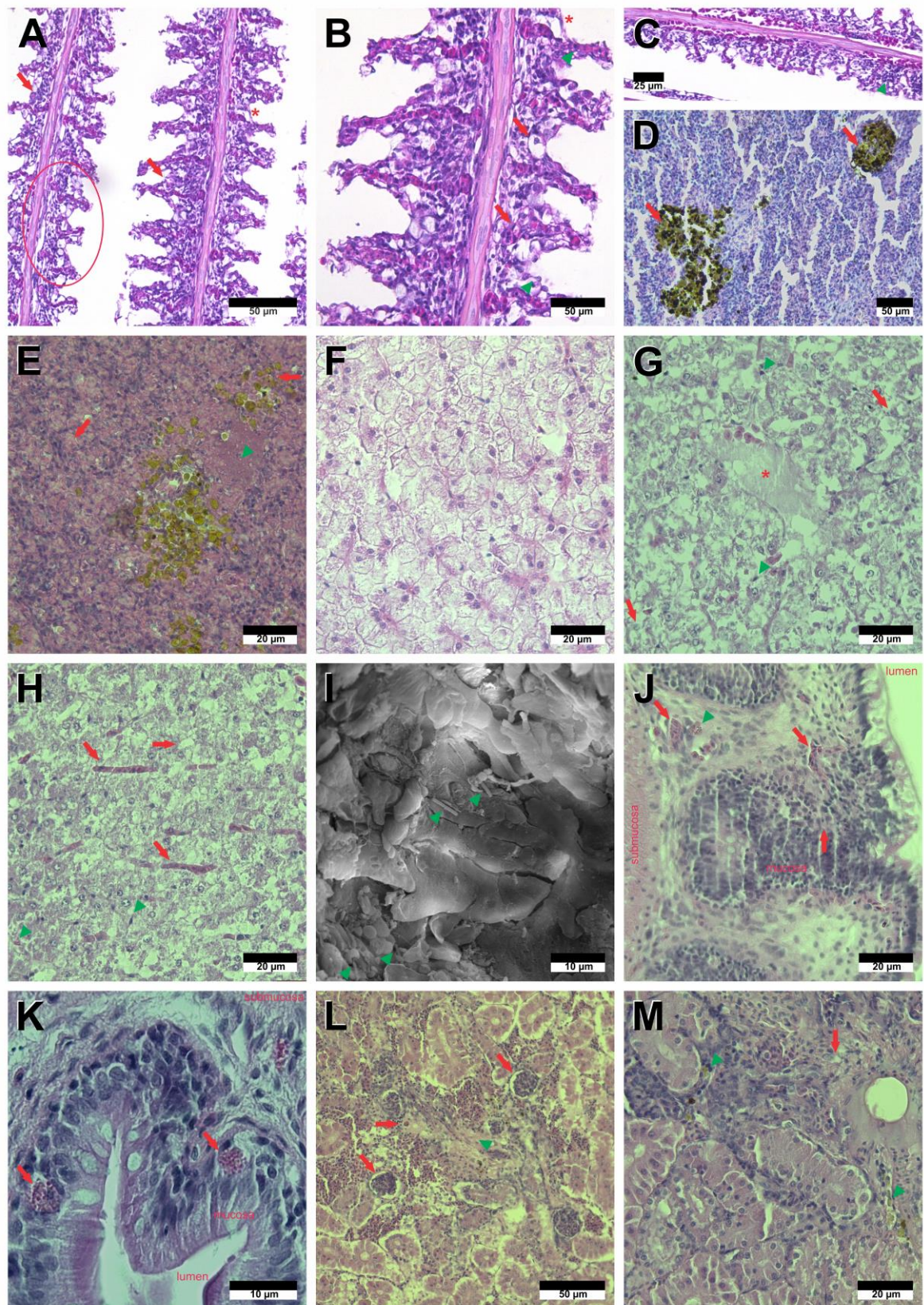


Figure 7. Histopathological findings in Nile tilapia juveniles naturally and experimentally infected with a pathogenic strain of *Aeromonas jandaei*. Cross-section of gills (A, B, C),

spleen (D, E), liver (F, G, H), intestine (J, K), kidney (L, M), and upper view of stomach lumen from fish of the first outbreak (I), showing a multifocal process of interlamellar hyperplasia (south-east thick arrow), epithelial cells elevation (asterisk), moderate heterophilic infiltrate (asterisk) and increased mucus production in the gills; melano-macrophage centres (MMCs, south-east thick arrow), vascular congestion with thrombus (green medium triangle centred), necrosis (south west thick arrow) in the spleen; hepatocyte degeneration and vacuolization, hemolyzed blood (asterisk), necrosis (green medium triangle centred), vacuolization (south east thick arrow), tissue degeneration (rightwards thick arrow), congestion (south east thick arrow), and necrosis (green medium triangle centred) in the liver; red blood cell infiltrate (south east thick arrow); congestion (green medium triangle centred, and south east thick arrow), change in the structure of the villi with a high number of inflammatory cells (upwards thick arrow) in the intestine; degeneration of the renal corpuscle (south east thick arrow); interstitial nephritis (rightwards thick arrow); degeneration of renal tubules (green medium triangle centred), MMCs (green medium triangle centred), and karyolysis of the epithelial cell nuclei of the renal tubule (downwards thick arrow) in the kidney; and lesion caused by parasitism in the stomach filled with bacilli (I). No abnormal changes were observed in the fish injected PBS (data not shown).

9. Supplementary Tables

Supplementary Table 1. Maximum Likelihood nucleotide substitution models applied in this study. Bayesian Information Criterion - BIC, Akaike Information Criterion, corrected - AICc, Maximum Likelihood value - lnL, Gamma distribution - +G, evolutionary invariable sites - +I, transition/transversion bias - R, nucleotide frequencies - f, and rates of base substitutions - r. K2: Kimura 2-parameter; T92: Tamura 3-parameter; TN93: Tamura-Nei.

Gene	Model	Parameters	BIC	AICc	lnL	(+I)	(+G)	R	f(A)	f(T)	f(C)	f(G)	r(AT)	r(AC)	r(AG)	r(TA)	r(TC)	r(TG)	r(CA)	r(CT)	r(CG)	r(GA)	r(GT)	r(GC)	No. sequences	No. positions
16S rRNA	K2+G+I	82	3119,075	2486,253	-1160,719	0,62	0,48	1,46	0,250	0,250	0,250	0,250	0,051	0,051	0,148	0,051	0,148	0,051	0,051	0,148	0,051	0,148	0,051	0,051	41	410
<i>gyrB</i>	T92+G	78	4434,115	3868,912	-1855,866	n/a	0,44	2,00	0,215	0,215	0,285	0,285	0,035	0,047	0,191	0,035	0,191	0,047	0,035	0,145	0,047	0,145	0,035	0,047	39	276
<i>rpoB</i>	K2+G	77	4368,427	3785,416	-1815,292	n/a	0,30	2,69	0,250	0,250	0,250	0,250	0,034	0,034	0,182	0,034	0,182	0,034	0,034	0,182	0,034	0,182	0,034	0,034	39	372
Multilocus	TN93+G	67	10666,626	10102,54	-4984,134	n/a	0,25	2,20	0,237	0,198	0,265	0,300	0,030	0,040	0,099	0,036	0,298	0,045	0,036	0,223	0,045	0,078	0,030	0,040	32	1058

Supplementary Table 2. Bayesian Inference nucleotide substitution models applied in this study. Akaike Information Criterion corrected - AICc, Maximum Likelihood value - lnL, Hasegawa-Kishino-Yano substitution model - HKY, symmetrical substitution model - SYM, transitional substitution model - TIM, transversional substitution model - TVM.

16S rRNA											
Model	lnL	K	AICc	freqA	freqC	freqG	freqT	Kappa 2.8190 (ti/tv = 1.4195)	p-inv		
HKY+I	-1149,234	85	2468,46806	0,2346	0,2397	0,3202	0,2056		0,786		
<i>gyrB</i>											
Model	lnL	K	AICc	R(a) [AC]	R(b) [AG]	R(c) [AT]	R(d) [CG]	R(e) [CT]	R(f) [GT]	gamma shape	pinvar
SYM+G	-1810,187	82	3854,902837	0,3357	2,8724	1,4690	2,3582	7,3524	1,000	0,5790	0
<i>rpoB</i>											
Model	lnL	K	AICc	R(a) [AC]	R(b) [AG]	R(c) [AT]	R(d) [CG]	R(e) [CT]	R(f) [GT]	gamma shape	pinvar
TIM2ef+G	-1788,046	80	3736,09128	0,5195	1,8320	0,5195	1,0000	6,2611	1,0000	0,3290	0
Multilocus											
lnL	-4746,199										
AICc	9701,3582										
K	95										
No. sites	1058										
No. subsets	5										

Subset	Best Model	# sites	Partition names
1	TIM+I+G	410	16S rRNA
2	TRNEF+G	92	<i>gyrB</i> 1
3	K81UF+G	216	<i>gyrB</i> 2, <i>rpoB</i> 2
4	TVM+G	216	<i>gyrB</i> 3, <i>rpoB</i> 3
5	TRN	124	<i>rpoB</i>

Supplementary Table 4. Physicochemical parameters of water during *in vivo* experiments. The data are presented as the arithmetic mean $\pm$ standard deviation (minimum-maximum values).

Temperature	pH	Dissolved oxygen	Conductivity	Salinity
28.17 $\pm$ 0.69 (27.2-29.2)	8.30 $\pm$ 0.11 (8.16-8.46)	9.25 $\pm$ 0.33 (8.90-9.80)	164.53 $\pm$ 0.19 (164.20-164.80)	0.08 $\pm$ 0.00 (0.08-0.08)

Genetic diversity, virulence, antimicrobial resistance genes, and antimicrobial susceptibility of group B *Streptococcus* (GBS) linked to mass mortalities of cultured Nile tilapia in Brazil

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Abstract

Streptococcus agalactiae, group B *Streptococcus* (GBS), is the main bacterial pathogen of cultured Nile tilapia in many countries, causing massive deaths in all stages of the farming cycle throughout the year. This study investigated the genetic diversity, virulence, presence of antibiotic resistance genes, and antimicrobial susceptibility of 72 GBS linked to mass mortalities of cultured Nile tilapia in Brazil. Isolate identity was confirmed by morphological, biochemical, and molecular analyses. Capsular serotype, multi-locus sequence typing (MLST) allelic profiles, and putative pathogenic factors were identified by polymerase chain reaction (PCR), gel electrophoresis, sequencing and molecular analyses of different genes. The presence of antimicrobial resistance genes and antimicrobial susceptibility to florfenicol (FFC), oxytetracycline (OTC), thiamphenicol (TAP) and their combination were evaluated by PCR, followed by gel electrophoresis, and broth microdilution antimicrobial susceptibility testing, respectively. All clinical isolates studied were confirmed to be GBS, one from serotype III (IA2022) and 71 from serotype Ib, suggesting that serotype Ib was the most prevalent between 2011 and 2016 in the south and southeast regions of Brazil. Eight different allelic profiles were identified for the first time, being *adhP*-52, *pheS*-2, *atr*-31, *glnA*-4, *sdhA*-2, *tkt*-19 the most predominant. Between one (*glcK*) and three (*adhP* and *glnA*) alleles were present at each locus. All strains, except IA2022, showed a partial gene deletion event on the *glcK* gene. Surface protein *Rib* and hypervirulent GBS adhesin *BibA* were detected in all strains, except for 18P, which was negative for *rib*. On the other hand, α and β antigens of the C protein were only detected in IA2022. All antimicrobials showed high minimum inhibitory concentration (MIC ≥ 16 μ g/mL) values against several strains with negative results for resistance genes. Despite indifference and antagonism being the most predominant activities in all combinations evaluated, the record of synergism, including in a strain with a resistance gene and phenotypic resistance, suggests that combination therapy can have therapeutic efficacy when well planned.

The combination involving OTC and TAP or FFC is an attractive candidate for improving the treatment of streptococcosis using combination therapy, even for strains showing phenotypic and genotypic resistance to OTC. This study provides pertinent data on pathogenic GBS genetic diversity, virulence, the presence of antibiotic resistance genes, and antimicrobial susceptibility, which may be useful in the development of effective vaccines and therapeutic strategies for the prevention and control of streptococcosis in the fish farm.

Keywords: capsular serotyping, *glcK*, minimum inhibitory concentration, multilocus sequence typing, *Streptococcus agalactiae*, virulence factors.

Highlights

- Eight different allelic profiles were identified for the first time.
- Serotype Ib was the most prevalent between 2011 and 2016 in the south and southeast regions of Brazil.
- All strains, except IA2022, showed a partial gene deletion event on the *glcK* gene.
- Surface protein *Rib* and hypervirulent GBS adhesin *BibA* were detected in all strains, except for 18P.
- There was no correlation between the resistance genotype determined by PCR and the antimicrobial susceptibility patterns.
- OTC and TAP or FFC combinations are attractive candidates for improving the treatment of streptococcosis using combination therapy.

Introduction

Nile tilapia, *Oreochromis niloticus*, is one of the major species produced in the world aquaculture. According to the Food and Agriculture Organization of the United Nations (FAO,

2022), in 2020, it was the third major species produced, after grass and silver carps (*Ctenopharyngodon idellus* and *Hypophthalmichthys molitrix*, respectively), sharing 9 % of finfish production. Due to its zootechnical characteristics, such as strong environmental adaptability, ease of breeding, disease resistance, high protein content, large size, rapid growth, and palatability, Nile tilapia is probably the most widely introduced species and economically important as food fish in many countries, being introduced in 114 countries or regions (FAO, 2021), including Brazil, where it represented, in 2020, 62,3% of fish production (IBGE, 2020).

Brazil has a good tilapia sector, which grows fast and represents over 74% of the production of Nile tilapia in the Americas. In the last decade, tilapia production increased from 150,000 to over 400,000 tonnes, making Brazil the 4th major Nile tilapia producer in the world, behind China, Indonesia, and Egypt (FAO, 2021; IBGE, 2020; Peixe BR, 2021). However, this sector is being negatively impacted by bacterial diseases (Assane, Prada-Mejia, et al., 2021; Chideroli et al., 2017; Delphino et al., 2019; Junior et al., 2020; Sebastião, Furlan, Hashimoto, & Pilarski, 2015; Sebastião, Pilarski, Kearney, & Soto, 2017) and the lack of effective therapies (Assane, de Sousa, et al., 2021; de Oliveira, Queiroz, Teixeira, Figueiredo, & Leal, 2018). According to Tavares-Dias and Martins (2017), in Brazil, the annual loss to the inland aquaculture sector due to parasitic and bacterial diseases is estimated to be USD 84 million.

Currently, *Streptococcus agalactiae*, group B *Streptococcus* (GBS), is the main bacterial pathogen of Nile tilapia, causing massive deaths in all stages of the farming cycle throughout the year (Chideroli et al., 2017), with higher frequency when the water temperature is > 28 °C (Delphino et al., 2019). On the other hand, only two antimicrobials (florfenicol-FFC, and oxytetracycline-OTC) are permitted for use in Brazilian aquaculture (SINDAN, 2022), despite the reports of vaccine failure and antibiotic ineffectiveness after a streptococcosis outbreak (Chideroli et al., 2017; de Oliveira et al., 2018).

Isolates of GBS show distinct genetic diversity, as well as variability in biochemical and physiological characteristics, whose knowledge is necessary for the correct diagnosis, development and implementation of effective prevention and treatment strategies. The polysaccharide capsule (CPS), encoded by *cps* gene (Imperi et al., 2010); the allelic profile of seven different loci (sequence type (ST)) (Jones et al., 2003), used to group strains into clonal complexes (CC) (Honsa et al., 2008); and the surface proteins (virulence proteins), such as the α and β antigens of the C protein (*bca* and *bac*, respectively), *BibA*, C5a peptidase (*scpB*), hyaluronate lyase (*hly*), *hvgA*, and *Rib* (Rajagopal, 2009) are the main sources of its diversity. There are ten structurally and serologically distinct CPS types (capsular serotypes: Ia, Ib and II-IX), >1800 ST (Jolley, Bray, & Maiden, 2018), several clonal complexes (CC) (Honsa et al., 2008), and putative virulence factors identified and associated with strains virulence (Marques, Kasper, Pangburn, & Wessels¹, 1992; Rajagopal, 2009) and host immune response (Bianchi-Jassir et al., 2020; Flaherty et al., 2019). Thus, capsular serotype, ST, CC, and virulence factors identification are necessary for both epidemiological studies and the development of effective prevention and treatment strategies.

To date, five capsular serotypes (Ia, Ib, II, III and IV) and six CC (CC7, CC19, CC23, CC103, CC283, CC552) have been linked to disease outbreaks in cultured fish in different countries (Chen et al., 2015; Delannoy, Samai, & Labrie, 2021; Sudpraseart, Wang, & Chen, 2021), including Brazil (Chideroli et al., 2017; Delphino et al., 2019; Godoy et al., 2013). Current data indicate that in Brazil, capsular serotype Ib and III, and ST-103, ST-260, ST-552, and ST-553 (CC552) are the most prevalent, although strains belonging to capsular serotype Ia and non-serotypeable strains have been isolated from disease outbreaks (Barony et al., 2017; Chideroli et al., 2017; Godoy et al., 2013). However, information about the geographical distribution of sequence types, virulence, antimicrobial susceptibility profile and the

mechanisms of antibiotic resistance of GBS isolated from Nile tilapia farmed in Brazil is still scarce.

This study aimed to enrich our current knowledge in GBS epidemiology and provide pertinent data for the development of effective GBS infection prevention and treatment methods. Here, we present the genetic diversity, virulence, mechanisms of antibiotic resistance, and antimicrobial susceptibility of GBS linked to mass mortalities of cultured Nile tilapia in Brazil. Capsular serotype, ST, and putative pathogenic factors were identified. The presence of antimicrobial resistance genes and antimicrobial susceptibility to FFC, thiamphenicol and their combination were evaluated.

Materials and methods

Clinical isolates

Seventy-two clinical isolates of *Streptococcus agalactiae* (group B Streptococcus, GBS) recovered between 2011 and 2018, from the brain, liver and kidney of diseased Nile tilapia, *Oreochromis niloticus*, farmed in different locations of Brazil (Figure 1) were used to determine the genetic diversity, virulence, the presence of antimicrobial resistance genes, and antimicrobial susceptibility to florfenicol (FFC), oxytetracycline (OTC), thiamphenicol (TAP), and their combinations.

Genetic diversity

The genetic diversity was determined by polymerase chain reaction (PCR) and sequencing of different genes used for identification, multi-locus sequence typing (MLST), capsular serotyping, and detection of virulence and antibiotic resistance factors in GBS. Seven housekeeping genes, alcohol dehydrogenase (*adhP*), phenylalanyl tRNA synthetase (*pheS*), amino acid transporter (*atr*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA*), glucose

kinase (*glcK*), and transketolase (*tkl*); the virulence genes, capsular polysaccharide (*cps*), β antigens of the C protein (*bac*), α antigens of C protein (*bca*), hyaluronate lyase (*hly*), surface protein Rib (*rib*), C5a peptidase (*scpB*), and hypervirulent GBS adhesins (*hvgA* and *BibA*); and the antibiotic resistance genes, the mobile genetic element (MGE) insertion sequence (IS) 1548; erythromycin Ribosomal Methylase (*ermB* and *ermTR*), erythromycin resistance efflux pump (*mefA*), lincomycin and clindamycin resistance gene-lincosamide nucleotidyltransferase (*linB*), tetracycline resistance proteins (*tetM* and *tetO*), and phenicol exporters (*fexA* and *fexB*) were amplified as described in Table 1. MLST allelic profiles and capsular serotype of each clinical isolate were determined as previously described by Jones et al. (2003) and Imperi et al. (2010), respectively.

Sample preparation for PCR

Genomic DNA was extracted from the bacteria of freshly grown culture plates using DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's instruction (Qiagen, 2020). DNA was quantified using a NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific™, Brazil) and used to perform Hot Start and Multiplex PCRs to amplify the *atr*, *cps*, *bac*, *bca*, *hly*, *rib*, *scpB*, IS1548, *ermB*, *ermTR*, *mefA*, *linB*, *tetM*, *tetO*, *fexA* and *fexB* genes, using the primers described in Table 1.

Hot Start PCR

Hot Start PCR (25 μ L reaction) was performed with 0.04 U μ L⁻¹ Invitrogen Platinum™ II Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific™ Brazil, Lot 00813145), 1X Platinum™ II PCR Buffer (Thermo Fisher Scientific™ Brazil), 10 mM dNTP mix (Thermo Fisher Scientific™ Brazil, Lot 1987090, 0.2 mM each), 0.2 μ M forward primer, 0.2 μ M reverse primer, extracted DNA (20–50 ng μ L⁻¹ at a ratio of 260/280⁻¹ between 1.8 and 2.0) and

ultrapure water. Reactions (25 μL) were incubated in a thermal cycler (ProFlex PCR System, Thermo Fisher Scientific™ Brazil) under the conditions described in Table 1.

Multiplex PCR

Multiplex PCR (25 μL reaction) was performed as described in Table 1, with 0.3 U μL^{-1} Invitrogen Platinum™ II *Taq* Hot-Start DNA Polymerase (Thermo Fisher Scientific™ Brazil, Lot 00813145), 1X Platinum™ II PCR Buffer (Thermo Fisher Scientific™ Brazil, Lot 01120395), 10 mM dNTP mix (Thermo Fisher Scientific™ Brazil, Lot 1987090, 0.2 mM each), 0.25 μM of each primer except for primer 1 and 16 that were used at a concentration of 0.4 μM , extracted DNA (20–50 ng μL^{-1} at a ratio of 260 280⁻¹ between 1.8 and 2.0), and ultrapure water. Reactions (25 μL) were incubated in a thermal cycler (ProFlex PCR System, Thermo Fisher Scientific™ Brazil) under the conditions described in Table 1.

Electrophoresis

All PCR products were analysed by electrophoresis in a 1.5% agarose gel stained with 1 μL fluorescent stain for dsDNA (Nancy 520®, Sigma-Aldrich, Brazil), visualized under UV light, and sequenced as described in our previous study (Assane et al. 2021a).

Antimicrobial Susceptibility Testing (AST)

To determine the antimicrobial activity of florfenicol (FFC, $\geq 99.0\%$ purity, Sigma-Aldrich), oxytetracycline (OTC, $\geq 95.0\%$ purity, Supelco), and thiamphenicol (TAF, $\geq 99.0\%$ purity, Sigma-Aldrich) against the clinical isolates, the broth microdilution method was performed as described in our previous studies (Assane et al. 2019, 2021b), following the Clinical Laboratory Standards Institute guideline VET03 (CLSI, 2020a) if possible. Briefly, antimicrobial stock solutions (1,024 $\mu\text{g mL}^{-1}$ FFC, OTC, or TAP) were prepared as described

in our previous study (Assane et al., 2021b). The ranges of concentrations of antimicrobials used in the tests were 128–0.125 $\mu\text{g mL}^{-1}$ FFC, OTC, or TAP. *Streptococcus pneumoniae* ATCC® 49619 and *Escherichia coli* ATCC 25922® were used as quality control.

Cation-adjusted Mueller-Hinton broth (CAMHB) supplemented with 4% (v/v) lysed horse blood (LHB) was used to perform the tests. For inoculum preparation, colony suspension (3–5 well-isolated colonies) of each strain tested was adjusted to $1\text{--}2 \times 10^8$ CFU/mL using a spectrophotometer (0.08–0.13 at 625 nm), and diluted 1:150 (1×10^6 CFU/mL). The diluted inoculum was immediately inoculated onto test plates containing the final twofold dilutions of each antimicrobial. The final inoculum concentration was $3.3\text{--}6.6 \times 10^5$ CFU/mL, and the growth control only had the inoculum and CAMHB supplemented with 4% LHB.

Test plates were incubated in duplicate at $28^\circ\text{C} \pm 2^\circ\text{C}$ for 44–48 h and minimum inhibitory concentration (MIC) endpoints were determined by visual inspection. Samples from three wells, the lowest concentration of antimicrobial without visible bacterial growth, were inoculated aseptically, in duplicate, onto CAMHA plates supplemented with sheep blood. Plates were incubated at $28^\circ\text{C} \pm 2^\circ\text{C}$ for 44–48 h to determine the MBC as the lowest concentration without bacterial growth.

The antimicrobial activity was categorized as bactericidal ($\text{MBC/MIC} < 4$) or bacteriostatic ($\text{MBC/MIC} \geq 4$), based on the MBC-to-MIC ratio (Assane et al., 2019; Giguère et al., 2013). Due to the lack of standardized interpretative criteria for susceptibility testing of GBS isolated from aquatic organisms (CLSI, 2020a, 2020b), the strain categorization as wild-type or non-wild-type was avoided.

Checkerboard Assay

To evaluate interactions between the studied antimicrobials against the clinical isolates, *in vitro* antimicrobial activity of their combinations was determined by checkerboard and time-

kill kinetic analysis, using the MIC values determined previously (Leber, 2016). Test conditions (culture medium, incubation temperature and time, and inoculum concentration) were identical to those described previous sections. Antimicrobial concentration ranges on the test were determined based on the MIC of each antimicrobial against the tested strain.

MIC endpoints were determined by visual inspection and the fractional inhibitory concentration (FIC) was calculated as follows: FIC of antimicrobial A = MIC of antimicrobial A in combination/MIC of antimicrobial A alone, and FIC of antimicrobial B = MIC of antimicrobial B in combination/MIC of antimicrobial B alone. The FIC index (FICI) was defined as the sum of FICs. The interactions were classified as synergy ($FICI \leq 0.5$), additive ($0.5 < FICI \leq 1$), indifference ($1 < FICI < 2$), and antagonism ($FICI \geq 2$) (EUCAST, 2000; Odds, 2003).

Results and discussion

Currently, *Streptococcus agalactiae*, group B *Streptococcus* (GBS), is the main bacterial pathogen of Nile tilapia, *Oreochromis niloticus*, causing massive deaths in all stages of the farming cycle throughout the year, with higher frequency when the water temperature is higher ($> 28^{\circ}\text{C}$) (Asencios et al., 2016; Chideroli et al., 2017; Delannoy et al., 2021; Delphino et al., 2019; Verner-Jeffreys et al., 2018). Considering that isolates of GBS show distinct genetic diversity (Barony et al., 2017), as well as variability in biochemical and physiological characteristics (Goodfellow et al., 2012), information about their genetic diversity, virulence, presence of antibiotic resistance genes, and antimicrobial susceptibility, are relevant for both epidemiological studies and the development of effective prevention and treatment strategies. In this study, we investigated the genetic diversity, virulence, presence of antibiotic resistance genes, and antimicrobial susceptibility of 72 GBS linked to mass mortalities of cultured Nile tilapia in Brazil.

All clinical isolates studied were confirmed to be GBS, one (IA2022) from serotype III and 71 from serotype Ib, suggesting that serotype Ib was the most prevalent between 2011 and 2016 in Paraná and São Paulo states (Brazil) (Table 2). Serotype III was only isolated from a disease outbreak in Bahia state (Brazil) in 2018, corroborating the results from previous studies that indicate that this serotype mostly occurs in fish farms located in the northeastern region of Brazil (Leal, Queiroz, Pereira, Tavares, & Figueiredo, 2019). These serotypes, along with serotype Ia, are the most reported serotypes associated with disease outbreaks in different countries (Chen et al., 2015; Delannoy et al., 2021; Sudpraseart et al., 2021), including Brazil (Chideroli et al., 2017; Delphino et al., 2019; Godoy et al., 2013). Current data indicate that in Brazil, capsular serotype Ib and III, and ST-103, ST-260, ST-552, and ST-553 (CC552) are the most prevalent, although strains belonging to capsular serotype Ia and non-serotypeable strains have been isolated from disease outbreaks (Barony et al., 2017; Chideroli et al., 2017; Godoy et al., 2013).

The allelic, virulence and antibiotic resistance factors profiles of each strain are presented in Table 2. The alleles defined for the MLST scheme were based on sequence lengths of between 242 and 652 bp of seven housekeeping genes (Table 3). Eight different allelic profiles (in the order alcohol dehydrogenase - *adhP*, phenylalanyl tRNA synthetase - *pheS*, amino acid transporter - *atr*, glutamine synthetase - *glnA*, serine dehydratase - *sdhA*, glucose kinase - *glcK*, and transketolase - *tkt*) were identified for the first time (2, 17, 31, 32, 2, -, 32; 2, 2, 31, 4, 2, -, 32; 52, 2, 31, 32, 2, -, 19; 52, 2, 31, 32, 2, -, 32; 52, 2, 31, 4, 2, -, 19; 52, 2, 31, 4, 2, -, 32; 52, 2, 31, 81, 2, -, 19; and 52, 2, 31, 81, 2, -, 32), being 52, 2, 31, 4, 2, -, 19 the most predominant. Due to the lack of reverse sequences of all alleles, required for sequence type (ST) assignment (Jolley et al., 2018), these profiles were not deposited in the MLST database. Between one (*glcK*) and three (*adhP* and *glnA*) alleles were present at each locus. All strains, except IA2022, showed a partial gene deletion event on the *glcK* gene. This phenomenon was also reported in

previous studies evaluating the genetic diversity of *S. agalactiae* isolated from fish cultured in Brazil (Assis, Tavares, Pereira, Figueiredo, & Leal, 2017), suggesting that most of our strains are genetically related to the strains previously reported in disease outbreaks in Brazil. This diversity of serotype Ib strains in the main tilapia production regions in Brazil, confirmed in the present study, may be behind the ineffectiveness of commercial vaccines in preventing streptococcosis, frequently reported by fish farmers.

Surface protein Rib (*rib* = resistance to proteases, immunity, group B) and hypervirulent GBS adhesin BibA (*BibA*) were detected in all strains, except for 18P, which was negative for *rib* (Table 2). On the other hand, α and β antigens of the C protein (*bca* and *bac*, respectively) were only detected in IA2022. These results agree with previous studies which demonstrated that these proteins are associated with invasive strains isolated from mammals (Fluegge et al., 2011; Persson et al., 2008; Stålhammar-Carlemalm, Stenberg, & Lindahl, 1993), and may be evidence of an important role of these proteins in GBS infection in Nile tilapia. Considering that the expression of these antigens is correlated with protection (Bianchi-Jassir et al., 2020; Santi et al., 2009; Stålhammar-Carlemalm et al., 1993), our result indicates that all studied strains are good candidates to be used for the development of a protein and whole-cell vaccine against GBS.

Oxytetracycline (OTC) showed high minimum inhibitory concentration ($MIC \geq 16 \mu\text{g/mL}$) values against several strains with negative results for tetracycline resistance genes evaluated (104FG, IP109, IP212, Z05, IP214, 43P, 64P, IP193, IP206, IP205, D174C, D147C, D149C, D207C, 110FG, D172C, IP107, D188R, D191R, D241R, D148C, D175R, D204R), indicating that the phenotypic antibiotic susceptibility patterns may not be similar to those obtained by genotyping. Similar results were observed with florfenicol (FFC) and thiamphenicol (TAP). However, the strain IA2022 was positive for tetracycline resistance protein Tet(M) (*tetM*) and had a high MIC ($32 \mu\text{g/mL}$) of OTC (Table 4), corroborating the correlation between the

resistance genotype determined by PCR assays and the antimicrobial susceptibility pattern. These results suggest that the higher MIC values of these strains found to be negative by PCR are probably associated with mechanisms not evaluated in this study, and highlight the importance of antibiotic susceptibility testing for appropriate therapy decision-making.

All antimicrobials showed bactericidal activity against the tested strains, despite the high MIC values ($\geq 16 \mu\text{g/mL}$) observed against some strains (Table 4). Most strains showed higher MIC values ($\geq 16 \mu\text{g/mL}$) of OTC (23 strains) than FFC (one strain) and TAP (11 strains), indicating that FFC and TAP are more active than OTC against GBS.

The *in vitro* antimicrobial activity of TAP in combination with FFC or OTC, and FFC in combination with OTC, against clinical isolates with higher MIC values ($\geq 16 \mu\text{g/mL}$) against at least one of the combined antimicrobial is presented in Tables 5-7, respectively. The MIC of TAP against 64P ($64 \mu\text{g/mL}$) and D115C ($128 \mu\text{g/mL}$) were reduced fourfold and eightfold, respectively, after the TAP and FFC combination, resulting in synergic effects ($\Sigma\text{FIC} = 0.375$). However, the MIC values remained high ($\geq 16 \mu\text{g/mL}$), suggesting a low probability of therapeutic efficacy of the combination against these strains. On the other hand, the MIC of OTC against IA2022 ($32 \mu\text{g/mL}$) was reduced fourfold after the OTC combination with TAP or FFC, resulting in synergic effects ($\Sigma\text{FIC} = 0.5$) and lower MIC ($8 \mu\text{g/mL}$). Oxytetracycline combination with TAP or FFC also showed antagonistic activity against some strains (IP214, D148C, and D241C). Despite indifference and antagonism being the most predominant activities in all combinations, the record of synergism, including in a strain with a resistance gene and phenotypic resistance, suggests that combination therapy can have therapeutic efficacy when well planned. Previous studies also demonstrated the potential of this form of therapy against bacterial diseases in cultured fish (Assane, Gozi, Valladão, & Pilarski, 2019; Assane, Santos-Filho, et al., 2021; Bandeira Junior et al., 2018; Bandeira Junior et al., 2021; de Abreu Reis Ferreira et al., 2022). Our results suggest that the combination involving OTC

and TAP or FFC is an attractive candidate for improving the treatment of streptococcosis using combination therapy (CT), even for strains showing phenotypic and genotypic resistance to OTC. However, for the application of these combinations in aquaculture, further *in vivo* studies should be performed to determine their pharmacokinetics-pharmacodynamics properties and environmental and economic impacts on the fish farm.

In conclusion, our findings reveal that genetically diverse virulent *S. agalactiae* serotype Ib is the most common and widely distributed in Nile tilapia cultured in the south and southeast regions of Brazil, and due to its economic impacts and high MIC values of the available antimicrobials against it, the authorities and aquaculture workers should be more concerned about the prudent use of antimicrobials in fish farm and find eco-friendly and effective preventive and therapeutic options. Therefore, further studies should be performed to develop effective prophylactic and therapeutic strategies against GBS.

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Tables

Table 3. Primers and cycling conditions used for PCR amplification of different genes used for identification, multi-locus sequence typing (MLST), capsular serotyping, and detection of virulence and antibiotic resistance factors of 72 clinical isolates of *Streptococcus agalactiae* recovered between 2011 and 2018, from the brain, liver and kidney of diseased Nile tilapia, *Oreochromis niloticus*, farmed in different locations of Brazil.

Gene	Primer	Sequence (5'→3')	Cycling conditions (temperature, time, cycles)					Amp licon (bp)	Reference	
			Initial	Denatur	Anneali	Extens	Hold			
			denaturation	ation	ng	ion				
Identification (Hot-Start PCR using Platinum <i>Taq</i> DNA Polymerase or Platinum II <i>Taq</i> Hot-Start DNA Polymerase)										
16S rRNA	F-16S	AGAGTTTGATYMYGGCTC	95 °C, 5 min.	95 °C, 1	54 °C,	72 °C,	72 °C,	900	(Sebastião et	
		AG		min.	1.5 min.	1 min.	10 min.			al., 2015)
	R-16S	CCGTCAATTCMTTTRAGTT	1 ×	30 ×			1 ×			
		T								
16S to 23S rRNA	F-STRA- AgI	AAGGAAACCTGCCATTTG	94 °C, 2 min.	94 °C,	60 °C,	68 °C,	4 °C, 2	270	(Forsman,	
				15 s.	30 s.	30 s.	min.		Tilsaia-	

intergenic spacer	R -STRA- AgII	TTAACCTAGTTTCTTTAAA ACTAGAA	1 ×	30 ×			1 ×		Timisjrvi, & Alatossava, 1997)
<i>atr</i>	F- <i>atr</i>	CAACGATTCTCTCAGCTTT GTAA	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	780	(Mudzana, Mavenyengw a, & Gudza- Mugabe, 2021)
	R- <i>atr</i>	TAAGAAATCTCTTGTGCGG ATTTC	1 ×	30 ×			1 ×		
Sequence typing amplification (Hot-Start PCR using Platinum II <i>Taq</i> Hot-Start DNA Polymerase)									
Alcohol dehydrogen ase (<i>adhP</i>)	FA- <i>adhP</i>	GTTGGTCATGGTGAAGCAC T	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	650	(Jones et al., 2003)
	RA- <i>adhP</i>	ACTGTACCTCCAGCACGAA C	1 ×	30 ×			1 ×		

Phenylalanine synthetase (<i>PheS</i>)	FA-PheS	GATTAAGGAGTAGTGGCA CG	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	650
	RA-PheS	TTGAGATCGCCCATTGAAA T	1 ×	30 ×			1 ×	
Glutamine transporter protein (<i>atr</i>)	FA- <i>atr</i>	CGATTCTCTCAGCTTTGTT A	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	650
	RA- <i>atr</i>	AAGAAATCTCTTGTGCGGA T	1 ×	30 ×			1 ×	
Glutamine synthetase (<i>glnA</i>)	FA- <i>glnA</i>	CCGGCTACAGATGAACAA TT	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	589
	RA- <i>glnA</i>	CTGATAATTGCCATTCCAC G	1 ×	30 ×			1 ×	
	FA- <i>sdhA</i>	AGAGCAAGCTAATAGCCA AC	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	646

Serine dehydratase (<i>sdhA</i>)	RA-sdhA	ATATCAGCAGCAACAAGT GC	1 ×	30 ×	1 ×	
Glucose kinase (<i>glcK</i>)	FA-glcK	CTCGGAGGAACGACCATT AA	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.
	RA-glcK	CTTGTAACAGTATCACCGT T	1 ×	30 ×	4 °C, 2 min.	607
Transketolase (<i>tkt</i>)	FA-tkt	CCAGGCTTTGATTTAGTTG A	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.
	RA-tkt	AATAGCTTGTTGGCTTGAA A	1 ×	30 ×	4 °C, 2 min.	859
Sequence typing sequencing (Sanger dideoxy sequencing)						
	FS-adhP	GGTGTGTGCCATACTGATT T	-			498 (Jones et al., 2003)

Alcohol	RS-adhP	ACAGCAGTCACAACCACT	
dehydrogenase (<i>adhP</i>)		CC	
Phenylalanine	FS-PheS	ATATCAACTCAAGAAAAG	501
yl tRNA synthetase (<i>PheS</i>)	RS-PheS	CT TGATGGAATTGATGGCTAT G	
Glutamine transporter protein (<i>atr</i>)	FS-atr	ATGGTTGAGCCAATTATTT	501
	RS-atr	C	
		CCTTGCTCAACAATAATGC	
		C	
	FS-glnA	AATAAAGCAATGTTTGATG	498
		G	

Glutamine synthetase (<i>glnA</i>)	RS- <i>glnA</i>	GCATTGTTCCCTTCATTAT C	
Serine dehydratase (<i>sdhA</i>)	FS- <i>sdhA</i>	AACATAGCAGAGCTCATG AT	519
	RS- <i>sdhA</i>	GGGACTTCAACTAAACCTG C	
Glucose kinase (<i>glcK</i>)	FS- <i>glcK</i>	GGTATCTTGACGCTTGAGG G	459
	RS- <i>glcK</i>	ATCGCTGCTTTAATGGCAG A	
Transketolase (<i>tkt</i>)	FS- <i>tkt</i>	ACACTTCATGGTGATGGTT G	480

	RS-tkt	TGACCTAGGTCATGAGCTT								
		T								
Capsular serotyping (Multiplex PCR using Platinum II <i>Taq</i> Hot-Start DNA Polymerase)										
<i>cpsI</i>	1-cpsI-Ia-	GAATTGATAACTTTTGTGG	1 × 94 °C, 2	30 × 94	30 × 60	30 × 68	1 × 4	470	(Imperi et al., 2010)	
	6-7-F	ATTGCGATGA	min.	°C, 15 s.	°C, 30 s.	°C, 30	°C, 2			
<i>cpsI</i>	2-cpsI-6-R	CAATTCTGTCGGACTATCC				s.	min.	470		
		TGATG								
<i>cpsI</i>	3-cpsI-7-R	TGTCGCTTCCCACTGAGT						179		
		GTTGA								
<i>cpsL</i>	4-cpsL-F	CAATCCTAAGTATTTTCGG						688		
		TTCATT								
<i>cpsL</i>	5-cpsL-R	TAGGAACATGTTTCATTAAC								
		ATAGC								

<i>cpsG</i>	6-cpsG-F	ACATGAACAGCAGTTCAA CCGT	352
<i>cpsG</i>	7-cpsG-R	ATGCTCTCCAAACTGTTCT TGT	
<i>cpsG</i>	8-cpsG-2-	TCCATCTACATCTTCAATC	272
	3-6-R	CAAGC	-352
<i>cpsN</i>	9-cpsN-5-F	ATGCAACCAAGTGATTATC ATGTA	582
<i>cpsN</i>	10-cpsN-5- R	CTCTTCACTCTTTAGTGTA GGTAT	582
<i>cpsJ</i>	11-cpsJ-8- F	TATTTGGGAGGTAATCAAG AGACA	438
<i>cpsJ</i>	12-cpsJ-8- R	GTTTGGAGCATTCAAGATA ACTCT	

<i>cpsJ</i>	13-cpsJ-2-	CATTTATTGATTCAGACGA	438-
	4-F	TTACATTGA	621
<i>cpsJ</i>	14-cpsJ-2-	CCTCTTTCTCTAAAATATT	465
	R	CCAACC	
<i>cpsJ</i>	15-cpsJ-4-	CCTCAGGATATTTACGAAT	538
	R	TCTGTA	
<i>cpsI</i>	16-cpsI-7-	CTGTAATTGGAGGAATGTG	179
	9-F	GATCG	-229
<i>cpsI</i>	17-cpsI-9-	AATCATCTTCATAATTTAT	229
	R	CTCCCATT	
<i>cpsJ</i>	18-cpsJ-Ib-	GCAATTCTTAACAGAATAT	621
	F	TCAGTTG	
<i>cpsJ</i>	19-cpsJ-Ib-	GCGTTTCTTTATCACATAC	
	R	TCTTG	

Virulence genes (Hot-Start PCR using Platinum II <i>Taq</i> Hot-Start DNA Polymerase)											
β antigens of the C protein (<i>bac</i>)	bac-F	CTATTTTGGATATTGACAA	94 °C, 2 min.	94 °C,	60 °C,	68 °C,	4 °C, 2	592	(Mudzana et al., 2021)		
		TGCAA		15 s.	30 s.	30 s.	min.				
	bac-R	GTCGTTACTTCCTTGAGAT	1 ×	30 ×				1 ×			
		GTAAC									
α antigens of C protein (<i>bca</i>)	bca-F	TAACAGTTATGATACTTCA	94 °C, 2 min.	94 °C,	60 °C,	68 °C,	4 °C, 2	535			
		CAGAC		15 s.	30 s.	30 s.	min.				
	bca-R	ACGACTTTCTTCCGTCCAC	1 ×	30 ×				1 ×			
		TTAGG									
Hyaluronate lyase (<i>hly</i>)	hly-F	TCCATTTAAAGCCCTTGGT	94 °C, 2 min.	94 °C,	60 °C,	68 °C,	4 °C, 2	199			
		G		15 s.	30 s.	30 s.	min.				
	hly-R	GGCGCCAGTATAAGCAAC	1 ×	30 ×				1 ×			
		AT									

Surface protein (rib)	rib-F	CAGGAAGTGCTGTTACGTT AAAC	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	369	
	rib-R	CGTCCCATTTAGGGTCTTC C	1 ×	30 ×			1 ×		
C5a peptidase (scpB)	scpB-F	ACAACGGAAGGCGCTACT GTTC	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	255	
	scpB-R	ACCTGGTGTGTTGACCTGAA CTA	1 ×	30 ×			1 ×		
hypervirulent GBS adhesion (Gbs2018C -)	bibA-F	AATCGAAAACAACGTTGG AAAG	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	2200	(Santi et al., 2009)
	biba-R	AAACCAGGCTTCATCAGTC ATT	1 ×	30 ×			1 ×		
	hvgA-F	ATACAAATTCTGCTGACTA CCG	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min.	210	(Teatero et al., 2014)

<i>hvgA</i> , Gbs20	<i>hvgA</i> -R	TTAAATCCTTCCTGACCAT	1 ×	30 ×	1 ×	
18A - <i>BibA</i>)		TCC				
Antibiotic resistance genes (Hot-Start PCR)						
MGE	IS1548-F	TTGCGCAGTTGAATTGGAT	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 4 °C, 2 min. 690 (Mudzana et al., 2021)
IS1548		AG				
	IS1548-R	TTCTCTAACTTCAATCTGT	1 ×	30 ×	1 ×	
		CCCCTA				
Erythromycin	<i>ermB</i> -F	GAAAAGGTACTCAACCAA	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 4 °C, 2 min. 640
in		ATA				
Ribosomal	<i>ermB</i> -R	AGTAACGGTACTTAAATTG	1 ×	30 ×	1 ×	
Methylase		TT TAC				
(<i>ermB</i>)						
Erythromycin	<i>ermTR</i> -F	GAAGTTTAGCTTTCCTAA	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 4 °C, 2 min. 400
in						

Ribosomal	ermTR-R	GCTTCAGCACCTGTCTTAA	1 ×	30 ×	1 ×
Methylase (<i>ermTR</i>)		TTGAT			
Erythromycin resistance	mefA-F	AGTATCATTAATCACTAGT	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.
efflux pump (<i>mefA</i>)	mefA-R	TTCTTCTGGTACTAAAAGT	1 ×	30 ×	68 °C, 30 s.
		GG			4 °C, 2 min.
Licosamide nucleotidyl transferases (<i>linB</i>)	linB-F	CCTACCTATTGTTTGTGGA	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.
	linB-R	ATAACGTTACTCTCCTATT	1 ×	30 ×	68 °C, 30 s.
		C			4 °C, 2 min.
	tetM-F	GTGGAGTACTACATTTACG	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.
		AG			68 °C, 30 s.
					4 °C, 2 min.

Tetracyclin	tetM-R	GAAGCGGATCACTATCTG	1 ×	30 ×		1 ×	
e resistance		AG					
(<i>tetM</i>)							
Tetracyclin	tetO-F	GCGGAACATTGCATTTGAG	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min. 538
e resistance		GG					
(<i>tetO</i>)							
	tetO-R	CTCTATGGACAACCCGACA	1 ×	30 ×		1 ×	
		GAAG					
Phenicol	fexA-F	GTACTTGTAGGTGCAATTA	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min. 1272 (Kehrenberg
exporter		CGGCTGA					& Schwarz,
	fexA-R	CGCATCTGAGTAGGACAT	1 ×	30 ×		1 ×	2005)
		AGCGTC					
	fexB-F	TTCCCACTATTGGTGAAAG	94 °C, 2 min.	94 °C, 15 s.	60 °C, 30 s.	68 °C, 30 s.	4 °C, 2 min. 816 (Liu et al.,
		GAT					2012)

fexB-R	GCAATTCCTTTTATGGAC	1 ×	30 ×	1 ×
	GTT			

Table 4. Allelic, virulence and antibiotic resistance factors profiles of 72 clinical isolates of *Streptococcus agalactiae* recovered between 2011 and 2018, from the internal organs of diseased Nile tilapia, *Oreochromis niloticus*, farmed in different locations of Brazil.

Strain	GenBank	Isolation/collection			Allelic Profile ²	Virulence genes ³							Antibiotic resistance genes ⁴								
		Date	Source	Location ¹		1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	9
18 P	KJ561063	2012	Liver	1	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
IP195	KU605552	2013	Kidney	2	2, 17, 31, 32, 2, -, 32	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
IP196	KU605553	2013	Kidney	2	-, -, 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
D115R	KU605572	2014	Kidney	3	52, -, 31, 4, 2, -, 32	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
78FG	KJ561053	2012	Brain	4	52, 2, 31, 32, 2, -, 32	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
IP213	KU605559	2013	Kidney	5	52, 2, 31, 32, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
112FG	KJ561072	2012	Brain	4	52, 2, 31, 81, 2, -, 32	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
IP207	KU605557	2013	Kidney	5	52, 2, 31, 81, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
IP202	KU605554	2013	Kidney	5	2, 2, 31, 4, 2, -, 32	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-

D151C	KX385295	2016	Brain	5	- , - , 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
104FG	KJ561057	2012	Brain	4	- , - , 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
IP109	KU605544	2011	Kidney	5	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
IP212	KU605558	2013	Kidney	5	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
Z05	KJ561051	2014	Brain	6	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
IP214	KU605560	2013	Kidney	5	52, 2, 31, 32, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
76FG	KJ561058	2012	Brain	4	- , - , 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
99FG	KJ561054	2012	Brain	4	- , - , 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
102FG	KJ561062	2012	Brain	4	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
103FG	KJ561055	2012	Brain	4	- , - , 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
105FG	KJ561059	2012	Brain	4	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	
106FG	KJ561061	2012	Brain	4	- , - , 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	

43P	KJ561068	2011	Brain	1	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
64P	KJ561071	2011	Brain	1	52, 2, 31, 32, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D115C	KU605571	2014	Brain	3	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D203R	KX385311	2016	Kidney	3	52, 2, 31, 4, 2, -, 32	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
100FG	KJ561056	2012	Brain	4	-, -, 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
77FG	KJ561060	2012	Brain	4	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
74P	KJ561064	2011	Brain	1	-, -, 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
M	KJ561070	2011	Brain	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
36P	KJ561067	2011	Liver	1	2, 2, 31, 4, 2, -, 32	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
IP193	KU605550	2013	Kidney	2	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D6C	KU605568	2014	Brain	7	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D234R	KX385319	2016	Kidney	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-

IP206	KU605556	2016	Kidney	5	52, 2, 31, 4, 2, -, 19	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D224C	KX385318	2016	Brain	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
IP205	KU605555	2013	Kidney	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D6R	KU605569	2014	Kidney	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
26P	KJ561065	2011	Kidney	1	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D234C	KX385318	2016	Brain	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D177C	KX385299	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D172R	KX385297	2016	Kidney	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D202C	KX385308	2016	Brain	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D193C	KX385305	2016	Brain	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D193R	KX385306	2016	Kidney	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D174C	KX385298	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-

D147C	KX385292	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D169C	KX385296	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D189R	KX385302	2016	Kidney	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D149C	KX385294	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Z09	KJ561052	2014	Brain	6	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D207C	KX385314	2016	Brain	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D203C	KX385310	2016	Brain	3	-, -, 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
110FG	KJ561069	2012	Brain	4	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D172C	-	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
IP107	KU605543	2011	Kidney	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D188R	KX385300	2016	Brain	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D145C	KX385290	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-

D204C	KX385312	2016	Brain	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D191R	KX385303	2016	Brain	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D241R	KX385321	2016	Kidney	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D241C	KX385320	2016	Brain	3	-, -, 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D148C	KX385293	2016	Brain	5	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D145R	KX385291	2016	Kidney	5	-, -, 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D208C	KX385315	2016	Brain	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D209C	KX385316	2016	Brain	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D189C	KX385301	2016	Brain	7	-, -, 31, -, -, -, -	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D204R	KX385313	2016	Kidney	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D5R	KU605567	2014	Kidney	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
D191C	KX385303	2016	Brain	7	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-

D202R	KX385309	2016	Kidney	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
124FG	KU605573	2015	Kidney	3	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
IA2022	ON500637	2018	Brain	8	95,-. 95, -, 89, 3, -	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-

¹Allelic profiles for each gene are presented in the order alcohol dehydrogenase (*adhP*), phenylalanyl tRNA synthetase (*pheS*), amino acid transporter (*atr*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA*), glucose kinase (*glcK*), and transketolase (*tkl*).

²Location 1 = Itambaracá (Paraná - PR), 2 = Reservoir of Canoas II, Palmital (São Paulo - SP), 3 = Nova Avanhandava Dam - Tietê River, Buritama (SP), 4 = Paranapanema River, Avaré (SP), 5 = Ilha Solteira Dam – Paraná River, Ilha Solteira (SP), 6 = Guaíra (SP), 7 = Chavantes reservoir, Chavantes (SP), 8 = Paulo Afonso (Bahia).

³Virulence gene 1 = β antigens of the C protein (*bac*), 2 = α antigens of C protein (*bca*), 3 = hyaluronate lyase (*hly*), 4 = surface protein Rib (*rib*), 5 = C5a peptidase (*scpB*), 6 = hypervirulent GBS adhesin BibA (*BibA*), 7 = hypervirulent GBS adhesin HvgA (*HvgA*).

⁴Antibiotic resistance gene 1 = mobile genetic element (MGE) insertion sequence (IS) 1548, 2 = erythromycin Ribosomal Methylase *ermB*, 3 = erythromycin Ribosomal Methylase *rmTR*, 4 = erythromycin resistance efflux pump (*mefA*), 5 = lincomycin and clindamycin resistance gene – lincosamide nucleotidyltransferase (*linB*), 6 = tetracycline resistance protein Tet(M) (*tetM*), 7 = tetracycline resistance protein Tet(O) (*tetO*), 8 = phenicol exporter gene *fexA*, 9 = phenicol exporter gene *fexB*.

Table 5. Characteristics of loci included in the GBS MLST system

Locus	Gene	Size of the sequenced fragment (bp)	Number of alleles identified
<i>adhP</i>	Alcohol dehydrogenase	336-562	3
<i>pheS</i>	Phenylalanyl tRNA synthetase	390-578	2
<i>atr</i>	Amino acid transporter	469-629	2
<i>glnA</i>	Glutamine synthetase	502-595	3
<i>sdhA</i>	Serine dehydratase	490-558	2
<i>glcK</i>	Glucose kinase	495	1
<i>tkt</i>	Transketolase	242-652	2

Table 6. Antimicrobial activity ($\mu\text{g/mL}$) (minimum inhibitory concentration - MIC, minimum bactericidal concentration - MBC, and MBC/MIC ration) of florfenicol (FFC), thiamphenicol (TAP) and oxytetracycline (OTC) against 72 clinical isolates of *Streptococcus agalactiae* recovered between 2011 and 2018, from the internal organs of diseased Nile tilapia, *Oreochromis niloticus*, farmed in different locations of Brazil.

Strain	GenBank	FFC		TAP		OTC		MBC/MIC			Interpretation*		
		MIC	MBC	MIC	MBC	MIC	MBC	FFC	TAP	OTC	FFC	TAP	OTC
18P	KJ561063	2	>8	16	16	0.5	2	-	1	4	-	a	a
IP195	KU605552	1	2	4	4	4	4	2	1	1	a	a	a
IP196	KU605553	2	>8	4	16	4	4	-	4	1	-	a	a
D115R	KU605572	2	>8	16	16	0.5	0.5	-	1	1	-	a	a
78FG	KJ561053	0.25	0.25	16	16	0.25	0.25	1	1	1	a	a	a
IP213	KU605559	0.25	0.25	16	>64	0.25	0.25	1	-	1	a	-	a
112FG	KJ561072	0.25	0.25	8	32	0.25	0.25	1	4	1	a	a	a
IP207	KU605557	1	>4	4	4	0.25	0.25	-	1	1	-	a	a

IP202	KU605554	1	>4	4	4	0.25	0.25	-	1	1	-	a	a
D151C	KX385295	<0.25	-	128	512	0.5	2	-	4	4	-	a	a
104FG	KJ561057	4	4	4	4	>64	-	1	1	-	a	a	-
IP109	KU605544	2	2	8	32	256	256	1	4	1	a	a	a
IP212	KU605558	2	2	8	32	256	256	1	4	1	a	a	a
Z05	KJ561051	2	8	8	32	256	256	4	4	1	a	a	a
IP214	KU605560	2	8	8	32	128	128	4	4	1	a	a	a
76FG	KJ561058	<0.25	-	<0.25	-	0.5	0.5	-	-	1	-	-	a
99FG	KJ561054	8	8	32	32	0.5	0.5	1	1	1	a	a	a
102FG	KJ561062	<0.25	-	<0.25	-	0.5	0.5	-	-	1	-	-	a
103FG	KJ561055	<0.25	-	<0.25	-	0.5	0.5	-	-	1	-	-	a
105FG	KJ561059	4	4	4	4	2	2	1	1	1	a	a	a

106FG	KJ561061	-	-	-	-	-	-	-	-	-	-	-	-
43P	KJ561068	4	4	4	8	32	32	1	2	1	a	a	a
64P	KJ561071	2	8	64	64	16	16	4	1	1	a	a	a
D115C	KU605571	4	8	128	512	4	16	2	4	2	a	a	a
D203R	KX385311	2	8	8	32	2	2	4	4	1	a	a	a
100FG	KJ561056	4	8	4	4	1	1	2	1	1	a	a	a
77FG	KJ561060	4	8	4	4	8	8	2	1	1	a	a	a
74P	KJ561064	2	8	4	16	4	4	4	4	1	a	a	a
M	KJ561070	64	-	>256	>256	8	8	-	-	1	-	-	a
36P	KJ561067	8	8	8	16	2	2	1	2	1	a	a	a
IP193	KU605550	4	8	32	128	64	64	2	4	1	a	a	a
D6C	KU605568	2	2	32	128	1	1	1	4	1	a	a	a

D234R	KX385319	-	-	-	-	-	-	-	-	-	-	-	-
IP206	KU605556	2	2	8	8	32	32	1	1	1	a	a	a
D224C	KX385318	-	-	-	-	-	-	-	-	-	-	-	-
IP205	KU605555	2	4	4	4	>256	-	2	1	-	a	a	-
D6R	KU605569	-	-	-	-	-	-	-	-	-	-	-	-
26P	KJ561065	-	-	-	-	-	-	-	-	-	-	-	-
D234C	KX385318	4	4	4	8	0.5	0.5	1	2	1	a	a	a
D177C	KX385299	2	2	4	8	2	2	1	2	1	a	a	a
D172R	KX385297	2	2	4	8	2	2	1	2	1	a	a	a
D202C	KX385308	2	4	4	8	1	1	1	2	1	a	a	a
D193C	KX385305	2	2	4	4	0.5	0.5	1	1	1	a	a	a
D193R	KX385306	2	2	4	4	1	1	1	1	1	a	a	a

D174C	KX385298	8	8	4	4	>256	-	1	1	-	a	a	-
D147C	KX385292	8	8	4	8	>256	-	-	-	-	-	-	-
D169C	KX385296	4	4	4	4	2	2	-	1	1	-	a	a
D189R	KX385302	2	2	4	4	0.5	0.5	-	1	1	-	a	a
D149C	KX385294	2	2	4	8	>256	-	-	2	-	-	a	-
Z09	KJ561052	2	2	4	8	8	8	-	2	1	-	a	a
D207C	KX385314	2	2	4	8	32	32	-	2	1	-	a	a
D203C	KX385310	2	2	4	16	0.5	0.5	-	4	1	-	a	a
110FG	KJ561069	2	2	4	4	32	32	-	1	1	-	a	a
D172C	-	2	2	4	16	16	16	-	4	1	-	a	a
IP107	KU605543	2	2	4	4	16	16	-	1	1	-	a	a
D188R	KX385300	2	2	4	4	16	16	-	1	1	-	a	a

D145C	KX385290	2	2	4	16	2	2	-	4	1	-	a	a
D204C	KX385312	2	2	4	8	1	1	-	2	1	-	a	a
D191R	KX385303	2	8	4	16	16	16	4	4	1	a	a	a
D241R	KX385321	2	4	4	16	32	32	2	4	1	a	a	a
D241C	KX385320	2	>8	4	16	8	>32	-	4	-	-	a	-
D148C	KX385293	2	>8	4	16	32	32	-	4	1	-	a	a
D145R	KX385291	2	8	4	16	32	32	4	4	1	a	a	a
D208C	KX385315	2	8	4	8	1	2	4	2	2	a	a	a
D209C	KX385316	2	8	4	16	4	8	4	4	2	a	a	a
D189C	KX385301	2	4	4	4	0.5	1	2	1	2	a	a	a
D204R	KX385313	2	4	4	16	32	32	2	4	1	a	a	a
D5R	KU605567	2	>8	4	16	4	4	-	4	1	a	a	a

D191C	KX385303	2	8	4	8	1	4	4	2	4	a	a	a
D202R	KX385309	2	4	4	4	0.5	0.5	2	1	1	a	a	a
124FG	KU605573	2	2	4	4	1	>2	1	1	-	a	a	-
IA2022	ON500637	4	16	2	2	32	64	4	1	2	a	a	a

* a = bactericidal, b = bacteriostatic.

Table 7. *In vitro* antimicrobial activity of thiamphenicol (TAP) in combination with florfenicol (FFC) against clinical isolates of *Streptococcus agalactiae* recovered between 2011 and 2014, from the internal organs of diseased Nile tilapia, *Oreochromis niloticus*, farmed in different locations of Brazil.

Strain	GenBank	MIC ¹ alone (µg/mL)		MIC in combination (µg/mL)		Fold MIC in combination		ΣFIC ²
		TAP	FFC	TAP	FFC	TAP	FFC	
18P	KJ561063	16.00	2.00	8.00	1.00	1/2	1/2	1.00
D115R	KU605572	16.00	2.00	8.00	0.25	1/2	1/8	0.63
78FG	KJ561053	16.00	0.25	4.00	0.25	1/4	1	1.25
IP213	KU605559	16.00	0.25	8.00	0.25	1/2	1	1.50
99FG	KJ561054	32.00	8.00	8.00	4.00	1/4	1/2	0.75
64P	KJ561071	64.00	2.00	16.00	0.25	1/4	1/8	0.38
D115C	KU605571	128.00	4.00	16.00	1.00	1/8	1/4	0.38
IP193	KU605550	32.00	4.00	16.00	2.00	1/2	1/2	1.00
D6C	KU605568	32.00	2.00	8.00	2.00	1/4	1	1.25

¹MIC = minimum inhibitory concentration.

²FIC = fractional inhibitory concentration.

Table 8. *In vitro* antimicrobial activity of thiamphenicol (TAP) in combination with oxytetracycline (OTC) against clinical isolates of *Streptococcus agalactiae* recovered between 2011 and 2018, from the internal organs of diseased Nile tilapia, *Oreochromis niloticus*, farmed in different locations of Brazil.

Strain	GenBank	MIC ¹ alone (ug/mL)		MIC in combination (µg/mL)		Fold MIC in combination		ΣFIC ²
		TAP	OTC	TAP	OTC	TAP	OTC	
18 P	KJ561063	16.00	0.50	8.00	0.50	1/2	1	1.50
D115R	KU605572	16.00	0.50	8.00	0.50	1/2	1	1.50
78FG	KJ561053	16.00	0.25	4.00	0.13	1/4	1/2	0.75
IP213	KU605559	16.00	0.25	8.00	0.25	1/2	1	1.50
D151C	KX385295	128.00	0.50	32.00	0.25	1/4	1/2	0.75
IP109	KU605544	8.00	256.00	4.00	128.00	1/2	1/2	1.00
IP212	KU605558	8.00	256.00	4.00	128.00	1/2	1/2	1.00
Z05	KJ561051	8.00	256.00	4.00	64.00	1/2	1/4	0.75
IP214	KU605560	8.00	128.00	4.00	64.00	1/2	1/2	1.00
99FG	KJ561054	32.00	0.50	8.00	0.25	1/4	1/2	0.75
43P	KJ561068	4.00	32.00	2.00	16.00	1/2	1/2	1.00

64P	KJ561071	64.00	16.00	16.00	8.00	1/4	1/2	0.75
D115C	KU605571	128.00	4.00	16.00	2.00	1/8	1/2	0.63
IP109	KU605544	8.00	256.00	8.00	128.00	1	1/2	1.50
IP212	KU605558	8.00	256.00	8.00	128.00	1	1/2	1.50
Z05	KJ561051	8.00	256.00	8.00	128.00	1	1/2	1.50
IP214	KU605560	8.00	128.00	8.00	128.00	1	1	2.00
IP193	KU605550	32.00	64.00	16.00	16.00	1/2	1/4	0.75
D6C	KU605568	32.00	1.00	8.00	0.50	1/4	1/2	0.75
IP206	KU605556	8.00	32.00	8.00	8.00	1	1/4	1.25
D207C	KX385314	4.00	32.00	2.00	16.00	1/2	1/2	1.00
110FG	KJ561069	4.00	32.00	2.00	8.00	1/2	1/4	0.75
D172C	-	4.00	16.00	2.00	8.00	1/2	1/2	1.00
D191R	KX385303	4.00	16.00	2.00	8.00	1/2	1/2	1.00
D241R	KX385321	4.00	32.00	2.00	32.00	1/2	1	1.50
D148C	KX385293	4.00	32.00	4.00	32.00	1	1	2.00
D145R	KX385291	4.00	32.00	2.00	16.00	1/2	1/2	1.00

D204R	KX385313	4.00	32.00	2.00	16.00	1/2	1/2	1.00
IA2022	ON500637	2.00	32.00	0.50	8.00	1/4	1/4	0.50

¹MIC = minimum inhibitory concentration.

²FIC = fractional inhibitory concentration.

Table 9. *In vitro* antimicrobial activity of florfenicol (FFC) in combination with oxytetracycline (OTC) against clinical isolates of *Streptococcus agalactiae* recovered between 2011 and 2018, from the internal organs of diseased Nile tilapia, *Oreochromis niloticus*, farmed in different locations of Brazil.

Strain	GenBank	MIC alone (µg/mL)		MIC in combination (µg/mL)		Fold MIC in combination		ΣFIC
		FFC	OTC	FFC	OTC	FFC	OTC	
IP109	KU605544	2.00	256.00	1.00	128.00	1/2	1/2	1.00
IP212	KU605558	2.00	256.00	0.25	128.00	1/8	1/2	0.63
Z05	KJ561051	2.00	256.00	1.00	128.00	1/2	1/2	1.00
IP214	KU605560	2.00	128.00	0.25	64.00	1/8	1/2	0.63
43P	KJ561068	4.00	32.00	2.00	16.00	1/2	1/2	1.00
64P	KJ561071	2.00	16.00	0.25	8.00	1/8	1/2	0.63
IP109	KU605544	2.00	256.00	1.00	128.00	1/2	1/2	1.00
IP212	KU605558	2.00	256.00	1.00	128.00	1/2	1/2	1.00
Z05	KJ561051	2.00	256.00	1.00	64.00	1/2	1/4	0.75
IP214	KU605560	2.00	128.00	1.00	16.00	1/2	1/8	0.63
IP193	KU605550	4.00	64.00	2.00	16.00	1/2	1/4	0.75

IP206	KU605556	2.00	32.00	1.00	8.00	1/2	1/4	0.75
D207C	KX385314	2.00	32.00	1.00	16.00	1/2	1/2	1.00
110FG	KJ561069	2.00	32.00	1.00	8.00	1/2	1/4	0.75
D172C	-	4.00	16.00	4.00	8.00	1	1/2	1.50
D191R	KX385303	2.00	16.00	2.00	8.00	1	1/2	1.50
D241R	KX385321	2.00	32.00	2.00	32.00	1	1	2.00
D148C	KX385293	2.00	32.00	2.00	32.00	1	1	2.00
D145R	KX385291	2.00	32.00	2.00	16.00	1	1/2	1.50
D204R	KX385313	2.00	32.00	2.00	16.00	1	1/2	1.50
IA2022	ON500637	4.00	32.00	1.00	8.00	1/4	1/4	0.50

¹MIC = minimum inhibitory concentration, ²FIC = fractional inhibitory concentration.

Figures

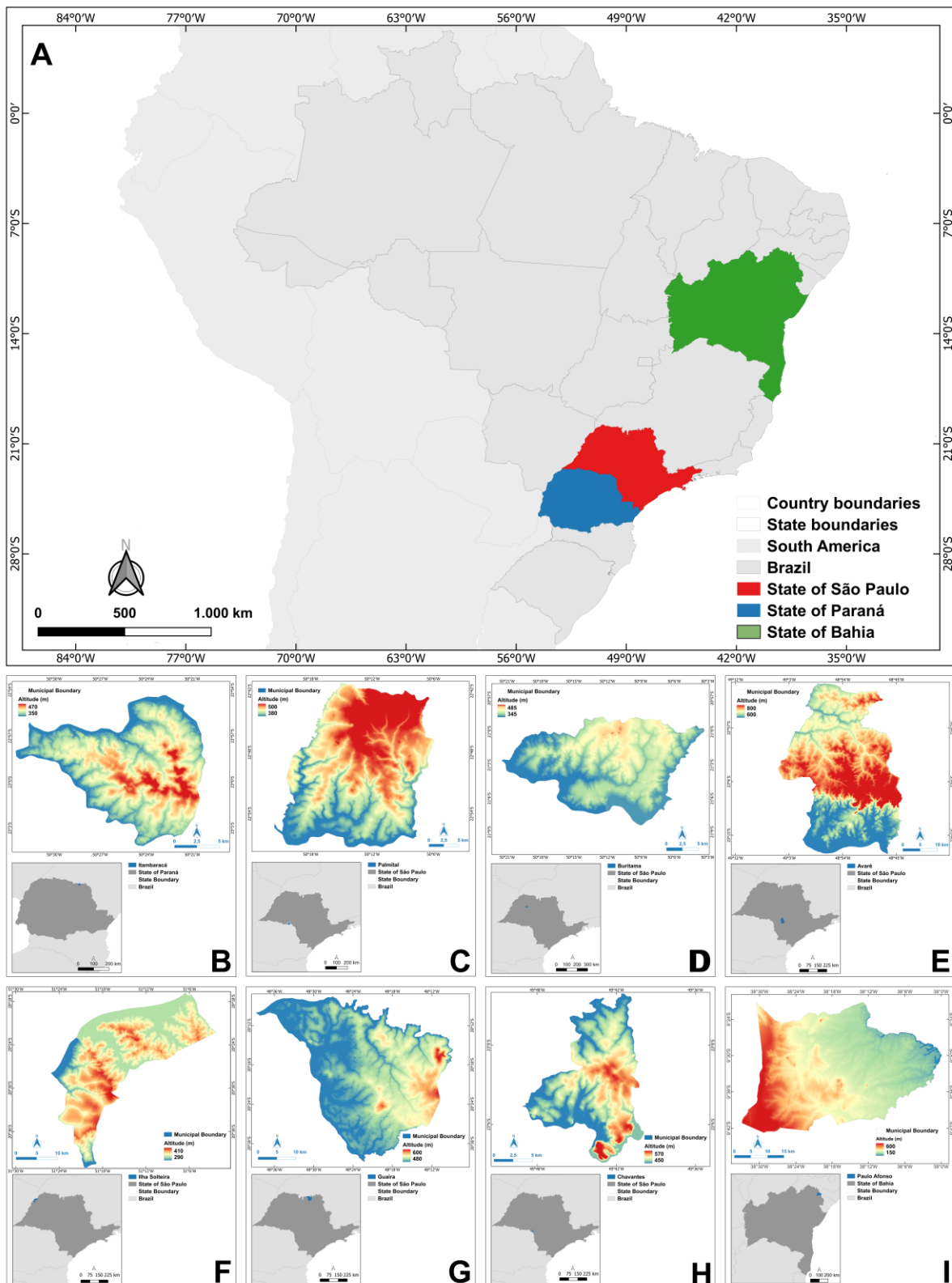


Figure 1. Map showing the geographical location of clinical isolates of *Streptococcus agalactiae* (group B Streptococcus, GBS) recovered between 2011 and 2018, from the brain,

liver and kidney of diseased Nile tilapia, *Oreochromis niloticus*, farmed in Brazil. Datum SIRGAS 2000. Data source: IBGE, 2021; SRTM/NASA (Earth Explorer USGS).

Cytotoxicity and antimicrobial activity of synthetic peptides alone or in combination with conventional antimicrobials against fish pathogenic bacteria

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Abstract

Aims: This study aimed to evaluate the *in vitro* cytotoxicity and efficacy of synthetic host defence peptides (HDPs), alone or in combination with florfenicol (FFC), oxytetracycline (OTC) or thiamphenicol (TAP), against different pathogenic bacteria isolated from diseased fish.

Methods and Results: Solid-phase synthesis, purification, and characterization of several HDPs were performed manually, using the fluorenylmethyloxycarbonyl protecting group in different resins, and *via* high-performance liquid chromatography-mass spectrometry, respectively. The *in vitro* cytotoxicity and antimicrobial activity of HDPs, FFC, OTC, and TAP against Nile tilapia red blood cells (RBCs), and relevant fish pathogenic bacteria (*Aeromonas*, *Citrobacter*, *Edwardsiella*, *Streptococcus*, *Lactococcus*, and *Vibrio*) was determined using the haemolysis assay and broth microdilution method, respectively. The checkerboard assay was used to evaluate the synergy between the most active HDPs and other antimicrobials against the tested strains. MUC 7 12-mer, FFC, OTC, and TAP were not cytotoxic to Nile tilapia RBCs, in all tested concentrations. LL-37, (p-BthTX-I)₂, and Hylin-a1 were not cytotoxic at concentrations up to 78.13, 19.53, and 9.77 µg mL⁻¹, respectively. Host defence peptides demonstrated potent antimicrobial activity (minimum inhibitory concentration ≤ 31.25 µg mL⁻¹) against *Aeromonas jandaei* (KR-12-a5), *Citrobacter freundii* (Kr12a5; (p-BthTX-I)₂; LL-37; and Hylin a1), *Streptococcus agalactiae* (Hylin a1; (p-BthTX-I)₂; and LL-37), *Lactococcus garviae* (Hylin-a1) and *Vibrio fluvialis* (KR-12-a5). The combinations of (p-BthTX-I)₂ with TAP and LL37 with FFC showed synergistic activity against *C. freundii* (fractional inhibitory concentration index of 0.25 and 0.50, respectively).

Conclusions: Synthetic HDPs have the potential as a good treatment option for bacterial diseases in aquaculture.

Significance and Impact of the Study: The *in vivo* effectiveness of synthetic HDPs such as KR-12-a5; LL-37; (p-BthTX-I)₂ and Hylin-a1 can be tested alone or in combination with conventional antimicrobials as a treatment option to reduce the use of antimicrobials in aquaculture.

Keywords: Cytotoxicity, antimicrobial activity, synthetic peptides, synergy, fish pathogenic bacteria

Introduction

There is an immediate requirement for novel antimicrobials in aquaculture, due to the widespread resistance of bacterial pathogens to many important antimicrobials and the ineffective administration strategies available for fish farmers. Most of the 47 currently available antimicrobials approved for use in aquaculture (food species) around the world (FDA, 2007; Health Canada, 2010; Yoshimizu *et al.*, 2016; Felleskatalogen, 2018; Miranda, Godoy and Lee, 2018; Lulijwa, Rupia and Alfaro, 2019) are becoming less effective due to the rapid emergence of antibiotic-resistant bacteria (Soto *et al.*, 2015; Chideroli *et al.*, 2017; de Oliveira *et al.*, 2018). Screening compounds for antimicrobial activity against fish pathogenic bacteria is a cost-effective and sustainable solution to address this challenge. In the last decade, several attempts have been made to explore new, environmentally-friendly antimicrobial drugs from natural sources, including virus (Culot, Grosset and Gautier, 2019), plant (Devi *et al.*, 2016; Bandeira Junior *et al.*, 2018; Assane, Valladão and Pilarski, 2021) and animal (Chettri *et al.*, 2017; Pan *et al.*, 2017; Buonocore *et al.*, 2019) products.

Host defence peptides (HDPs), also known as antimicrobial peptides (AMPs), are a member of the promising new eco-friendly generation of antimicrobials for meeting the demand of antimicrobials in aquaculture (Li *et al.*, 2017; Zermeño-Cervantes *et al.*, 2020; de Sousa *et al.*, 2021). Host defence peptides are short polypeptide sequences (~12-50 residues) produced by all living organisms as a host defence mechanism against infection (Yeaman and Yount, 2003; Haney, Straus and Hancock, 2019). They are important components of the innate and adaptive immune system of fish and help to protect fish from microbial infection. The mechanism of action of HDPs varies between types (Zasloff, 2002; Yeaman and Yount, 2003; Li *et al.*, 2017; Haney *et al.*, 2019; Raheem and Straus, 2019). However, most HDPs act by disrupting the cytoplasmic membrane of bacteria *via* disruption of the lipid bilayer, or thinning of membrane lipid bilayer followed by the dissolution of the membrane (Kang *et al.*, 2017),

depolarization and cell death (Li *et al.*, 2017), and direct interference on membrane-associated targets or cytoplasmatic targets (Haney *et al.*, 2019; Raheem and Straus, 2019).

Although new peptide-based antimicrobial would be prone to similar patterns of bacterial resistance as those observed for conventional antimicrobials (Haney *et al.*, 2019), bacteria are less likely to develop resistance against HDPs due to their immunomodulatory properties (Chettri *et al.*, 2017; Scheenstra *et al.*, 2019), direct action (bind and neutralization) on bacterial lipopolysaccharides (LPS) (Ciornei *et al.*, 2005; Sigurdardottir *et al.*, 2006), and the low probability of bacterial membrane mutations (Zasloff, 2002). According to Spohn *et al.* (2019) evolution of resistance against HDPs is limited, and the resistance level provided by point mutations and gene amplification is very low, with antibiotic-resistant bacteria displaying no cross-resistance to HDPs. Moreover, HDPs can kill antibiotic-resistant bacteria (Ageitos *et al.*, 2017), and enhance the antimicrobial activity of conventional antimicrobials in combination therapies (Yeaman and Yount, 2003; Carneiro *et al.*, 2020).

Within the last three decades, the number of studies on the potentialities of synthetic and naturally occurring HDPs found in bacteria, insects, plants, vertebrates, including alligator (Barksdale *et al.*, 2016), cattle, pig, fish (Chia *et al.*, 2010; Masso-Silva and Diamond, 2014; Shin *et al.*, 2017; Valero *et al.*, 2020), and humans (Zasloff, 2002; Yeaman and Yount, 2003; Scheenstra *et al.*, 2019; Mookherjee *et al.*, 2020) has grown intensely. There are currently at least 28 HDPs in clinical trials and two peptide-derive treatments (PAC-113 in Taiwan and dalbavancin in the USA) approved for use in medicine around the world (Mookherjee *et al.*, 2020). Several HDPs have been isolated and reported to have a strong antimicrobial effect against Gram-positive, Gram-negative, and alcohol acid-resistant bacteria isolated from different organisms (Turner *et al.*, 1998; Ciornei *et al.*, 2005; Ouhara *et al.*, 2005; Ji *et al.*, 2007; Rivas-Santiago *et al.*, 2013; Kreling *et al.*, 2016), and negligible toxicity against fish (de Sousa *et al.*, 2021). Nevertheless, the antimicrobial activity of HDPs and their combinations

with conventional antimicrobials against *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Streptococcus*, *Lactococcus*, and *Vibrio* isolated from fish has not been widely reported.

Based on the above information, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of synthetic HDPs alone or in combination with conventional antimicrobials (florfenicol, oxytetracycline, thiamphenicol) was determined against Gram-positive (*Lactococcus garviae*, and *Streptococcus agalactiae*) and Gram-negative (*Aeromonas caviae*, *A. hydrophila*, *A. jandaei*, *Citrobacter freundii*, *Edwardsiella tarda* and *Vibrio fluvialis*) pathogenic bacteria, isolated from diseased freshwater fish in Brazil. In addition, the *in vitro* cytotoxic effect against Nile tilapia *Oreochromis niloticus* red blood cells (RBCs) was also determined.

Materials and methods

Antimicrobials

Host defence peptides (HDPs) were synthesized in the Department of Biochemistry and Technological Chemistry at the Institute of Chemistry of São Paulo State University (UNESP), Araraquara, São Paulo, Brazil. Analytical standards of florfenicol (FFC, Lot: MKCF7029, $\geq 99.0\%$ purity), oxytetracycline (OTC, Lot: BCBG9599V, $\geq 95.0\%$ purity), and thiamphenicol (TAP, Lot: BCBP3008V, $\geq 99.0\%$ purity) were obtained commercially from Sigma–Aldrich.

HDPs synthesis

Solid-phase peptide synthesis was performed manually *via* a previously described method (Merrifield, 1963), using the Fmoc (9-fluorenylmethyloxycarbonyl) protecting group in different resins (Table S1). Deprotection of the Fmoc group was performed in a 20% 4-methylpiperidine solution in dimethylformamide (DMF). In all cases, the amino acids were coupled in 2× excess, using diisopropylcarbodiimide (DIC)/N-hydroxybenzotriazole (HOBt)

in 50% (v v⁻¹) solution of methylene chloride (DCM)/DMF. After 2 h of incubation, the success of the coupling was evaluated by the ninhydrin test (Kaiser *et al.*, 1970). The cleavage of the resin and removal of the protecting groups from the side chains of the amino acid residues were carried out simultaneously for 2 h, using a cocktail of reagents (Table S1) in the proportion of 10 ml g⁻¹ resin. After this procedure, the crude peptides were precipitated with anhydrous ethyl ether and separated from the soluble non-peptide material by centrifugation. The peptide content was then extracted with 0.045% (v v⁻¹) TFA/H₂O (solvent A), and lyophilized for further purification and characterization *via* high-performance liquid chromatography (HPLC) and mass spectrometry (MS).

Purification and characterization of HDPs by HPLC

Purification of synthetic peptides was carried out in a semipreparative way using a HPLC System Gold BECKMAN, in a C18 reverse-phase column (Phenomenex 2.1 x 25 cm). The degree of purity of the fractions was determined on a Shimadzu chromatograph, on an analytical column (0.46 x 25 cm) of C18 reverse-phase (Kromassil). The solvents used for the purification and verification of the purity content of the peptides were 0.045% TFA in deionized water (Solvent A) and 0.036% TFA in acetonitrile (Solvent B). The purification and purity analysis methods are shown in Table S1.

Mass spectrometry

The mass spectrum was obtained using an LCQ FLEET ThermoScientific mass spectrometer, with electrospray ionization (ESI) operated in the positive ionization mode (ESI⁺) *via* direct injection, located at the Institute of Chemistry of Araraquara at Unesp, in the Department of Organic Chemistry.

Cytotoxicity studies by haemolytic activity

The *in vitro* cytotoxic effect of seven synthesized HDPs, FFC, OTC, and TAP against fish RBCs was studied by examining the haemolytic activity of each antimicrobial against Nile tilapia *Oreochromis niloticus* RBCs following the method described in our study (Assane *et al.*, 2021). Briefly, fresh blood was collected from healthy juvenile Nile tilapia by puncture of caudal blood vessels, then washed three times by centrifugation ($500\times g$ for 5 min, 4°C) with phosphate-buffered saline (PBS, pH 7.4).

A suspension of 1% (v v^{-1}) erythrocyte (1% ER) was made with packed RBC's suspended in PBS. 2 mL Eppendorf tubes containing 1% ER and different concentrations of each antimicrobial (or control solution) were incubated for 1 h at 28°C , followed by centrifugation at $500\times g$ for 5 min (4°C). 100 μl aliquots of the supernatant were transferred to 96-well plates, and the absorbance at 540 nm was determined.

The assay was performed in triplicate, and 1% (v v^{-1}) Triton X-100 solution and PBS were used as positive (100% lysis) and negative (0% lysis) controls, respectively. The ranges of antimicrobial concentrations in the tests were 1,250–2.44 $\mu\text{g ml}^{-1}$ HDP, 512–1 $\mu\text{g ml}^{-1}$ FFC, 512–1 $\mu\text{g ml}^{-1}$ OTC, and 128–0.25 $\mu\text{g ml}^{-1}$ TAP.

The percentage of lysis was determined for each concentration of antimicrobial evaluated using the following equation: $\% \text{ of lysis} = \frac{(A_s - A_{\text{PBS}})}{(A_{\text{Triton}} - A_{\text{PBS}})} \times 100$, where A_s , A_{PBS} , and A_{Triton} represent absorbance of the sample (antimicrobial), PBS and 1% (v v^{-1}) Triton X-100, respectively.

The average value and standard deviation for each concentration of antimicrobial were calculated from triplicate assays. Data were examined for normality and homogeneity of variance, via one-way ANOVA or t-test ($\alpha = 0.05$). Significant results ($P < 0.05$) from dependent variables were tested using the Holm-Sidak multiple comparison test of means, at a

significance level of 0.05. Rare non-normal data were normalised using a square root transformation.

Antimicrobial susceptibility testing (AST)

Bacterial isolates

Antimicrobial sensitivity to each HDP, FFC, OTC, and TAP was tested in Gram-negative (*Aeromonas caviae*, *A. hydrophila*, *A. jandaei*, *Citrobacter freundii*, *Edwardsiella tarda*, and *Vibrio fluvialis*) and Gram-positive (*Lactococcus garvieae*, and *Streptococcus agalactiae*) clinical isolates. Isolates were previously recovered from the internal organs of Nile tilapia, cachara (*Pseudoplatystoma fasciatum*), or freshwater angelfish (*Pterophyllum scalare*) positively diagnosed with a bacterial infection, sampled during disease outbreaks at fish farms or facilities located in Brazil. Two reference strains, *Escherichia coli* (ATCC 25922) and *Aeromonas hydrophila* (ATCC 7966) were included in the studies for quality control (QC) and comparative purposes, respectively.

Bacteria identities were confirmed by morphological, biochemical, and molecular (16S rRNA, DNA gyrase subunit B–*gyrB*, and RNA-polymerase subunit B–*rpoB* gene sequencing) tests. The obtained sequences from molecular analyses were deposited in GenBank.

Minimum inhibitory and bactericidal concentrations (MIC and MBC)

Antimicrobial activity (MIC and MBC) was determined *via* a method inspired by the microdilution broth method, recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014) when possible. Briefly, peptides were dissolved and diluted in sterile distilled water to prepare 5,000 µg mL⁻¹ antimicrobial stock solutions. Florfenicol (FFC), OTC, and TAP were dissolved in 95% ethanol (FFC, TAP) or 100% methanol (OTC), and diluted in sterile distilled water to prepare 1,280 µg mL⁻¹ FFC, 1,280 µg mL⁻¹ OTC, 5,120 µg mL⁻¹ TAP,

and 20,480 $\mu\text{g ml}^{-1}$ TAP antimicrobial stock solutions. The ranges of antimicrobial concentrations in these tests were 250–0.4883 $\mu\text{g ml}^{-1}$ HDP, 64–0.125 $\mu\text{g ml}^{-1}$ FFC, 64–0.125 $\mu\text{g ml}^{-1}$ OTC, 256–0.5 $\mu\text{g ml}^{-1}$ TAP, and 1,024–2 $\mu\text{g ml}^{-1}$ TAP.

All isolates were recovered and cultured with media specific to each strain prior to use. Cation-adjusted Mueller-Hinton Broth (BD Difco, Lot: 8096574) (CAMHB) was used for susceptibility testing of *Aeromonas*, *Citrobacter*, *Edwardsiella*, and *Lactococcus*. Cation-adjusted Mueller-Hinton Broth supplemented either with 5% (v v⁻¹) lysed horse blood (LHB) or 1.5% NaCl was used for the susceptibility testing of *Streptococcus* and *Vibrio*, respectively. Bacteria at a final concentration of 5×10^5 colony-forming units (CFU) ml^{-1} were added to each well and incubated at 28 °C for 24–28 h. Minimum inhibitory concentrations were determined in triplicate, with two wells in each MIC test plate used as a control for growth and sterility. Quality control ranges for *E. coli* ATCC 25922 and FFC were determined at 28 °C.

Endpoints were determined using the standard MIC method (visual inspection). The lowest concentration of each antimicrobial at which no bacterial growth was observed in the culture suspension was defined as the MIC. Culture suspensions without visible bacterial growth were then spread on Mueller Hinton Agar (Sigma–Aldrich, Lot: BCBS4007V) plates and incubated for 24 h in triplicate. The lowest concentration at which no growth was observed visually on the plate was regarded as the MBC.

Interpretative criteria

The antimicrobial activity categorization of HDPs was avoided, due to a lack of standardized interpretative criteria for AST of HDPs (tested in this study) against fish pathogens, and an absence of studies on their pharmacokinetics (PK) and pharmacodynamics (PD) in fish.

Following the alternative interpretative criteria suggested by CLSI (2014), breakpoints derived from microbiological, pharmacokinetic (using accepted clinical doses) and pharmacodynamic data were used to categorise bacterial isolates as susceptible to standard dose regimen, susceptible to increased exposure or resistant to FFC, OTC, and TAP. For FFC, isolates were considered susceptible to the standard dose regimen, susceptible to increased exposure or resistant if the value of MIC was ≤ 4 , 8 or $> 8 \mu\text{g ml}^{-1}$, respectively (Feng and Jia, 2009; Kosoff *et al.*, 2009; Gaunt *et al.*, 2010; Lim *et al.*, 2010; Fang *et al.*, 2013; Yang *et al.*, 2013; Assane *et al.*, 2019). For OTC, isolates were considered susceptible, standard dose regimen; susceptible, increased exposure or resistant if the value of MIC was ≤ 1 , 2 or $> 2 \mu\text{g ml}^{-1}$, respectively (Chen and Bowser, 2005; Chen, Getchell, Wooster, Craigmill, and Bowser, 2004; Rogstad, Hormazabal, Ellingsen, and Rasmussen, 1991). For TAP, isolates were considered susceptible, standard dose regimen; susceptible, increased exposure or resistant if the value of MIC was ≤ 4 , 8 or $> 8 \mu\text{g ml}^{-1}$, respectively (Castells *et al.*, 2000; Ho *et al.*, 2000; Intorre *et al.*, 2002; Malvisi *et al.*, 2002; Fang *et al.*, 2013; Assane *et al.*, 2019).

The MBC to MIC ratio was used to identify agents as either bacteriostatic or bactericidal. If this ratio was greater than 4, the agent was considered bacteriostatic; otherwise, bactericidal (Pankey and Sabath, 2004; Giguère, Prescott and Dowling, 2013).

***In vitro* synergism testing**

The checkerboard assay (Garcia and Isenberg, 2007) was used to evaluate synergy between the combination of the most active HDPs with FFC, OTC or TAP against *Citrobacter*. The range of concentration of each antimicrobial in the checkerboard assay was determined based on the results of the prior AST described in section 2.3, being: $[249.92\text{--}0.244 \mu\text{g ml}^{-1}]$ (p-BthTX-I)₂ + $[512\text{--}8 \mu\text{g ml}^{-1}]$ TAP; $[125.12\text{--}0.122 \mu\text{g ml}^{-1}]$ KR-12-a5 + $[512\text{--}8 \mu\text{g ml}^{-1}]$ TAP;

[125.04–0.122 $\mu\text{g ml}^{-1}$] LL-37 + [512–8 $\mu\text{g ml}^{-1}$] TAP; [125.04–0.122 $\mu\text{g ml}^{-1}$] LL-37 + [1,024–16 $\mu\text{g ml}^{-1}$] FFC; and [125.04–0.122 $\mu\text{g ml}^{-1}$] LL-37 + [32–0.5 $\mu\text{g ml}^{-1}$] OTC.

Endpoints were determined in duplicate for each combination using 2,3,5–triphenyl tetrazolium chloride (TTC) staining, by adding 20 μL of 5 mg ml^{-1} TTC (Sigma–Aldrich, Lot: BCBR5461, $\geq 95.0\%$ purity) solution to all test wells and incubating for 15 min after 24 h incubation at 28 $^{\circ}\text{C}$.

The fractional inhibitory concentration (FIC) of each antimicrobial was calculated for each bacteria as follows:
$$\text{FIC of antimicrobial A} = \frac{\text{MIC of antimicrobial A in combination}}{\text{MIC of antimicrobial A alone}};$$
$$\text{FIC of antimicrobial B} = \frac{\text{MIC of antimicrobial B in combination}}{\text{MIC of antimicrobial B alone}}.$$
 The summation of FIC (ΣFIC) index ($\text{FIC}_{\text{antimicrobial A}} + \text{FIC}_{\text{antimicrobial B}}$) was determined, and used to interpret the interaction following the recommendations of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Disease (ESCMID) as follows: synergy ($\Sigma\text{FIC} \leq 0.5$); additive ($> 0.5–1$); indifference (> 1 to < 2); and antagonism (≥ 2) (EUCAST, 2000; Odds, 2003).

Results

Antimicrobial peptide synthesis and purification

In the current study, several peptides were synthesized, to evaluate their antimicrobial properties against fish pathogenic bacteria and some possible synergism with conventional antimicrobials. Peptide synthesis was performed in solid-phase (SPPS), according to the method described by Merrifield (1963). After synthesis, the peptides were purified in a semi-preparative system in HPLC, using different methods (Table S1) and the degree of purity of the fractions was determined in an analytical column with a linear gradient of 5–95% solvent B in 30 min (Figure S1). The tubes containing the material with a degree of purity greater than 95% were collected and lyophilized for later use. Then, the electrospray ionization (ESI) mass

spectrometry technique was performed *via* direct injection, to confirm the molecular weight of each peptide before use.

Haemolytic effect

The positive control [1% (v v⁻¹) Triton X-100 solution] showed 100% lysis, whereas PBS showed no lysis of Nile tilapia *Oreochromis niloticus* RBCs. Haemolytic activity of MUC 7 12-mer, (p-BthTX-I)₂, LL-37, Hylin-a1, FFC, OTC, and TAP, as a function of concentration, is shown in Figure 1. All antimicrobials had low haemolytic activity compared to the positive control, in all tested concentrations. The commercial antimicrobials (FFC, OTC, and TAP) and MUC 7 12-mer were not cytotoxic to Nile tilapia RBCs in any tested concentration. Hylin-a1 was the most cytotoxic antimicrobial. LL-37, (p-BthTX-I)₂, and Hylin-a1 were not toxic to the tested cells at concentrations up to 78.13, 19.53, and 9.77 µg ml⁻¹, respectively.

Antimicrobial activity

The antimicrobial activity of HDPs, FFC, OTC, and TAP against Gram-negative (*Aeromonas*, *Citrobacter*, *Edwardsiella*, and *Vibrio*) and Gram-positive (*Lactococcus*, and *Streptococcus*) clinical isolates, and two reference strains, *Aeromonas hydrophila* ATCC[®] 7966[™], and *Escherichia coli* ATCC[®] 25922[™], is presented in Table 1 and Table 2.

The MIC and MBC of most HDPs against *Aeromonas* were above the highest concentration tested (250 µg ml⁻¹). Nevertheless, two strains of *Aeromonas jandaei* (KJ561033, and MH155229) were sensitive to KR-12-a5 (Table 1). Those strains were also sensitive to FFC, OTC and TAP. All strains of *A. jandaei* were sensitive to FFC and TAP (Table 2). *A. caviae* and *A. hydrophila* were sensitive to at least one commercial antimicrobial (Table 2).

Citrobacter and *Streptococcus* were sensitive to most of the HDPs tested, with HDPs showing antimicrobial activity considered bactericidal to most sensitive strains. In contrast,

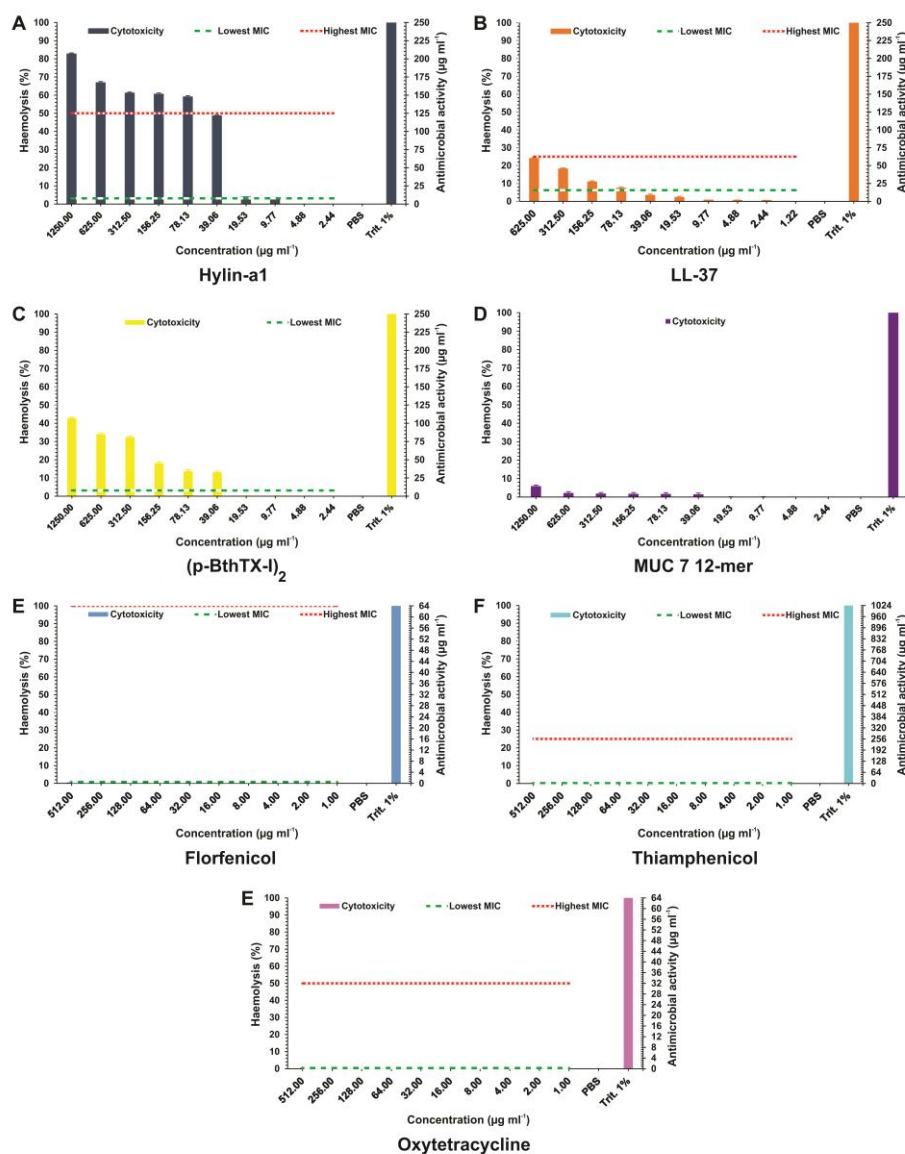


Figure 1. Haemolytic effect of Hylin-a1 (a), LL-37 (b), (p-BthTX-I)₂ (c), MUC 7 12-mer (d), florfenicol (e), thiamphenicol (f), and oxytetracycline (g) as a function of concentration. Note that the lowest minimum inhibitory concentration (MIC) of most antimicrobials against the tested bacterial strains were below the concentrations with significant haemolytic activity. MIC values greater than the highest concentration tested are not presented (MIC > 250).

Citrobacter was resistant to commercial antimicrobials (except to OTC). *Edwardsiella tarda* was resistant to all antimicrobial tested, and *Vibrio fluvialis* was only sensitive to the commercial antimicrobials and KR-12-a5.

The antimicrobial activity of des-Cys¹¹, Lys¹², Lys¹³-(pBthTX-I)₂K, and MUC 7 12-mer was above the highest concentration tested (250 µg mL⁻¹) for all strains tested (Table 1). As shown in Table 1, KR-12-a5; LL-37; and Hylin-a1 were the most active HDPs, being active against most of the strains tested (nine out of 19, seven out of 18, and seven out of 19).

Combination of HDPs with FFC, OTC or TAP

The combination of (p-BthTX-I)₂ and TAP resulted in synergism [fractional inhibitory concentration index (ΣFIC) = 0.25, and ΣFIC = 0.31] against *C. freundii* MH155228, with (p-BthTX-I)₂ at 0.98 or 1.95 µg ml⁻¹ in combination with TAP at 8 or 4 µg ml⁻¹, respectively. The combination of LL-37 and FFC also resulted in synergism (ΣFIC = 0.5) against *C. freundii* MH155228 with LL-37 at 7.82 in combination with FFC at 8 µg ml⁻¹. No synergism or antagonism was observed in other combinations tested (Table 3).

Table 1. Antimicrobial activity ($\mu\text{g mL}^{-1}$) of Hylin-a1; LL-37; des-Cys¹¹, Lys¹², Lys¹³-(pBthTX-I)₂K; (p-BthTX-I)₂; MUC 7 12-mer and KR-12-a5 against Gram-negative, Gram-positive fish pathogenic bacteria.

Strain	Hylin-a1		LL-37		des-Cys ¹¹ , Lys ¹² , Lys ¹³ -(pBthTX-I) ₂ K		(p-BthTX-I) ₂		MUC 7 12-mer		KR-12-a5	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Aeromonas caviae</i>												
KU605565	>250	-	>250	-	>250	-	>250	-	>250	-	>250	-
<i>A. hydrophila</i>												
ATCC 7966	>250	-	>125	-	>250	-	>250	-	>250	-	>250	-
MH591949	>250	-	>125	-	>250	-	>250	-	>250	-	>250	-
<i>A. jandaei</i>												
KJ561033	>250	-	>125	-	>250	-	>250	-	>250	-	15.63	62.50

MH155229	>250	-	>125	-	>250	-	>250	-	>250	-	62.50	62.50
MN133840	>250	-	>250	-	>250	-	>250	-	>250	-	>250	-
MN134409	>250	-	>250	-	>250	-	>250	-	>250	-	>250	-
<i>Citrobacter freundii</i>												
KJ561092	125	>250	15.63	15.63	>250	-	7.81	15.63	>250	-	3.91	3.91
MH155228	125	>250	15.63	15.63	>250	-	7.81	15.63	>250	-	3.91	3.91
<i>Edwardsiella tarda</i>												
I3	>250	-	>125	-	>250	-	>250	-	>250	-	>250	-
<i>Lactococcus garvieae</i>												
KJ560984	15.63	31.25	>125	-	>250	-	>250	-	>250	-	>250	-
KJ560992	15.63	31.25	>125	-	>250	-	>250	-	>250	-	>250	-
<i>Streptococcus agalactiae</i>												

KJ561054	62.50	62.5	62.5	62.5	>250	-	-	-	>250	-	62.5	62.5
KJ561057	15.63	15.63	31.25	31.25	>250	-	>250	-	>250	-	15.63	15.63
KU605552	31.25	31.25	62.5	62.5	>250	-	7.81	7.81	>250	-	7.81	7.81
KU605571	7.81	15.63	31.25	31.25	>250	-	>250	-	>250	-	>250	-
KX385295	7.81	15.63	31.25	62.50	>250	-	>250	-	>250	-	>250	-
<i>Vibrio fluvialis</i>												
MH155230	125	125	>125	-	>250	-	>250	-	>250	-	62.5	62.5

Table 2. Antimicrobial activity ($\mu\text{g ml}^{-1}$) of four commercial antimicrobials (florfenicol – FFC, oxytetracycline – OTC, and thiamphenicol - TAP) against Gram-negative, Gram-positive fish pathogenic bacteria, and two reference strains, *Aeromonas hydrophila* ATCC 7966, and *Escherichia coli* ATCC 25922.

Strain	Host	FFC		OTC		TAP	
		MIC	MBC	MIC	MBC	MIC	MBC
<i>Aeromonas caviae</i>							
KU605565	<i>O. niloticus</i>	-	-	-	-	8	8
<i>A. hydrophila</i>							
ATCC 7966	-	8	16	0.5	2	1	4
MH591949	<i>O. niloticus</i>	0.5	0.5	32	32	1	1
<i>A. jandaei</i>							
KJ561033	<i>O. niloticus</i>	2	4	≤0.125	≤0.125	1	1
MH155229		0.5	1	0.25	0.25	2	2
MN133840		0.5	2	16	32	1	4
MN134409		0.5	2	32	32	1	2
<i>Citrobacter freundii</i>							
KJ561092	<i>P. fasciatum</i>	32	64	1	>4	64	128
MH155228	<i>P. scalare</i>	32	64	1	2	64	64

<i>Edwardsiella tarda</i>							
I3	<i>O. niloticus</i>	64	>64	32	64	64-256	> 1024
<i>Lactococcus garvieae</i>							
KJ560984	<i>O. niloticus</i>	-	-	-	-	4	>16
KJ560992	<i>P. fasciatum</i>	-	-	-	-	2	>8
<i>Streptococcus agalactiae</i>							
KJ561054	<i>O. niloticus</i>	8	8	0.5	0.5	32	32
KJ561057		4	4	>64	-	16	16
KU605552		2	2	2	4	4	4
KU605571		-	-	4	16	128	512
KX385295		-	-	0.5	2	128	512
<i>Vibrio fluvialis</i>							
MH155230	<i>C. macropomum</i>	1	2	0.25	1	2	2
<i>Escherichia coli</i> (Quality control)							
ATCC 25922	-	8	32	1	>4	128	512

Table 3. *In vitro* antimicrobial activity of (p-BthTX-I)₂, KR-12-a5 and LL-37 in combination with florfenicol–FFC, oxytetracycline–OTC or thiamphenicol–TAP against *Citrobacter freundii* MH155228.

MIC alone (µg/ml)		MIC in combination (µg/ml)	Fold MIC in combination		ΣFIC	Interpretation
(p-BthTX-I) ₂	TAP	(p-BthTX-I) ₂ + TAP	(p-BthTX-I) ₂	TAP		
7.81	64	1.95 + 4	1/4	1/16	0.31	Synergism
7.81	64	0.98 + 8	1/8	1/8	0.25	Synergism
7.81	64	0.49 + 16	1/16	1/4	0.31	Synergism
7.81	64	0.24 + 32	1/32	1/2	0.53	Additivism
MIC alone (µg/ml)		MIC in combination (µg/ml)	Fold MIC in combination		ΣFIC	Interpretation
KR-12-a5	TAP	KR-12-a5 + TAP	KR-12-a5	TAP		
3.91	64	1.96 + 32	1/2	1/2	1.00	Additivism
LL-37	TAP	LL-37 + TAP	LL-37	TAP		
15.63	256	1.95 + 128	1/8	1/2	0.63	Additivism
15.63	256	3.91 + 128	1/4	1/2	0.75	Additivism
15.63	256	7.82 + 128	1/2	1/2	1.00	Additivism

LL-37	FFC	LL-37 + FFC	LL-37	FFC		
31.26	32	1.95 + 16	1/16	1/2	0.56	Additivism
31.26	32	3.91 + 16	1/8	1/2	0.63	Additivism
31.26	32	7.82 + 8	1/4	1/4	0.50	Synergism
31.26	32	15.63 + 8	1/2	1/4	0.75	Additivism
LL-37	OTC	LL-37 + OTC	LL-37	OTC		
31.26	1	1.95 + 0.5	1/16	1/2	0.56	Additivism
31.26	1	3.91 + 0.5	1/8	1/2	0.63	Additivism
31.26	1	7.82 + 0.5	1/4	1/2	0.75	Additivism
MIC alone (µg/ml)		MIC in combination (µg/ml)	Fold MIC in combination		ΣFIC	Interpretation
LL-37	OTC	LL-37 + OTC	LL-37	OTC		
31.26	1	15.63 + 0.25	1/2	1/4	0.75	Additivism

Discussion

Due to the requirement for novel antimicrobials in aquaculture, as a result of widespread resistance to many important antimicrobials, and consequent consumer health risk by bio-magnification (Gautam *et al.*, 2016), several attempts have been made to explore new, environmentally-friendly antimicrobial drugs from natural sources, including virus (Culot *et al.*, 2019), plant (Devi *et al.*, 2016; Bandeira Junior *et al.*, 2018) and animal (Chettri *et al.*, 2017; Pan *et al.*, 2017; Buonocore *et al.*, 2019) products.

In this study, the HDPs Hylin-a1 (IFGAILPLALGALKNLIK) (Castro *et al.*, 2009; Crusca *et al.*, 2011), LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRI-TES) (Ji *et al.*, 2007), KR-12-a5 (LL-37 analogue - KRIVKLILKWLR-NH₂) (Jacob *et al.*, 2013; Caiaffa *et al.*, 2017), MUC 7 12-mer (RKSYSKCLHKRCR, amino acids 40–51 of the parent human salivary mucin, MUC7) (Situ *et al.*, 2003), (p-BthTX-I)₂ (a disulfide-linked dimeric form of p-BthTX-I, KKYRYHLKPFCKK) (Santos-Filho *et al.*, 2015, 2017), and des-Cys¹¹, Lys¹², Lys¹³-(p-BthTX-I)₂K [(KKYRYHLKPF)₂K] (a (p-BthTX-I)₂ analogue, where Lysines 12 and 13 and Cysteine 11 were deleted and dimerization was done through a Lys residue at position 11) were synthesized and studied.

The haemolytic activity against Nile tilapia *Oreochromis niloticus* RBCs and *in vitro* antimicrobial activity of synthesized HDPs, alone or in combination with florfenicol (FFC), oxytetracycline (OTC) or thiamphenicol (TAP), against different pathogenic bacteria isolated from diseased fish were investigated.

Although the MIC and MBC of some HDPs against several strains tested were above the highest HDP concentration used, all HDPs were bactericidal to most of the sensitive strains, and had negligible cytotoxicity against Nile tilapia RBCs, except for LL-37 and Hylin-a1 which had considerable cytotoxicity.

Information regarding the risk of toxic effects to the host is of great importance during the characterization of HDPs toward the development of novel antimicrobials for aquaculture (Aoki and Ueda, 2013). Several HDPs have been reported to be toxic against different cell lines from animals (Aoki and Ueda, 2013), even at relatively low dosages (lower than their MIC against most pathogens). There are reports of strong cytotoxicity of LL-37 and Hylin-a1 against different cell lines from various organisms (Johansson *et al.*, 1998; Castro *et al.*, 2009; Bridle *et al.*, 2011; da Silva *et al.*, 2013). However, in this work only strong haemolytic activity of Hylin-a1 against the Nile tilapia RBCs was observed. The cytotoxicity of LL-37 was considerable, but still very low compared to that of Hylin-a1, and similar to that of TVEO (data not shown), which is known to be safe for Nile tilapia (Valladão *et al.*, 2019). Moreover, LL-37 *in vivo* toxicity appears to be attenuated by one or several factors present in the serum (Johansson *et al.*, 1998; Bridle *et al.*, 2011). Based on these aspects and the results from our previous studies (de Sousa *et al.*, 2021), LL-37 *in vivo* toxicity against the Nile tilapia can be expected to be negligible.

The number of studies assessing the antimicrobial effects of HDPs against fish pathogens has rapidly increased over the last two decades, and there is evidence of HDPs' strong activity against many Gram-positive and Gram-negative bacteria, such as *Bacillus mycoides*, *Lactococcus garviae*, *Leucothrix mucor*, *Renibacterium salmoninarum*, *Streptococcus iniae*, *A. caviae*, *Aeromonas hydrophila*, *A. salmonicida*, *A. sobria*, *Edwardsiella ictaluri*, *E. tarda*, *Flavobacterium columnare*, *F. meningosepticum*, *Morganella morganii*, *Pasteurella multocida*, *Photobacterium damsela*, *Photobacterium damsela* subsp. *piscidida*, *Plesiomonas shigelloides*, *Salmonella enterica*, *Shewanella* spp., *Vibrio alginolyticus*, *V. anguillarum*, *V. campbellii*, *V. damsela*, *V. fluvialis*, *V. harvei*, *V. ordalii*, *V. parahaemolyticus*, *V. penaeicida*, *V. vulnificus*, and *Yersinia ruckeri* (Valero *et al.*, 2020). Most studies are based on HDPs derived from aquatic organisms, mainly piscidins, hepcidins, defensins, cathelicidins

and histone-derived peptides (Valero *et al.*, 2020; Raju *et al.*, 2021). To the best of our knowledge, this is the first report of antimicrobial activity of Hylin-a1; LL-37; (p-BthTX-I)₂; des-Cys¹¹, Lys¹², Lys¹³-(pBthTX-I)₂K; MUC 7 12-mer; and KR-12-a5 against fish clinical isolates of *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Vibrio*, *Lactococcus*, and *Streptococcus*.

Our results indicated that KR-12-a5; LL-37; and Hylin-a1 were the most promising of the studied synthetic HDPs, showing strong *in vitro* antimicrobial activity against both Gram-negative (*Aeromonas jandaei*, *Citrobacter freundii*, and *Vibrio fluvialis*), and Gram-positive (*Lactococcus garvieae*, and *Streptococcus agalactiae*) bacteria pathogenic to fish. Furthermore, our results are in agreement with previous reports showing that KR-12-a5, LL-37, and Hylin-a1 have strong, broad-spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria isolated from different organisms (Johansson *et al.*, 1998; Castro *et al.*, 2009; Aka, 2015; Caiaffa *et al.*, 2017; Kim, Rajasekaran and Shin, 2017).

The clear advantages of HDPs over conventional antimicrobials, which include the slower emergence of resistance, broad-spectrum antibiofilm activity, the ability to favourably modulate the host immune response (Magana *et al.*, 2020), and the high frequency of collateral sensitivity (increased sensitivity) to HDPs in antibiotic-resistant bacteria (Lázár *et al.*, 2018) highlights their great potential to be used as a new class of antimicrobials, to either replace conventional antimicrobials or as adjuvants in combination antimicrobial therapy (CT).

Notwithstanding that CT against bacterial pathogens is frequently used in medicine to enhance the bacterial effects of both new and existing antimicrobials (Tamma, Cosgrove and Maragakis, 2012; Zumla *et al.*, 2014), its application in aquaculture is limited. Most studies evaluating the *in vitro* and *in vivo* synergism between different antimicrobials against fish pathogenic bacteria are based on CT involving EOs, their major components and conventional antimicrobials (de Souza *et al.*, 2017; Bandeira Junior *et al.*, 2018; Assane *et al.*, 2019; Hayatgheib *et al.*, 2020; Assane *et al.*, 2021).

In this study, the combination of (p-BthTX-I)₂ or LL-37 with commercial antimicrobials (TAP, and FFC, respectively) resulted in synergism against *C. freundii* MH155228 resistant to the commercial antimicrobials, suggesting the potential of using CT incorporating (p-BthTX-I)₂ or LL-37 with existing antimicrobials to reduce the use of antimicrobials in aquaculture while reducing antimicrobial resistance.

Despite the uncertain clinical relevance of *in vitro* studies (Tängdén, 2014), our findings can be explored for *in vivo* and clinical studies, and to support therapeutic decisions in situations where there are no evidence-based treatment options.

In conclusion, synthetic HDPs such as Kr-12-a5; (p-BthTX-I)₂; LL 37; and Hylin-a1 have the potential to be good treatment options for bacterial diseases in aquaculture, and HDPs can be tested alone or in combination against fish pathogenic bacteria in *in vivo* studies.

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Conflict of interest

The author declares that they have no conflict of interest.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

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Supporting information

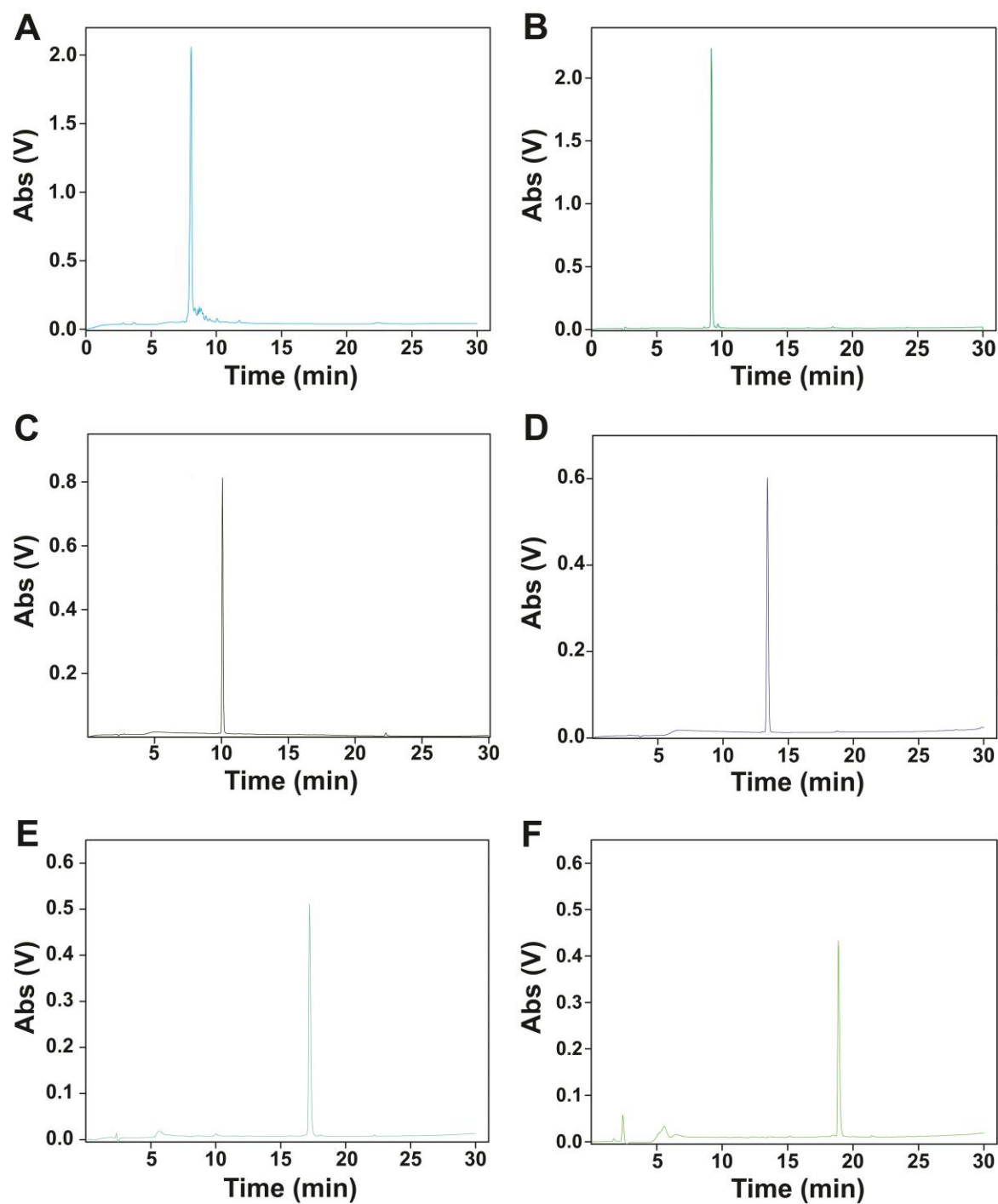


Figure S1. Chromatogram of synthesized and purified peptides determined by HPLC. a) MUC 7 12-mer; b) (p-BthTX-I)₂; c) des-Cys¹¹, Lys¹², Lys¹³-(pBthTX-I)₂K; d) KR-12-a5; e) LL-37; and f) Hylin-a1.

Table S1. The method used in peptide synthesis.

Peptide	Sequence	Resin	Cleavage	Peptide Purification method*
Hylin-a1	IFGAILPLALGALKNLIK	Rink MBHA resin	amide 95% TFA, 2.5% H ₂ O, 2.5% TIS	30-70% in 170 minutes
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	Rink MBHA resin	amide 95% TFA, 2.5% H ₂ O, 2.5% TIS	30-70% in 90 minutes
(p-BthTX-I) ₂ [†]	(KKYRYHLKPFCKK) ₂	Fmoc-Lys(boc)- wang resin	94% TFA, 2.5% H ₂ O, 2.5% EDT, 1% TIS	5-45% in 90 minutes
des-Cys ¹¹ , Lys ¹² , Lys ¹³ - (pBthTX-I) ₂ K [‡]	(KKYRYHLKPF) ₂ K	Rink MBHA resin	amide 95% TFA, 2.5% H ₂ O, 2.5% TIS	5-50% in 90 minutes

Table S1 (*continued*)

MUC 7 12-mer	RKSYKCLHKRCR	Fmoc-Arg(Pmc)- wang resin	94% TFA, 2.5% H ₂ O, 2.5% EDT, 1% TIS	5-25% in 90 minutes
KR-12-a5	KRIVKLILKWLR	Rink MBHA resin	amide 95% TFA, 2.5% H ₂ O, 2.5% TIS	18-58% in 120 minutes

*The method used for the purification of each peptide on HPLC semi-prepared. For example, a gradient of 30 to 70% (of solvent B) in 170 minutes.

†The peptide (p-BthTX-I)₂ was dimerized by disulphide bridge through the oxidation of Cys residues.

‡The des-Cys¹¹, Lys¹², Lys¹³-(pBthTX-I)₂K peptide is an analogue of the (p-BthTX-I)₂ peptide. The difference is that des-Cys¹¹, Lys¹², Lys¹³-(pBthTX-I)₂K suffered the deletion of lysines 12 and 13 and cysteine 11. Dimerization was done through a Lys residue at position 11.

Chemical composition, cytotoxicity and antimicrobial activity of selected plant-derived essential oils against fish pathogens

Abbreviated running headline: **Antibacterial and toxic effects of EOs**

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Abstract

The use of essential oils (EOs) is a promising strategy for reducing antimicrobial use in aquaculture. In this study, we investigated the chemical composition, *in vitro* cytotoxicity and antimicrobial activity of 16 commercial plant-derived EOs against *Aeromonas hydrophila*, *A. jandaei* and *Citrobacter freundii* isolated from diseased freshwater fishes (*Oreochromis niloticus* and *Pterophyllum scalare*). Efficacy of treatment with EOs and four antimicrobials used in aquaculture; enrofloxacin, florfenicol, oxytetracycline, and thiamphenicol, were compared. The chemical compositions of EOs were determined via high-resolution gas chromatography, and the *in vitro* cytotoxicity and antimicrobial activity were assessed using a hemolysis assay and the microdilution method, respectively. Ninety-four components of EOs were identified. The principal components of most EOs were oxygenated monoterpene hydrocarbons. Most EOs possessed non-cytotoxic effects against fish erythrocytes at concentrations with strong ($\text{MIC} \leq 500 \mu\text{g/mL}$) and moderate ($600 \mu\text{g/mL} \leq \text{MIC} \leq 1,500 \mu\text{g/mL}$) antimicrobial activity. *A. sativum*, *C. verum*, *O. basilicum*, *S. aromaticum*, and *T. vulgaris* EOs were the most active against microbial strains assessed. The present study highlights the potential of using EO to control bacterial disease in cultured fish.

Keywords: fish disease, antimicrobials, phytochemicals, solvents, haemotoxicity, drug synergism

Introduction

Indiscriminate use of antimicrobials in aquaculture has significantly accelerated the development of antimicrobial resistance and the accumulation of antimicrobial residues throughout the environment (Valladão, Gallani, & Pilarski, 2015). Therefore, it is necessary to develop environmentally friendly and effective treatments of bacterial disease in farmed fish. Screening non-cytotoxic compounds for antimicrobial activity against fish pathogens, and the study of different therapeutic strategies for treating fish are cost-effective and sustainable solutions that are being investigated due to their potential to address this challenge in the long term. Several researchers have previously identified new antimicrobial compounds from natural sources, including animals (Kumari, Tyor, & Bhatnagar, 2019), plants (Harikrishnan et al., 2010; H. Liu et al., 2020; Suphoronski et al., 2019), and a virus (Culot, Grosset, & Gautier, 2019).

Treatment with plant essential oils (EOs) is an environmental-friendly alternative to antimicrobial use in aquaculture (Culot et al., 2019; Romero, Gloria, & Navarrete, 2012). In addition to reducing resistance phenomenon (Dugo & Di Giacomo, 2002) and broad-spectrum antimicrobial activity against fish pathogens, they are considered safe substances with several potential benefits for fish farming, which include the improvement of fish welfare, health and zootechnical performance indices, such as growth performance, feed conversion rate, and protein efficiency rate (Acar, Kesbiç, İnanan, & Yılmaz, 2019; De Souza et al., 2019; Sutili, Gatlin, Heinzmann, & Baldisserotto, 2018). The antibacterial (Vaseeharan, Sivalingam, & Palaniappan, 2013), antiparasitic (Da Costa et al., 2017; Metin, Diler, Didinen, Terzioglu, & Gormez, 2015; Soares et al., 2016) and immunomodulatory (Acar et al., 2019; Sutili et al., 2018; Valladão et al., 2017) effects of EOs help to prevent disease outbreaks in fish farming, therefore minimizing economic loss due to disease and help combat the indiscriminate use of antimicrobials, and therefore, the emergence of antimicrobial resistance. Moreover, the

reduction of antimicrobial utilization in aquaculture has the potential to improve the quality of aquaculture products by reducing antimicrobial residue in both the environment and animals intended for human consumption (X. Liu, Steele, & Meng, 2017).

Use of varying antimicrobial susceptibility testing (AST) protocols to assess the *in vitro* antimicrobial activities of EOs and the lack of *in vivo* studies have been barriers for compiling useful information regarding fish toxicity and the chemical characterization of many EOs, and have therefore minimized EO use (Culot et al., 2019). First, a precise comparison of AST results of different studies is difficult, and fragmentary information regarding the antimicrobial activity of a given EO is common (Thielmann, Muranyi, & Kazman, 2019). Second, *in vivo* studies are indispensable for evaluating the effectiveness of EO therapy in the field and facilitate the use of EO treatment on a large scale. Third, to determine the most appropriate EO dose for a given fish species it is crucial to have information regarding EO toxicity. Some well-studied essential oils are toxic to fish at relatively low doses that are often below their minimum inhibitory concentration (MIC) against their target pathogen (Soares et al., 2016; Tavares-Dias, 2018). Finally, the antimicrobial activity of a given EO is not solely determined by its principal active components (Da Cunha, Heinzmann, & Baldisserotto, 2018; Dugo & Di Giacomo, 2002), and it is necessary to specify the chemical composition of an EO in order to better understand AST results. This is because the chemical composition of a given EO depends on features including plant harvesting and its sampling process, including edaphoclimatic conditions, origin, plant organ used, age and vegetative cycle stage at harvest (Adams, 2017; Dugo & Mondello, 2011; Romero et al., 2012; Wang, Chittiboyina, Avonto, Parcher, & Khan, 2014).

This study was performed to improve available information regarding the chemical composition, antibacterial activity, and toxicity of plant-derived EOs, and it will provide

insights regarding the potential of EOs to be used both as antimicrobial crude drugs and sources of natural active compounds to improve bacterial disease control in aquaculture.

Materials and methods

Antimicrobials

Sixteen plant-derived essential oils (EOs) and 4 antimicrobial agents approved for use in aquaculture in different countries were studied (Table 1). EOs were purchased from Phytoterapica[®], Brazil and stored in tightly closed, dark vials at 4°C for further tests and analyses. Antimicrobials analytical standards were purchased from Sigma–Aldrich[®] (Brazil) and stored according to dealer recommendations.

Identification of chemical components of EOs

The chemical compositions of EOs were determined via gas chromatography (GC) with flame ionization detector (FID) and an Agilent HP 780A system (Agilent Technologies Inc., USA). Results were confirmed via GC/mass spectrometry (MS), as previously described by Valladão et al. (2019).

Cytotoxicity studies by haemolytic activity

The *in vitro* cytotoxic activities of AVEO, BCEO, COEO, CAEO, CBEO, CCEO, CSEO, EGEO, MPEO, OBEO, PGEO, and TVEO against fish red blood cells (RBCs) were studied by examining the haemolytic activity of each EO against Nile tilapia *Oreochromis niloticus* RBCs using 1% (v/v) Triton X–100 solution as a positive control (100% lysis) and phosphate-buffered saline (PBS, pH 7.4) as a negative control (0% lysis) (Ahmad, Khan, Manzoor, & Khan, 2010). Briefly, fresh blood was collected from healthy juvenile Nile tilapia to perform the haemolysis assay. RBCs were washed three times by centrifugation (500x *g* for 5 min, 4

°C) with PBS. A suspension of 1% (v/v) erythrocyte (1% ER) was made with packed RBCs suspended in PBS. 2 mL Eppendorf tubes containing 1% ER and different concentration of each EO (4–0.01% (v/v)) or control solution (1% (v/v) Triton X-100 or PBS) were incubated for 1h at 28 °C, and then the samples centrifuged at 500x *g* for 5 min (4°C). 100 µL aliquots of the supernatant were transferred to 96-well microplates, and the absorbance was determined at 540 nm. The assay was performed in triplicates.

The percentage of lysis was determined for each concentration of EO evaluated using the following equation: % of lysis = $\frac{(A_s - A_{PBS})}{(A_{Triton} - A_{PBS})} \times 100$, where A_s , A_{PBS} , and A_{Triton} represents the absorbance of sample, PBS, and 1% (v/v) Triton X–100, respectively. The average value and standard deviation for each concentration of EO were calculated from triplicate assays. Data normality and the homogeneity of variance were evaluated, and rare non-normal data were normalized using square root transformation. Data were analysed using one–way ANOVA, followed by the Holm-Sidak multiple comparison test at a significance level of 0.05.

Antimicrobial susceptibility testing

Bacterial isolates

Six isolates of clinically relevant bacterial pathogens, *Aeromonas hydrophila* (1), *A. jandaei* (4), and *Citrobacter freundii* (1), from diseased freshwater fishes cultured in Brazil and two reference strains, *A. hydrophila* ATCC® 7966™, and *Escherichia coli* ATCC® 25922™, were used to determine *in vitro* susceptibility to sixteen plant-derived EOs (ASEO, AVEO, BCEO, COEO, CVEO, CAEO, CBEO, CCEO, CSEO, LGEO, EGEO, MPEO, OBEO, PGEO, SAEO, and TVEO) and four antimicrobial agents approved for use in aquaculture in different countries (ENRO, FFC, OTC, and TAP). Clinical isolates were previously recovered from internal organs (kidney or brain) of Nile tilapia and freshwater angelfish *Pterophyllum scalare* positively diagnosed with a bacterial infection and stored at the Strain Bank of the Laboratory

Table 1. Chemical and physical properties of commercial antimicrobials evaluated in this study.

Essential oil					
Pant botanical name	Common name-abbreviation	Extraction	Density (ρ)	Purity (%)	Origin
<i>Allium sativum</i>	Garlic-ASEO	Steam distillation (SD) of the stalk	1.083	≥ 99.9	China
<i>Artemisia vulgaris</i>	Mugwort - AVEO	SD of leaves	0.931	≥ 99.9	Morocco
<i>Boswellia carterii</i>	Olibanum-BCEO	SD of resin	0.848	≥ 99.9	India
<i>Cananga odorata</i>	Ylang-ylang-COEO	SD of flowers	0.915	≥ 99.9	Comoros
<i>Cinnamomum verum</i>	Cinnamon-CVEO	SD of shells	1.060	≥ 99.9	India
<i>Citrus aurantium</i> var. <i>amara</i>	Bitter orange tree-CAEO	SD of leaves, branches and fruits	0.889	≥ 99.9	-
<i>Citrus bergamia</i>	Bergamot-CBEO	SD of fruit shells	0.874	≥ 99.9	Brazil
<i>Corymbia citriodora</i>	Lemon-scented gum-CCEO	SD of leaves	0.863	≥ 99.9	Brazil
<i>Cupressus sempervirens</i>	Italian cypress-CSEO	SD of leaves	0.863	≥ 99.9	France
<i>Cymbopogon citratus</i>	Lemon grass-LGEO	SD of leaves	0.874	≥ 99.9	India
<i>Eucalyptus globulus</i>	Tasmanian blue gum-EGEO	SD of leaves	0.912	≥ 99.9	Brazil

<i>Mentha x piperita</i>	Peppermint-MPEO	SD of leaves	0.898	≥ 99.9	India
<i>Ocimum basilicum</i>	Basil - OBEO	SD of leaves	0.889	≥ 99.9	India
<i>Pelargonium graveolens</i>	Geranium - PGEO	SD of leaves	0.889	≥ 99.9	India
<i>Syzygium aromaticum</i>	Clove - SAE0	SD of buds	1.060	≥ 99.9	Brazil
<i>Thymus vulgaris</i>	Thyme - TVEO	SD of leaves	0.941	≥ 99.9	Spain

Antimicrobial						
CAS Number	Generic name-abbreviation	Therpeutic category	Solvent	MW (g/mol)	Purity (%)	Brand
93106-60-6	Enrofloxacin - ENRO	Fluoroquinolone	Water	359.39	≥98.0	Fluka
73231-34-2	Florfenicol - FFC	Amphenicol	Ethanol	358.21	≥99.0	Sigma
6153-64-6	Oxytetracycline - OTC	Tetracycline	Methanol	496.46	≥95.0	Supelco
15318-45-3	Thiamphenicol - TAP	Amphenicol	Ethanol	356.22	≥99.0	Sigma

of Microbiology and Parasitology of Aquatic Organisms at the Aquaculture Centre of São Paulo State University. Fish were sampled during disease outbreaks at different fish farms or facilities throughout Brazil. FFC and *E. coli* (ATCC® 25922™) were used to monitor the performance (quality control) of antimicrobial susceptibility testing (AST).

***In vitro* antimicrobial activity**

Minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively) were determined using the microdilution broth method inspired by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2014) if possible. Briefly, ENRO, FFC, OTC, and TAP, were dissolved in sterile distilled water (ENRO), 95% ethanol (FFC, and TAP) or 100% methanol (OTC), and diluted in water to prepare 38.4 µg/mL ENRO, 1,280 µg/mL FFC, 1,280 µg/mL OTC, and 20,480 µg/mL TAP antimicrobial stock solutions. EOs were dissolved in either polyethylene glycol (PEG-400) or dimethyl sulfoxide (DMSO) and diluted using sterile distilled water to prepare 16% (v/v) EO stock solutions. The greatest final concentration of solvent used in tests was 2% (v/v) for PEG-400 and DMSO solutions. The antimicrobial activity of the solvents (range of concentrations: 25–0.05% (v/v)) against *A. hydrophila*, *A. jandaei*, and *E. coli* was determined prior to AST. The effects of PEG-400 and DMSO on EO antimicrobial activity were evaluated. The ranges of concentrations of antimicrobials used in tests were 1.92–0.00375 µg/mL ENRO, 128–0.25 µg/mL FFC, 128–0.25 µg/mL OTC, 1,024–0.25 µg/mL TAP, and 4–0.0078% (v/v) EO.

Bacteria at a final concentration of 5×10^5 colony-forming units (CFU) per mL were plated within individual wells and incubated at 28°C for 24 h. MICs were determined in triplicate, with two wells of each MIC test plate used as growth and sterility controls, respectively. All isolates were recovered and cultured with media appropriate for the strain

assessed. Cation-adjusted Mueller-Hinton Broth, CAMHB (BD™ Difco™, Lot: 8096574), was used to test the susceptibility testing of all strains.

Endpoints were determined using the standard MIC method (visual inspection). The lowest concentration of each antimicrobial at which no bacterial growth was observed in the culture suspension was defined as the MIC. Suspensions without visible bacterial growth were then spread on Mueller Hinton Agar (Sigma-Aldrich®, Lot: BCBS4007V) plates and incubated for 24 h in triplicate. The lowest concentration at which no growth was observed visually on the plate was regarded as the MBC.

Interpretative criteria

To facilitate the interpretation of results, EO concentrations expressed % (v/v) were converted to µg/mL using the EO density (ρ) of each EO. Due to the lack of standardized criteria for interpreting the AST of EOs against fish pathogens, breakpoint data available in the literature to classify the antimicrobial activity of EOs depending on MIC values (Da Cunha et al., 2018) were used to categorize bacterial isolates as susceptible ($\text{MIC} \leq 500 \text{ } \mu\text{g/mL}$), intermediately susceptible ($600 \text{ } \mu\text{g/mL} \leq \text{MIC} \leq 1,500 \text{ } \mu\text{g/mL}$) or resistant ($\text{MIC} > 1,600 \text{ } \mu\text{g/mL}$) to EOs assessed.

For antimicrobials commonly used in aquaculture, ENRO, FFC, OTC, and TAP, the isolates were classified as susceptible ($\text{MIC} \leq 0.096 \text{ } \mu\text{g/mL}$, $\leq 4 \text{ } \mu\text{g/mL}$, $\leq 1 \text{ } \mu\text{g/mL}$, and $\leq 8 \text{ } \mu\text{g/mL}$ respectively), intermediately susceptible ($0.096 < \text{MIC} \leq 1.92$, $\leq 4 \text{ } \mu\text{g/mL}$, $8 \text{ } \mu\text{g/mL}$, and $2 \text{ } \mu\text{g/mL}$, respectively) or resistant ($\text{MIC} > 1.92 \text{ } \mu\text{g/mL}$, $> 8 \text{ } \mu\text{g/mL}$, $> 2 \text{ } \mu\text{g/mL}$, and $> 8 \text{ } \mu\text{g/mL}$, respectively) following the alternative interpretative criteria suggested by CLSI (2014) (Agustín et al., 2019; Assane, Gozi, Valladão, & Pilarski, 2019; Teles, Castello Branco, Del Bianchi, Pilarski, & Reyes, 2015). MBC to MIC ratio was used to identify antimicrobial agents as either bactericidal or bacteriostatic. Agents were considered bactericidal if the MBC was no

more than 4 times greater than MIC; otherwise, agents were classified as bacteriostatic (Giguère, Prescott, & Dowling, 2013; Pankey & Sabath, 2004).

***In vitro* synergism testing**

The *in vitro* effects of EOs with relatively high haemolytic activity ($P < 0.05$) at 0.0625% (v/v) (588.1 µg/mL) against *O. niloticus* RBCs (TVEO) in combination with antimicrobial agents approved for use in aquaculture in different countries (FFC, OTC, and TAP) was assessed *via* the broth microdilution checkerboard method described by Leber (2016). Previously determined MIC values were used to select the concentration range of each antimicrobial for treating each strain of bacteria tested. The ranges of concentrations of antimicrobials used were: *A. hydrophila* ATCC®7966™: [64–1 µg/mL] FFC + [37,640–36.80 µg/mL] TVEO, [4–0.0625 µg/mL] OTC + [37,640–36.80 µg/mL] TVEO; *A. hydrophila* MH591949.1: [4–0.0625 µg/mL] FFC + [37,640–36.80 µg/mL] TVEO, [256–4 µg/mL] OTC + [37,640–36.80 µg/mL] TVEO, [4–0.0625 µg/mL] TAP + [37,640–36.80 µg/mL] TVEO; *C. freundii* MH155228.1: [256–4 µg/mL] FFC + [37,640–36.80 µg/mL] TVEO; *E. coli* ATCC®25922™: [64–1 µg/mL] FFC + [37,640–36.80 µg/mL] TVEO, [1,024–16 µg/mL] TAP + [37,640–36.80 µg/mL] TVEO.

Endpoints were determined using 2,3,5–triphenyl tetrazolium chloride (TTC) staining. Briefly, 20 µL 5 mg/mL TTC (Sigma-Aldrich®, Lot: BCBR5461, ≥95.0% purity) solution were added to all test wells and plates were incubated 15 min after 24 h incubations at 28°C. Endpoints were determined in duplicate.

The fractional inhibitory concentration (FIC) of each antimicrobial was calculated for each bacteria, as follows:
$$\text{FIC of antimicrobial A} = \frac{\text{MIC of antimicrobial A in combination}}{\text{MIC of antimicrobial A alone}};$$

$$\text{FIC of antimicrobial B} = \frac{\text{MIC of antimicrobial B in combination}}{\text{MIC of antimicrobial B alone}}.$$
 The summation of FIC (ΣFIC) index ($\text{FIC}_{\text{antimicrobial A}} + \text{FIC}_{\text{antimicrobial B}}$) was determined and the value was used to interpret the

interaction in accordance with recommendations of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Disease (ESCMID), as follows: synergy ($\Sigma\text{FIC} \leq 0.5$); additive ($> 0.5-1$); indifference (> 1 to <2); and antagonism (≥ 2).

Results

Chemical composition of essential oils (EOs)

Ninety-four chemical compounds were identified from EOs analysed. Sixteen were monoterpene hydrocarbons, forty-eight were oxygenated monoterpenes, two were oxygenated diterpenes, fifteen were sesquiterpene hydrocarbons, eight were oxygenated sesquiterpene, three were esters, and two were ketones. The percentage of the total identified components found in each EO ranged from 89.3% to 99.9% (Supplementary Table 1). Most EOs contained mostly oxygenated monoterpene hydrocarbons (Supplementary Table 1). The lowest (2%) and highest (96.2%) percentages of oxygenated monoterpene hydrocarbons in a single EO were observed in COEO and OBEO, respectively (Supplementary Table 1). High percentages of sesquiterpene hydrocarbons were observed in AVEO (20.5%) and COEO (75.1%). Esters were only observed in COEO (benzyl benzoate: 7.3%, benzyl salicylate: 3.9%), and PGEO (phenethyl butyrate: 1.5%). Ketones, 6-methyl-5-hepten-2-one, and methyl nonyl ketone were observed only in LGEO at low concentrations ($\leq 1.1\%$).

The main components of EOs identified and their highest percentage observed were 1,8-cineole (73.0% within EGEO), camphor (17.3% within AVEO), citronellal (77.2% within CCEO), citronellol (47.0% within PGEO), estragole (77.4% within OBEO), geranial (40.0% within LGEO), germacrene D (24.9% within COEO), linalyl acetate (35.3% within CAEO), menthol (70.0% within MPEO), thymol (40.4% within TVEO), and α -pinen (65.1% within BCEO). The most predominantly identified components were limonene, linalool (both

observed in 10 out of 13 EOs), myrcene and β -caryophyllene (observed in eight out of 13 EOs), and α -pinen and α -terpineol (observed in seven out of 13 EOs).

Haemolytic activity

Treatment with the positive control (1% Triton X-100 solution) resulted in 100% lysis, whereas treatment with phosphate buffer saline (PBS) did not lyse Nile tilapia *O. niloticus* red blood cells (RBCs). The haemolytic activity of each EO, which depended on its concentration (4–0.0313% (v/v)), is shown in Figure 1. Compared with the positive control, all EOs had decreased levels of haemolytic activity at all tested concentrations. Further, $\leq 1\%$ lysis occurred at 0.0156% (v/v) (169.22–132.44 $\mu\text{g/mL}$), and 0.0078% (v/v) (84.61–66.22 $\mu\text{g/mL}$) concentrations. However, compared with the negative control, all EOs produced a large % lysis at 4% (v/v) (43,320–33,904 $\mu\text{g/mL}$). COEO and TVEO had the lowest (11.98%) and highest (90.51%) % lysis values at 4% (v/v) (36,600 $\mu\text{g/mL}$ and 37,640 $\mu\text{g/mL}$, respectively). Haemolysis of RBCs induced by EOs was concentration-dependent.

AVEO, COEO, and CAEO were not toxic to erythrocytes at concentrations ranging from 2% (v/v) (18,610 $\mu\text{g/mL}$, 18,300 $\mu\text{g/mL}$, and 17,780 $\mu\text{g/mL}$, respectively) to 0.0078% (v/v) (72.70 $\mu\text{g/mL}$, 71.48 $\mu\text{g/mL}$, and 69.45 $\mu\text{g/mL}$, respectively). CCEO, CSEO, and OBEO were toxic from 1% (v/v) (8,630 $\mu\text{g/mL}$, 8,630 $\mu\text{g/mL}$, and 8,890 $\mu\text{g/mL}$, respectively) to 0.0078% (v/v) (67.42 $\mu\text{g/mL}$, 67.42 $\mu\text{g/mL}$, and 69.45 $\mu\text{g/mL}$, respectively).

Antimicrobial activity

The minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of polyethylene glycol (PEG-400) and dimethyl sulfoxide (DMSO) solvents for *Aeromonas hydrophila*, *A. jandaei*, and *E. coli* were above solvent concentrations used for AST of EOs

(Table 2). The MIC of florfenicol (FFC), against a quality control strain, *E. coli* (ATCC® 25922™), was within the concentration range established in CLSI guidelines (Table 3).

The MIC and MBC of the four antimicrobial agents approved for use in aquaculture (enrofloxacin–ENRO, florfenicol–FFC, oxytetracycline–OTC, and thiamphenicol–TAP) and sixteen selected plant-derived EOs, dissolved in PEG-400 or DMSO, against six clinical isolates of fish pathogenic bacteria and two reference strains are presented in Table 3. Most EOs had weak (MIC > 1,600 µg/mL) antimicrobial activity against clinical isolates, and bactericidal activity within the range of concentrations tested was sometimes not observed (Table 4). The antimicrobial activity was not solvent-dependent.

On the other hand, ASEO, SAEO, and TVEO were highly active against the *A. hydrophila* ATCC® 7966™ reference strain (MIC = 338.44 µg/mL, 331.25 µg/mL, and 294.06 µg/mL, respectively). TVEO was also highly active against *A. hydrophila* MH591949 (MIC = 294.06 µg/mL), and moderately active against *E. coli* ATCC® 25922™ (MIC = 1,176.25 µg/mL). SAEO was moderately active against *A. hydrophila* MH591949 (MIC = 662.5 µg/mL) and *E. coli* ATCC® 25922™ (MIC = 1,325 µg/mL). CVEO and OBEO were moderately active against the *A. hydrophila* clinical isolate and reference strain (MIC = 662.5 µg/mL, and 1,111.3 µg/mL, respectively). Among the sixteen selected plant-derived EOs tested, ASEO, CVEO, OBEO, SAEO, and TVEO were the most active and presented bactericidal activity against all tested strains (Table 4).

All strains tested were sensitive to ENRO, except to *A. jandaei* MN134409 and *C. freundii*, which were resistant (Table 3). *C. freundii* was resistant to all tested antimicrobials, except OTC. OTC also had good antimicrobial activity against *A. hydrophila* ATCC® 7966™, *A. jandaei* MH155229 and MT735587, and *E. coli* ATCC® 25922™. FFC was highly active against all tested *Aeromonas*, except the *A. hydrophila* ATCC® 7966™ reference strain. *C. freundii* and *E. coli* ATCC® 25922™ were also resistant to FFC and TAP. TAP was highly

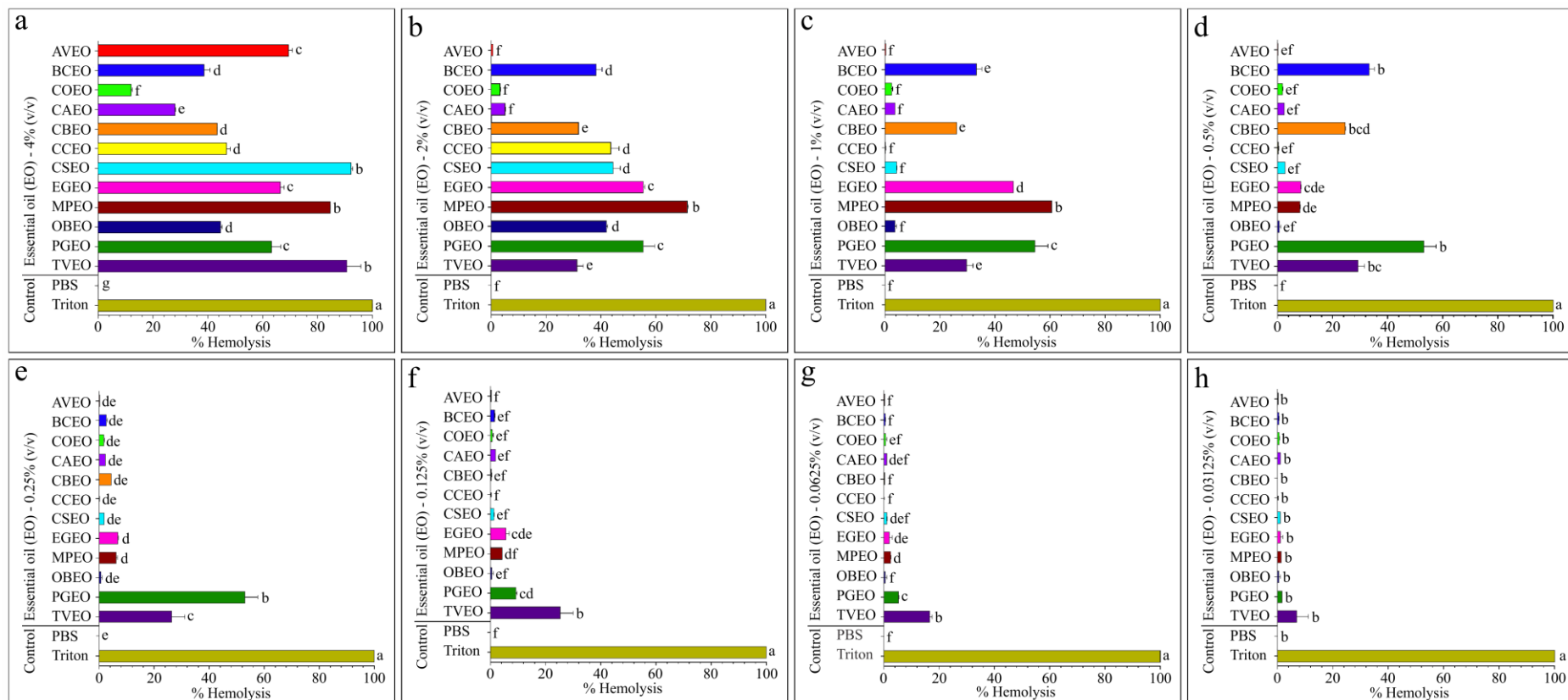


Figure 1. Haemolytic effect (%) of 16 selected plant-derived essential oils (mugwort *Artemisia vulgaris*–AVEO, olibanum *Boswellia carterii*–BCEO, ylang-ylang *Cananga odorata*–COEO, bitter orange tree *Citrus aurantium* var. *amara*–CAEO, bergamot *Citrus bergamia*–CBEO, lemon-scented gum *Corymbia citriodora*–CCEO, Italian cypress *Cupressus sempervirens*–CSEO, Tasmanian blue gum *Eucalyptus globulus*–EGEO, peppermint *Mentha x piperita*–MPEO, basil *Ocimum basilicum*–OBEO, geranium *Pelargonium graveolens*–PGEO, and thyme *Thymus vulgaris*–TVEO) at different concentrations (% (v/v)) (a: 4, b: 2, c: 1, d: 0.5, e: 0.25, f: 0.125, g: 0.0625, and h: 0.03125) against Nile tilapia *Oreochromis niloticus* red blood cells. Different letters indicate differences between % lysis ($P < 0.05$). Values are expressed as the mean $\pm$ SEM.

Table 2. Antimicrobial activity of polyethylene glycol (PEG-400), dimethyl sulfoxide (DMSO), and methanol against fish pathogenic bacteria and one reference strain *Escherichia coli* ATCC® 25922™.

Strain	Host	Isolation	Antimicrobial activity (% (v/v))					
			PEG400		DMSO		Methanol	
			MIC	MBC	MIC	MBC	MIC	MBC
<i>Aeromonas hydrophila</i>								
MH591949	<i>Oreochromis niloticus</i>	2016	3.13	3.13	12.5	12.5	12.5	12.5
<i>Aeromonas jandaei</i>								
MH155229	<i>O. niloticus</i>	2017	3.13	3.13	12.5	12.5	12.5	12.5
MT735587			3.13	3.13	12.5	12.5	12.5	12.5
MN133840		2019	3.13	3.13	12.5	12.5	12.5	12.5
MN134409			3.13	3.13	12.5	12.5	12.5	12.5
<i>Escherichia coli</i>								
ATCC® 25922™	-	-	3.13	3.13	12.5	12.5	12.5	12.5

Table 3. Antimicrobial activity (µg/mL) of enrofloxacin (ENR), florfenicol (FFC), oxytetracycline (OTC), thiamphenicol (TAP), and sixteen selected essential oils (EOs), dissolved in polyethylene glycol (PEG-400) and dimethyl sulfoxide (DMSO), against six clinical isolates of fish pathogenic bacteria and two reference strains, *Aeromonas hydrophila* ATCC® 7966™ and *Escherichia coli* ATCC® 25922™.

Strain	Host	Garlic <i>Allium sativum</i> EO				Mugwort <i>Artemisia vulgaris</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Aeromonas hydrophila</i>									
ATCC®7966™	-	338.44	1,353.8	338.44	1,353.8	9,305	9,305	18,610	18,610
MH591949	<i>O. niloticus</i>	5,415	10,830	5,415	10,830	18,610	18,610	37,220	37,220
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	10,830	21,660	10,830	21,660	18,610	18,610	37,220	37,220
MT735587		10,830	21,660	10,830	21,660	18,610	37,220	4,652.5	4,652.5
MN133840		10,830	21,660	10,830	21,660	18,610	18,610	18,610	18,610

Strain	Host	Garlic <i>Allium sativum</i> EO				Mugwort <i>Artemisia vulgaris</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MN134409		10,830	21,660	10,830	21,660	18,610	18,610	9,305	9,305
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	21,660	43,320	21,660	>43,320	37,220	>37,220	>37,220	-
<i>Escherichia coli</i>									
ATCC® 25922™		10,830	21,660	10,830	21,660	18,610	18,610	37,220	37,220
		Olibanum <i>Boswellia carterii</i> EO				Ylang-ylang <i>Cananga odorata</i> EO			
<i>Aeromonas hydrophila</i>									
ATCC®7966™	-	8,476	8,476	16,952	16,952	36,600	36,600	>36,600	-
MH591949	<i>O. niloticus</i>	16,952	16,952	33,904	33,904	36,600	>36,600	>36,600	-
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	16,952	16,952	8,476	16,952	18,300	18,300	>36,600	-

Strain	Host	Olibanum <i>Boswellia carterii</i> EO				Ylang-ylang <i>Cananga odorata</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MT735587		16,952	16,952	16,952	16,952	18,300	18,300	>36,600	-
MN133840		8,476	8,476	4,238	4,238	36,600	36,600	>36,600	-
MN134409		8,476	8,476	8,476	8,476	36,600	36,600	>36,600	-
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	33,904	>33,904	33,904	>33,904	36,600	>36,600	>36,600	-
<i>Escherichia coli</i>									
ATCC® 25922™		33,904	33,904	33,904	33,904	>36,600	-	>36,600	-
Cinnamon <i>Cinnamomum verum</i> EO					Bitter orange <i>Citrus aurantium</i> var. <i>amara</i> EO				
<i>Aeromonas hydrophila</i>									
ATCC® 7966™	-	662.5	1,325	0.0625	0.125	35,560	35,560	>35,560	-

Strain	Host	Cinnamon <i>Cinnamomum verum</i> EO				Bitter orange <i>Citrus aurantium</i> var. <i>amara</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MH591949	<i>O. niloticus</i>	662.5	1,325	1,325	2,650	35,560	35,560	>35,560	-
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	2,650	2,650	2,650	2,650	35,560	35,560	>35,560	-
MT735587		2,650	2,650	2,650	2,650	35,560	35,560	>35,560	-
MN133840		2,650	2,650	2,650	2,650	17,780	17,780	35,560	35,560
MN134409		2,650	2,650	2,650	2,650	17,780	35,560	>35,560	-
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	5,300	10,600	5,300	10,600	35,560	>35,560	>35,560	-
<i>Escherichia coli</i>									

Strain	Host	Bergamot <i>Citrus bergamia</i> EO				Lemon-scented gum <i>Corymbia citriodora</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ATCC® 25922™		2,650	5,300	2,650	5,300	35,560	35,560	>35,560	-
<i>Aeromonas hydrophila</i>									
ATCC®7966™	-	8,740	8,740	17,480	17,480	4,315	4,315	17,260	34,520
MH591949	<i>O. niloticus</i>	8,740	8,740	17,480	17,480	4,315	4,315	17,260	17,260
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	17,480	17,480	8,740	8,740	17,260	17,260	34,520	34,520
MT735587		17,480	17,480	17,480	17,480	8,630	8,630	8,630	8,630
MN133840		4,370	4,370	2,185	2,185	17,260	17,260	4,315	17,260
MN134409		8,740	8,740	4,370	4,370	8,630	8,630	2,157.5	2,157.5
<i>Citrobacter freundii</i>									

Strain	Host	Bergamot <i>Citrus bergamia</i> EO				Lemon-scented gum <i>Corymbia citriodora</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MH155228	<i>P. scalare</i>	34,960	34,960	34,960	34,960	34,520	>34,520	>34,520	-
<i>Escherichia coli</i>									
ATCC® 25922™		34,960	34,960	34,960	>34,960	34,520	34,520	17,260	34,520
Italian cypress <i>Cupressus sempervirens</i> EO					Lemongrass <i>Cymbopogon citratus</i> EO				
<i>Aeromonas hydrophila</i>									
ATCC®7966™	-	17,260	17,260	34,520	34,520	2,185	4,370	2,185	4,370
MH591949	<i>O. niloticus</i>	17,260	17,260	34,520	34,520	2,185	4,370	2,185	4,370
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	17,260	17,260	4,315	4,315	4,370	4,370	4,370	4,370
MT735587		8,630	8,630	8,630	17,260	4,370	4,370	4,370	4,370

Strain	Host	Italian cypress <i>Cupressus sempervirens</i> EO				Lemongrass <i>Cymbopogon citratus</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MN133840		8,630	8,630	4,315	4,315	4,370	4,370	4,370	4,370
MN134409		8,630	8,630	4,315	4,315	4,370	4,370	4,370	4,370
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	34,520	>34,520	>34,520	-	17,480	17,480	17,480	17,480
<i>Escherichia coli</i>									
ATCC® 25922™		34,520	34,520	34,520	34,520	17,480	17,480	17,480	17,480
		Tasmanian blue gum <i>Eucalyptus globulus</i> EO				Peppermint <i>Mentha x piperita</i> EO			
<i>Aeromonas hydrophila</i>									
ATCC® 7966™	-	18,240	18,240	18,240	18,240	4,490	8,980	8,980	17,960
MH591949	<i>O. niloticus</i>	18,240	18,240	18,240	36,480	2,245	4,490	8,980	17,960

Strain	Host	Tasmanian blue gum <i>Eucalyptus globulus</i> EO				Peppermint <i>Mentha x piperita</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	18,240	18,240	18,240	18,240	17,960	17,960	17,960	17,960
MT735587		18,240	18,240	18,240	18,240	8,980	17,960	8,980	17,960
MN133840		18,240	18,240	18,240	18,240	8,980	8,980	8,980	8,980
MN134409		18,240	18,240	9,120	9,120	8,980	17,960	17,960	17,960
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	36,480	36,480	36,480	36,480	17,960	17,960	17,960	17,960
<i>Escherichia coli</i>									
ATCC® 25922™		36,480	36,480	36,480	>36,480	4,490	8,980	8,980	17,960

Strain	Host	Basil <i>Ocimum basilicum</i> EO				Geranium <i>Pelargonium graveolens</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Aeromonas hydrophila</i>									
ATCC®7966™	-	1,111.3	2,222.5	1,111.3	2,222.5	4,445	4,445	8,890	8,890
MH591949	<i>O. niloticus</i>	1,111.3	2,222.5	555.63	1,111.3	2,222.5	2,222.5	4,445	4,445
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	8,890	8,890	8,890	8,890	4,445	4,445	4,445	4,445
MT735587		8,890	8,890	8,890	8,890	8,890	17,780	2,222.5	2,222.5
MN133840		8,890	8,890	8,890	8,890	4,445	4,445	2,222.5	4,445
MN134409		8,890	8,890	8,890	8,890	4,445	4,445	2,222.5	2,222.5
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	17,780	17,780	17,780	17,780	35,560	>35,560	>35,560	-

Strain	Host	Basil <i>Ocimum basilicum</i> EO				Geranium <i>Pelargonium graveolens</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Escherichia coli</i>									
ATCC® 25922™		8,890	8,890	8,890	8,890	8,890	35,560	17,780	35,560
Clove <i>Syzygium aromaticum</i> EO					Thyme <i>Thymus vulgaris</i> EO				
<i>Aeromonas hydrophila</i>									
ATCC®7966™	-	331.25	662.5	331.25	662.5	588.13	1176.25	294.06	588.13
MH591949	<i>O. niloticus</i>	662.5	1,325	662.5	1,325	294.06	588.13	294.06	588.13
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	2,650	2,650	2,650	2,650	2,352.5	2,352.5	2,352.5	2,352.5
MT735587		2,650	2,650	2,650	2,650	2,352.5	2,352.5	2,352.5	2,352.5
MN133840		2,650	2,650	2,650	2,650	2,352.5	2,352.5	2,352.5	2,352.5
MN134409		2,650	2,650	2,650	2,650	2,352.5	2,352.5	2,352.5	2,352.5

Strain	Host	Clove <i>Syzygium aromaticum</i> EO				Thyme <i>Thymus vulgaris</i> EO			
		PEG400		DMSO		PEG400		DMSO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	10,600	10,600	10,600	10,600	2,352.5	2,352.5	2,352.5	2,352.5
<i>Escherichia coli</i>									
ATCC® 25922™		1,325	2,650	662.5	1,325	1176.25	2,352.5	588.13	1176.25
		ENRO		FFC		OTC		TAP	
<i>Aeromonas hydrophila</i>									
ATCC® 7966™	-	<0.004	-	8	16	0.5	2	1	4
MH591949	<i>O. niloticus</i>	-	-	0.5	0.5	32	32	1	1
<i>Aeromonas jandaei</i>									
MH155229	<i>O. niloticus</i>	0.0075	0.015	0.5	>2	<0.125	-	2	8
MT735587		0.0075	0.015	0.5	1	0.25	>1	2	2

Strain	Host	ENRO		FFC		OTC		TAP	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MN133840		<0.004	-	0.5	2	32	32	1	2
MN134409		0.48	0.48	0.5	2	16	32	1	4
<i>Citrobacter freundii</i>									
MH155228	<i>P. scalare</i>	0.48	0.48	32	64	1	2	64	64
<i>Escherichia coli</i>									
ATCC® 25922™		0.015	-	8	>64	1	>4	128	512

active against all other strains assessed. All strains tested were sensitive to at least one antimicrobial. *C. freundii* was more resistant to EOs than other strains tested. Most of the antimicrobial strains tested did not inhibit bacterial growth in the range of concentrations tested or inhibited growth only when very high concentrations of antimicrobial were provided (Table 3).

***In vitro* synergism**

The *in vitro* antimicrobial activity of TVEO in combination with FFC, OTC, or TAP against *A. hydrophila*, *C. freundii*, and *E. coli* is presented in Table 5. The MIC of TVEO against *C. freundii* was fourfold lower than that of FFC at 1/2 MIC and TAP at 1/8 MIC, resulting in additive ($\Sigma\text{FIC} = 0.75$) and synergic effects ($\Sigma\text{FIC} = 0.375$), respectively (Table 5). In contrast, a combination of TVEO with FFC was ineffective against *A. hydrophila* ATCC®7966TM ($\Sigma\text{FIC} = 2$).

Discussion

The increasing emergence of antimicrobial resistance (Di Cesare et al., 2013; Watts, Schreier, Lanska, & Hale, 2017) in aquaculture is accompanied by reports of bad clinical outcomes or therapy failures using approved antimicrobials (Chideroli et al., 2017). Therefore, it is becoming clearer that new therapies, including new antimicrobials and administration strategies, are urgently needed to maintain, or increase, our current supply of aquaculture products. This work revealed that plant-derived EOs are great sources of crude antimicrobial drugs, alone or in combination with other antimicrobials, as well as sources of natural active compounds, which have the potential to enhance bacterial disease control in aquaculture.

In this study, the chemical compositions of EOs derived from different species varied greatly. Most EO components identified (55 of 94) were detected within a single EO.

Table 4. MBC/MIC ratio of sixteen selected plant-derived essential oils (EOs) against six clinical isolates of fish pathogenic bacteria and two reference strains, *Aeromonas hydrophila* ATCC® 7966™ and *Escherichia coli* ATCC® 25922™.

Strain	Essential oil ^a MBC/MIC															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>Aeromonas hydrophila</i>																
ATCC7966	4	1	1	-	2	-	1	2	1	2	1	2	2	1	2	2
MH591949	2	1	1	-	2	-	1	1	1	2	2	2	2	1	2	2
<i>Aeromonas jandaei</i>																
MH155229	2	1	2	-	1	-	1	1	1	1	1	1	1	1	1	1
MT735587	2	2	1	-	1	-	1	1	2	1	1	2	1	2	1	1
MN133840	2	1	1	-	1	1	1	4	1	1	1	1	1	2	1	1
MN134409	2	1	1	-	1	-	1	1	1	1	1	2	1	1	1	1
<i>Citrobacter freundii</i>																
MH155228	2	-	-	-	2	-	1	-	-	1	1	1	1	-	1	1
<i>Escherichia coli</i>																
ATCC 25922	2	1	1	-	2	-	-	2	1	1	-	2	1	2	2	2

^aEssential oils: 1- mugwort *Artemisia vulgaris* (AVEO), 2- olibanum *Boswellia carterii* (BCEO), 3- ylang-ylang *Cananga odorata* (COEO), 4- bitter orange tree *Citrus aurantium* var. *amara* (CAEO), 5-bergamot *Citrus bergamia* (CBE0), 6-lemon-scented gum *Corymbia citriodora* (CCEO), 7- Italian cypress *Cupressus sempervirens* (CSEO), 8- lemongrass *Cymbopogon citratus* (LGEO), 9- Tasmanian blue gum *Eucalyptus globulus* (EGEO), 10- peppermint *Mentha x piperita* (MPEO), 11- basil *Ocimum basilicum* (OBEO), 12- geranium *Pelargonium graveolens* (PGEO), and 13- thyme *Thymus vulgaris* (TVEO).

The chemical compositions of EOs vary according to several plant characteristics (location, age, climate, cultivars, temperature, and growth regulators) and sampling methods (plant part, drying, comminution, distillation, and storage) (Wang et al., 2014). However, EOs analyzed in this study (BCEO, COEO, CBEO, CCEO, CSEO, LGEO, EGEO, OBEO, and TVEO) had a similar chemical profile to those described in the literature from plants of the same species, even when plants were collected from different locations, extracted from a different plant organ, and analyzed using the same methods (Aguilar et al., 2014; Anil, Dosler, & Mericli, 2016; Bouzouita, Abdelfatteh, Kachouri, Hervé, & Moncef, 2010; Harkat-Madouri et al., 2015; Jin et al., 2015; Jordán, Martínez, Goodner, Baldwin, & Sotomayor, 2006; Khadidja, Nassima, Chahrazed, & Xavier, 2010; Kiran, Kujur, Patel, Ramalakshmi, & Prakash, 2017; Kirbaslar, Kirbaslar, & Dramur, 2001; Maher, Ghayth, Thabet, Sami, & Mohamed, 2012; Mohamed Hanaa, Sallam, El-Leithy, & Aly, 2012; Padalia, Verma, Upadhyay, Chauhan, & Singh, 2017; Phan Minh & Phan Tong, 2016; Reyes-Jurado, Cervantes-Rincón, Bach, López-Malo, & Palou, 2019; Selim, Adam, Hassan, & Albalawi, 2014; Soliman, Salaheldin, & Amer, 2017). For most of the EOs considered, differences were observed regarding percentages of components. On the other hand, the chemical compositions of some EOs (AVEO, MPEO) differed than those previously described in the literature, even those collected from the same country, extracted from the same plant organ, and analysed using the same methods (Bassolé et al., 2010; Schmidta et al., 2009; Sun, Wang, Wang, Zhou, & Yang, 2014).

When searching for antimicrobials of natural origin, one cannot ignore the possibility that they may cause toxic effects to the host. This information is needed for the EO to be used in the aquaculture of species intended for human consumption. Although most EOs and their major components are either considered safe or have low toxicity compared with chemotherapeutic products, there have been reports that some EOs and their major components are toxic against

Table 5. *In vitro* antimicrobial activity of thyme *Thymus vulgaris* essential oil (TVEO) in combination with florfenicol (FFC), oxytetracycline (OTC), or thiamphenicol (TAP) against *Aeromonas hydrophila*, *Citrobacter freundii*, and *Escherichia coli*.

Strain	MIC alone (µg/mL)		MIC in combination (µg/mL)	Fold MIC in combination		ΣFIC
	TVEO	FFC	TVEO + FFC	TVEO	FFC	
<i>Aeromonas hydrophila</i>						
ATCC 7966	588.13	8	588.13 + 8	1	1	2
MH591949	294.06	0.5	147.03 + 0.25	1/2	1/2	1
<i>Citrobacter freundii</i>						
MH155228	2,352.5	32	588.13 + 16	1/4	1/2	0.75
<i>Escherichia coli</i>						
ATCC 25922	1,176.25	8	588.13 + 1	1/2	1/8	0.625
	TVEO	OTC	TVEO + OTC	TVEO	OTC	
<i>Aeromonas hydrophila</i>						
ATCC 7966	588.13	0.5	36.76 + 0.5	1/16	1	1.06
MH591949	294.06	32	147.03 + 16	1/2	1/2	1

Table 5 (continued)

Strain	MIC alone (µg/mL)		MIC in combination (µg/mL)	Fold MIC in combination		ΣFIC
	TVEO	TAP		TVEO	TAP	
<i>Aeromonas hydrophila</i>						
MH591949	294.06	1	147.03 + 0.125	1/2	1/8	0.625
<i>Citrobacter freundii</i>						
MH155228	2,352.5	64	588.13 + 8	1/4	1/8	0.375
	TVEO	TAP	TVEO + TAP	TVEO	TAP	
<i>Escherichia coli</i>						
ATCC 25922	1,176.25	128	588.13 + 64	1/2	1/2	1

different animals (Malekmohammad, Rafieian-Kopaei, Sardari, & Sewell, 2019), including fishes (Spanghero et al., 2019; Tavares-Dias, 2018). It has often proven impossible to use the best concentrations that were obtained from *in vitro* tests on EOs used for the control of fish disease since the compounds possess a degree of toxicity (Tavares-Dias, 2018). In this study, an erythrocyte lysis assay widely used to estimate antimicrobial toxicity (Atterwill, 1995; Eschbach, Scharsack, John, & Medlin, 2001; Santos-Filho et al., 2017, 2015) revealed that hemolysis induced by EOs in fish red blood cells is concentration-dependent, and plasmatic concentrations of AVEO, BCEO, COEO, CAEO, CBEO, CCEO, EGEO, MPEO, OBEO, PGEO, and TVEO above 33,904–43,320 µg/mL can be toxic to red blood cells of Nile tilapia (*O. niloticus*).

Studies reporting the *in vitro* cytotoxicity of most of the EOs tested in this study are scarce. Nonetheless, existing published data showed that depending on the chemical profile, some EOs investigated here (AVEO, OBEO, EGEO, MPEO, and TVEO) can have *in vitro* (Jakovljević et al., 2020) and *in vivo* (De Souza et al., 2019; Malheiros, Maciel, Videira, & Tavares-Dias, 2016) toxic effects against different vertebrate cell lines at concentrations a thousand-fold lower than those reported here. Therefore, it is important to ensure that the plasma concentrations of these EOs do not exceed the values reported in this study whenever they are used to treat diseases in Nile tilapia.

Although the number of studies assessing the antimicrobial activity of EOs and their components has rapidly increased over the last three decades, the comparability of studies is often critical, mainly because of the different methods used for antimicrobial susceptibility testing (AST) and different definitions of antimicrobial activity (Thielmann et al., 2019). Thus, to facilitate inter-study comparisons, we tested multiple (sixteen) EOs using a single AST method, which was recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014), against six clinical isolates of fish pathogenic bacteria and two reference strains.

Additionally, we investigated the antimicrobial activity of four antimicrobial agents approved for use in aquaculture in different countries, enrofloxacin–ENRO, florfenicol–FFC, oxytetracycline–OTC, and thiamphenicol–TAP, and their combinations with TVEO against the clinical isolates and reference strains, using the same AST method and the broth microdilution checkerboard assay described by Leber (2016).

Some EOs and some major components of EOs identified in this study have already been reported to possess potent antimicrobial activity, either alone or in combination with other antimicrobials, against different bacteria (Alizadeh Behbahani & Imani Fooladi, 2018; Chouhan, Sharma, & Guleria, 2017; Malik et al., 2019; Tariq et al., 2019; Thielmann et al., 2019), including strains isolated from fish (Ismail & Yoshida, 2017). However, based on interpretative criteria proposed by Aligiannis et al. (2001), most EOs from this study were considered to have weak ($\text{MIC} > 1600 \mu\text{g/mL}$) antimicrobial activity against the clinical isolates tested. Further, some EOs were considered to lack bactericidal activity within the range of concentration tested. Similarly, weak and lack of antibacterial activity of several EOs, including BCEO, COEO, CAEO, CBEO, CCEO, CSEO, EGEO, MPEO, and PGEO, were previously reported by Thielmann et al. (2019).

The antimicrobial activity of a given EO against bacteria is determined by its mechanism of action, which is determined by the chemical composition (Chouhan et al., 2017; Cui, Zhang, Li, & Lin, 2019; Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). Thus, differing antimicrobial activities of EOs with different chemical profiles are expected.

ASEO, CVEO, OBEO, SAEO, and TVEO were considered active and possessed bactericidal activity against all tested strains. The MIC of ASEO ($338.44 \mu\text{g/mL}$), SAEO ($331.25 \mu\text{g/mL}$), and TVEO ($294.06 \mu\text{g/mL}$) against the reference strain, *Aeromonas hydrophila* ATCC[®] 7966[™], was hundred-fold lower than its hemotoxic concentration ($33,904\text{--}43,320 \mu\text{g/mL}$). The MICs of CVEO ($662.5 \mu\text{g/mL}$), SAEO ($662.5 \mu\text{g/mL}$), and

TVEO (294.06 µg/mL) against *A. hydrophila* MH591949 were also below their hemotoxic concentrations (33,904–43,320 µg/mL). Likewise, there are reports of strong antibacterial activity of these EOs against several Gram-negative (*Aeromonas caviae*, *A. hydrophila*, *Campylobacter jejuni*, *Edwardsiella tarda*, *Enterobacter aerogenes*, *Escherichia coli*, *Helicobacter pylori*, *Myxococcus piscicola*, *Salmonella enteritidis*, and *S. typhi*), Gram-positive (*Bacillus subtilis*, *Klebsiella* sp., *Listeria monocytogenes*, *Proteus* sp., *Staphylococcus aureus*, and *Streptococcus agalactiae*), and alcohol-acid resistant (*Mycobacterium marinum*) bacteria (Hyldgaard, Mygind, & Meyer, 2012; Karuppiyah & Rajaram, 2012; Lee & Gao, 2012; Luigina, Di Campli, Masulli, Di Bartolomeo, & Allocati, 1996; Semeniuc, Pop, & Rotar, 2017). However, little is known about their potential against fish pathogens. Such knowledge could contribute to selecting more potent plant-derived EOs for *in vivo* studies.

A limitation of this study is the absence of *in vivo* experiments. We cannot, therefore, be certain that good clinical outcomes and low toxicity can be observed under field conditions. We plan to address this limitation with appropriate *in vivo* experiments in future studies. Despite this limitation, our results strongly suggest that ASEO, CVEO, SAEO, and TVEO, which have a similar chemical profile to those from this study, are attractive candidates for improving the treatment of aeromoniosis in Nile tilapia. Moreover, it was observed that treatment with a combination of TVEO and commercially available antimicrobials (FFC and TAP) can be performed to reduce the dose of antimicrobial needed to treat the disease caused by antimicrobial-resistant *Citrobacter freundii* (Σ FIC = 0.75 and 0.375, respectively).

Interestingly, earlier studies examining the *in vitro* antimicrobial activity of EOs (Mousavi, Wilson, Raftos, Mirzargar, & Omidbaigi, 2011) and their components alone or in combination with different antimicrobials (Hayatgheib, Fournel, Calvez, Pouliquen, & Moreau, 2020), including FFC and OTC (Bandeira Junior et al., 2018) against different fish bacterial pathogens, highlighted the potential of using combination therapy (CT) in

aquaculture. Bandeira Junior et al. 2018 reported *in vitro* synergism between linalool and FFC (Σ FIC = 0.5) or OTC (FICI = 0.5) against *A. hydrophila* ATCC[®] 7966[™] and citral with OTC (Σ FIC = 0.5) against *C. freundii* MF565839. Indeed, CT is a promising strategy that has great potential to contribute to a reduction in the use of antimicrobials in aquaculture (Assane et al., 2019). However, this form of therapy should be studied more extensively to confirm its applicability under field conditions, and avoid the opposite effect to that intended, since synergism and antagonism could be found simultaneously (Pena-Miller et al., 2013), and experiments on drug interactions can lead to opposite conclusions by different methodologies (Odds, 2003).

Unfortunately, no reliable minimum inhibitory concentration breakpoints have been determined for EOs AST, nor have interpretative criteria of AST been specified. Current criteria are only based on MIC (Da Cunha et al., 2018) and possible toxic effects observed in the host. Reliable criteria that can be used to predict the probability of *in vivo* bacterial eradication and good clinical outcomes must also consider pharmacokinetic (PK) and pharmacodynamic (PD) properties of EOs within the host (Kohlert et al., 2000). Therefore, further studies involving studied EOs and their components that assess the *in vivo* efficacy of treatments and determine PK and PD properties of the host are strongly recommended to determine the most suitable dosage and treatment regimens needed, and to ensure the safety and effectiveness of EO therapy.

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Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supplementary Table 1. Chemical composition of thirteen selected plant-derived essential oils determined by GC–FID analysis.

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Monoterpene hydrocarbon														
	(<i>E</i>)-β-Ocimene	-	-	-	1.4	-	-	-	0.2	-	-	-	-	-
	(<i>Z</i>)-β-Ocimene	-	-	-	-	-	-	-	0.2	-	-	-	-	-
	δ-3-Carene	-	1.2	-	-	-	-	20.2	-	-	-	-	-	-
	Camphene	1.9	0.4	-	-	-	-	-	-	-	-	-	-	-
	Limonene	14.2	6.4	-	13.5	5.4	0.5	4.7	0.5	12.1	3.2	-	-	1.7
	Myrcene	0.5	3.2	-	4	-	0.3	1.9	13.8	-	0.6	-	-	0.6
	<i>p</i> -Cymene	0.8	6.8	-	-	6.3	-	0.7		4.4	-	-	-	34
	Sabinene	-	2.5	-	-	-	-	0.8	-	-	0.3	-	-	-
	Terpinolene	0.6	0.1	-	-	-	-	2.2	-	0.2	-	-	-	-
	Tricyclene	-	0.6	-	-	-	-	-	-	-	-	-	-	-
	α-Phellandrene	0.5	0.2	-	-	-	-	-	-	1.8	-	-	-	-
	α-Pinen	1.6	65.1	-	8.6	-	-	52.5	0.2	4.1	0.7	-	-	-

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
	α -Terpinen	-	0.1	-	-	-	-	-	-	-	-	-	-	-
	β -Myrcene	-	-	-	-	-	-	-	-	0.5	-	-	-	-
	β -Pinen	1.1	4.9	-	-	0.9	-	2.2	-	1.9	0.8	-	-	-
	γ -Terpinen	1.6	-	-	-	-	-	0.4	-	0.7	-	-	-	-
Oxygenated monoterpene														
Alcohol	Borneol	-	-	-	-	2	-	-	-	-	-	-	-	-
	Carvacrol	-	-	-	-	-	-	-	-	-	-	-	-	6.6
	Carveol	-	-	-	-	-	-	-	-	-	-	0.5	-	-
	Cis-Sabinene hydrate	-	-	-	-	-	-	-	-	-	-	0.2	-	-
	Citronellol	-	-	-	-	-	7.7	-	0.2	-	-	-	47	-
	Geraniol	-	-	-	-	-	-	-	3.2	-	-	-	10.1	-
	Isoborneol	-	-	-	-	-	1.3	-	-	-	-	-	-	-
	Isopulegol	-	-	-	-	-	0.5	-	-	0.1	-	-	-	-
	Levomenthol	-	-	-	-	-	-	-	-	-	1.9	-	-	-

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Aldehyde	Linalool	2.9	0.5	2	16.4	10.7	-	-	1.1	-	6.9	16.9	3.8	6.7
	Menthol	-	-	-	-	-	-	-	-	-	70	-	-	-
	Sabinene hydrate	-	-	-	-	-	-	-	-	-	1.2	-	-	-
	Terpinen-4-ol	-	-	-	-	-	-	1	-	-	-	-	-	-
	α -Terpineol	8.1	-	-	3.8	-	-	0.4	0.2	-	0.5	0.2	-	1.2
	Citronellal	-	-	-	-	-	77.2	-	0.2	-	-	-	-	-
	Geranial	-	-	-	-	-	-	-	40	-	-	-	1.5	-
	Neral	-	-	-	-	-	-	-	30.4	-	-	-	1.7	-
	Bornyl acetate	-	-	-	-	-	-	0.9	-	-	-	-	-	-
	Citronellyl acetate	-	-	-	-	-	7.0	-	-	-	-	-	-	-
Esters	Citronellyl formate	-	-	-	-	-	-	-	-	-	-	-	10.6	-
	Citronellyl tiglate	-	-	-	-	-	-	-	-	-	-	-	1	-
	Geranyl acetate	-	-	-	2.7	6.1	-	-	0.5	-	-	-	-	-
	Geranyl formate	-	-	-	-	-	-	-	-	-	-	-	1.9	-

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Ethers	Geranyl hexanoate	-	-	-	-	-	-	-	-	-	-	-	0.7	-
	Geranyl methyl	-	-	-	-	-	-	-	0.2	-	-	-	-	-
	Geranyl tiglate	-	-	-	-	-	-	-	-	-	-	-	1.5	-
	Linalyl acetate	-	-	-	35.3	33.1	-	-	-	-	-	-	-	-
	<i>L</i> -Menthyl acetate	-	-	-	-	-	-	-	-	-	1	-	-	-
	Neryl acetate	-	0.1	-	1.6	3.7	-	-	-	-	-	-	-	-
	Terpinyl acetate	-	-	-	-	-	-	2.2	-	-	-	-	-	-
	Anethole	-	-	-	-	-	-	-	-	-	-	0.7	-	-
	Estragole	-	5.2	-	-	-	-	-	-	-	-	77.4	-	-
	Myrcene epoxide	-	-	-	-	-	-	-	0.2	-	-	-	-	-
Ketones	Camphor	17.3	-	-	-	-	-	-	-	-	-	-	1.6	-
	Carvone	-	-	-	-	2.4	-	-	-	-	-	-	-	-
	Dihydrocarvone	-	-	-	-	-	-	-	-	-	-	-	-	0.9

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Oxydes	Isomenthone	-	-	-	-	-	-	-	-	-	3.8	-	6	-
	Menthone	-	-	-	-	-	-	-	-	-	6.5	-	0.9	-
	Pulegone	-	-	-	-	-	-	-	-	0.4	-	-	-	-
	α -Thujone	-	1.3	-	-	-	-	0.5	0.2	0.1	-	-	-	-
	cis-Linalool oxide	-	-	-	-	4.8	-	-	-	-	-	-	-	-
	(E)-Rose oxide	-	-	-	-	-	-	-	-	-	-	-	2.5	-
	(Z)-Rose oxide	-	-	-	-	-	-	-	-	-	-	-	0.5	-
	1,8-Cineole	14.2	-	-	-	-	0.7	-	-	73	-	0.3	-	1
	Trans-Linalool oxide	-	-	-	-	4.4	-	-	-	-	-	-	-	-
	β -Pinen oxide	-	-	-	-	-	-	-	1.5	-	-	-	-	-
Phenols	Eugenol	-	-	-	-	5.3	-	-	-	-	-	-	0.9	-
	Thymol	-	-	-	-	-	-	-	-	-	-	-	-	40.4
Oxygenated diterpene														
Alcohol	Incensol	-	0.1	-	-	-	-	-	-	-	-	-	-	-

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Esters	Incensol acetate	-	0.1	-	-	-	-	-	-	-	-	-	-	-
Sesquiterpene hydrocarbons														
	Aromadendrene	2.1	-	5.2	-	-	-	-	-	-	-	-	-	-
	Bisabolene	-	-	-	-	4.2	-	-	-	-	-	-	-	-
	Germacrene D	-	-	24.9	-	-	-	-	0.1	-	-	-	-	-
	<i>trans</i> - β -Bergamotene	-	-	-	-	-	-	-	0.1	-	-	-	-	-
	α -Bergamotene	-	-	-	-	-	-	-	-	-	-	0.2	-	-
	α -Curcumene	-	-	3.3	-	-	-	-	-	-	-	-	-	-
	α -Gurjunene	-	-	-	-	-	-	0.6	-	-	-	0.5	-	-
	α -Himachalene	2.7	-	-	-	-	-	-	-	-	-	-	-	-
	α -Muurolene	-	-	-	-	-	-	-	0.1	-	-	0.1	-	-
	β -Caryophyllene	8.4	-	19.8	10.5	-	0.7	0.4	0.2	-	-	0.4	1.4	-
	β -Farnesene	-	-	10.2	-	-	-	-	-	-	-	-	-	-
	β -Himachalene	7.3	-	-	-	-	-	-	-	-	-	-	-	-

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
	β -Ylangene	-	-	6.4	-	-	-	-	-	-	-	-	-	-
	γ -Muurolene	-	-	-	-	-	-	-	-	-	0.5	-	-	-
	δ -Cadinene	-	0.3	5.3	-	-	-	-	0.2	0.6	-	-	-	-
Oxygenated sesquiterpene														
Alcohol	Cedrol	-	-	-	-	-	-	2.7	-	-	-	-	-	-
	Nerolidol	-	-	-	-	-	-	-	-	-	-	1.6	-	-
	Selina-6-en-4-ol	-	-	-	-	-	-	-	0.2	-	-	-	-	-
	<i>T</i> -Muurolol	-	-	3.2	-	-	-	-	-	-	-	-	-	-
Esters	α -Turmerone	1.9	-	-	-	-	-	-	-	-	-	-	-	-
	β -Turmerone	1.8	-	-	-	-	-	-	-	-	-	-	-	-
Ethers	Bisabolene epoxide	3.5	-	-	-	-	-	-	-	-	-	-	-	-
Oxydes	Caryophyllene oxide	-	-	-	-	-	-	-	0.1	-	-	-	-	-
Esters														
	Benzyl benzoate	-	-	7.3	-	-	-	-	-	-	-	-	-	-

Chemical Group	Components (%)	Essential oil ^a												
		1	2	3	4	5	6	7	8	9	10	11	12	13
	Benzyl salicylate	-	-	3.9	-	-	-	-	-	-	-	-	-	-
	Phenethyl butyrate	-	-	-	-	-	-	-	-	-	-	-	1.5	-
Ketones														
	6-Methyl-5-hepten-2-one	-	-	-	-	-	-	-	1.1	-	-	-	-	-
	Methyl nonyl ketone	-	-	-	-	-	-	-	0.1	-	-	-	-	-
Identified components [%]		93.0	99.1	91.5	97.8	89.3	95.9	94.3	95.0	99.9	97.9	99.0	95.1	93.1
Monoterpene hydrocarbon [%]		22.8	91.5	0.0	27.5	12.6	0.8	85.6	14.9	25.7	5.6	0.0	0.0	36.3
Oxygenated monoterpene [%]		42.5	7.1	2.0	59.8	72.5	94.4	5.0	77.9	73.6	91.8	96.2	92.2	56.8
Oxygenated diterpene [%]		0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sesquiterpene hydrocarbons [%]		20.5	0.3	75.1	10.5	4.2	0.7	1.0	0.7	0.6	0.5	1.2	1.4	0.0
Oxygenated sesquiterpene [%]		7.2	0.0	3.2	0.0	0.0	0.0	2.7	0.3	0.0	0.0	1.6	0.0	0.0

^aEssential oils: 1- mugwort *Artemisia vulgaris* EO (AVEO), 2- olibanum *Boswellia carterii* EO (BCEO), 3- ylang-ylang *Cananga odorata* EO (COEO), 4- bitter orange tree *Citrus aurantium* var. *amara* EO (CAEO), 5-bergamot *Citrus bergamia* EO (CBEO), 6-lemon-scented gum *Corymbia citriodora* EO (CCEO), 7- Italian cypress *Cupressus sempervirens* EO (CSEO), 8- lemongrass *Cymbopogon citratus* EO (LGEO), 9- Tasmanian blue gum *Eucalyptus globulus* EO (EGEO), 10- peppermint *Mentha x piperita* EO (MPEO), 11- basil *Ocimum basilicum* EO (OBEO), 12- geranium *Pelargonium graveolens* EO (PGEO), and 13- thyme *Thymus vulgaris* EO (TVEO).

Thiamphenicol and florfenicol combination in Nile tilapia: simultaneous detection and quantification in plasma and muscle plus skin samples, and pharmacokinetics following single oral administration

Abstract

Thiamphenicol (TAP) and florfenicol (FFC) combination have *in vitro* and *in vivo* synergistic activity against fish bacterial pathogens. However, a lack of information on its pharmacokinetics (PK) and pharmacodynamics (PD) in fish limits its further application. Therefore, this study aimed to develop and validate an analytical method for simultaneous detection and quantification of TAP and FFC in tilapia plasma and muscle samples and determine the PK in freshwater tilapia at $30 \pm 0.32^{\circ}\text{C}$ after a single oral dose of 5 mg kg^{-1} TAP + 3.75 mg kg^{-1} FFC. Antimicrobial concentrations were analyzed by HPLC-MS. The PK characteristics were estimated by a non-compartmental model (linear up/log down). A rapid, precise, and sensitive method was developed using ethyl acetate (plasma) and a modified QuEChERS (muscle) for extraction. The method was validated according to the requirements of the Brazilian Health Regulatory Agency, the U.S. Department of Health and Human Services, and the VICH GL49. All antimicrobials were determined in a single run, and chromatographic separation was achieved using a reversed-phase Waters Xterra MS C₁₈ column and a Supelco guard column, using an isocratic mobile phase composed of acetonitrile in ultrapure water (55:45, by volume). The method was successfully applied to analyze the plasma and muscle samples in the PK study. The highest concentration of TAP and FFC was detected at 0.5 h (6.34 and $3.34 \mu\text{g mL}^{-1}$) and 16h (8.20 and $11.37 \mu\text{g mL}^{-1}$) post administration in plasma and muscle, respectively. There was no significant difference between the peak concentration and apparent volume of distribution during the terminal phase of each antimicrobial in different tissues. However, the peak time, the area under the concentration-time curve, and mean residence time was higher in the muscle, for both antimicrobials ($P < 0.05$). The calculated PK parameters and the curve obtained for both antimicrobials emphasize their fast absorption and distribution even when used in combination. The results from this

study could help establish antimicrobial susceptibility testing interpretative categories and design rational dosing regimens for TAP and FFC combination.

Keywords: Aquaculture, Florfenicol, HPLC-MS, *Oreochromis niloticus*, Pharmacokinetics, Thiamphenicol.

Highlights

- The developed and validated HPLC–MS/MS method is a rapid, simple, precise, and sensitive method for the simultaneous determination of TAP and FFC in fish plasma and muscle.
- Plasma and muscle plus skin pharmacokinetics of TAP and FFC were determined in tilapia.
- TAP and FFC had fast absorption and distribution in freshwater Nile tilapia reared at $30 \pm 0.32^{\circ}\text{C}$.
- TAP and FFC pharmacokinetics in tilapia could help design rational dosing regimens.

1. Introduction

Nile tilapia, *Oreochromis niloticus* L., is the most widely introduced species and economically important food fish in many countries, being introduced in 114 countries or regions (FAO, 2021). Currently, is the third major aquaculture species, after grass and silver carps (*Ctenopharyngodon idellus* and *Hypophthalmichthys molitrix*, respectively) (FAO, 2022a). China, Indonesia, Egypt, Brazil, Bangladesh, Viet Nam, Philippines, Thailand, Colombia, and Uganda are the main producing countries (FAO, 2022b). Despite its strong environmental adaptability and disease resistance, Nile tilapia aquaculture is highly impacted by bacterial diseases caused by both Gram-negative and Gram-positive bacteria, such as *Aeromonas hydrophila*, *A. jandaei*, *A. veronii*, *Edwardsiella tarda*, *Flavobacterium columnare*, *Francisella orientalis*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *S. iniae*, *S. dysgalactiae* and *Lactococcus garviae* (Assane et al., 2021b, 2019; de Abreu Reis Ferreira et al., 2022; Delphino et al., 2019; Dong et al., 2017; Vaneci-Silva et al., 2022).

Due to the high economic impacts of bacterial infections in Nile tilapia worldwide, several strategies are being evaluated and/or used to prevent bacterial disease outbreaks and reduce the economic impacts when they occur. Breeding Nile tilapia that are resistant to bacterial infections (Shoemaker et al., 2016; Suebsong et al., 2019), the use of commercial and autogenous vaccines (Barnes et al., 2021), dietary supplementation with prebiotics (Pilarski et al., 2017) and probiotics (Xia et al., 2020; Yamashita et al., 2017), and the use of new effective and environmentally friendly treatments (Assane et al., 2021c; de Sousa et al., 2021; Luo et al., 2018) are among the main strategies being evaluated. However, the use of antimicrobials remains the main strategy to improve fish health and disease outbreak control, despite the growing concerns about the negative impacts of its misuse on both human and animal health (di Cesare et al., 2013; Higuera-Llantén et al., 2018; Limbu et al., 2021). Thus, the approval of new effective antimicrobials for use in fish farming is relevant, mainly in countries like Brazil,

where there is only one antimicrobial (florfenicol – FFC) approved for use in the main cultured species (Nile tilapia) (SINDAN, 2022).

Florfenicol is an antimicrobial of the phenicol drug family, derived from thiamphenicol (TAP), and considered a critically important antimicrobial for fish disease control (OIE, 2015). It inhibits bacterial protein synthesis and has a broad spectrum of action against bacteria (Gram-negative and positive, aerobic, and anaerobic) (Fukui et al., 1987; Syriopoulou et al., 1981), with no prolonged post-antimicrobial effect (Rairat et al., 2019b), low toxicity to Nile tilapia, and low environmental risk (Mattioli et al., 2020). Due to its short half-life (Kogiannou et al., 2021), it is considered primarily time-dependent bacteriostatic. Its lipophilic characteristic allows rapid absorption (Feng and Jia, 2009; Kogiannou et al., 2021) and wide tissue distribution (Feng et al., 2016), ensuring that concentrations are high enough to treat intracellular pathogens and cross anatomical barriers, such as the barrier blood-brain (Papich, 2016). Its main metabolite is florfenicol amine (FFCA), which is considered a marker residue to determine the withdrawal period in food fish.

The bioavailability of FFC is high in several fish species, with reports of 91% and 99% of bioavailability after oral administration of 10 mg/kg.bw in cod (Samuelsen et al., 2003) and in Atlantic salmon (Horsberg et al., 1996), respectively. The recommended dose and administration regimen vary between countries and fish species (i.e., the recommended dose in Brazil is 10 - 20 mg/kg/day for 10 days, depending on the fish species, while in the USA is 10 - 15 mg/kg/day for 10 days, and in Peru, Taiwan, and the Republic of Korea is 10 mg/kg/day for 3 - 5 days) (FAO, 2016; FDA, 2007; Rairat et al., 2019a; SINDAN, 2022).

Despite its wide use in aquaculture and strong antimicrobial activity against the main fish bacterial pathogens, such as *A. hydrophila* (Assane et al., 2019; Bandeira Junior et al., 2018; Godoy et al., 2008), *A. jandei* (Assane et al., 2021a), *A. veronii* (Assane et al., 2019; Bandeira Junior et al., 2018), *F. columnare* (de Abreu Reis Ferreira et al., 2022), *Vibrio fluvialis* (Assane

et al., 2019), and *S. agalactiae* (Chideroli et al., 2017; de Oliveira et al., 2018), there are reports of resistant strains (Assane et al., 2019; Bandeira Junior et al., 2018; Gallani et al., 2016), treatment failure, and recurrent infection in Nile tilapia after treatment using the recommended dose and regimen (de Oliveira et al., 2018). Therefore, new antimicrobials or therapies that are both environmentally friendly and effective must be conceived for the treatment of the increasing number of bacterial diseases that affect cultured fish.

The use of combination therapy (CT) is among the promising strategies to reduce the use of antimicrobials in aquaculture while reducing the economic impacts of bacterial disease outbreaks. This form of therapy is promising for solving the aforementioned problems; as it reduces the emergence of bacterial resistance (Milatovic and Braveny, 1987; Xu et al., 2018), and it is effective against multiresistant pathogens (Allen et al., 2002; Guner et al., 2011; Lin et al., 2015; Xu et al., 2018). Furthermore, it can give satisfactory therapeutic results using lower doses of antimicrobials (Assane et al., 2019; Bandeira Junior et al., 2021).

Currently, there are at least four combinations of antimicrobials approved for use in aquaculture in different countries (FDA, 2007; Felleskatalogen, 2018; Health Canada, 2010) and new promising combinations are being studied (Assane et al., 2021c, 2019; Bandeira Junior et al., 2021; de Abreu Reis Ferreira et al., 2022; Huang et al., 2022). The combination of TAP with FFC stands out among the promising combinations for use in aquaculture. There are reports of a synergistic effect of this combination against both Gram-negative and Gram-positive pathogenic bacteria recovered from diseased cattle, chickens (Wei et al., 2016), swine (Wei et al., 2016) and fish (Assane et al., 2019). Moreover, the differences in the mechanisms of bacterial resistance to these antimicrobials of the same class usually make bacteria resistant to one of them susceptible to the other (van Hoek et al., 2011), allowing the occurrence of a synergistic interaction when CT is used.

Despite these known benefits of the TAP + FFC combination, there is little information about TAP and FFC simultaneous detection and quantification in Nile tilapia plasma and muscle plus skin samples, and their pharmacokinetics (PK) and pharmacodynamics (PD) in cultured fish. Most studies about TAP and FFC simultaneous detection are focused on amphenicol maximum residue limits (MRL) in fish muscle (Barreto et al., 2016; Jung et al., 2022). The cost of antimicrobial research and development is high, so all drug candidates selected for clinical trials in animals must be evaluated as efficiently and cost-effectively as possible. It is proven that the application of *in vitro* antimicrobial susceptibility testing, and PK-PD principles to this process improves the selection of appropriate doses and clinical trial design (CLSI, 2020a, 2013; Kazakevich and LoBrutto, 2006; Rosenbaum, 2017) necessary for antimicrobial registration (MAPA, 2004).

Considering this scenario, understanding PK-PD proprieties of promising antimicrobials are cost-effective and sustainable solutions to address, in the long term, the issue of the emergence of bacterial diseases in a scenario of limited availability of effective antimicrobials. Thus, this study aims to develop an analytical method for simultaneous detection and quantification of TAP and FFC in Nile tilapia plasma and muscle plus skin (in natural proportion) samples and determine the PKs in freshwater Nile tilapia after a single oral dose of TAP + FFC combination.

2. Materials and methods

2.1. Reagents and analytical standards

Chloramphenicol (CAP, ≥ 98.0 purity) (internal standard), thiamphenicol (TAP, ≥ 99.0 purity) and florfenicol (FFC, ≥ 99.0 purity) analytical standards, magnesium sulfate anhydrous (MgSO_4 , ≥ 97 purity), methanol (CH_3OH , ≥ 99.9 purity) and acetonitrile (CH_3CN , ≥ 99.9 purity) were purchased from Merck (Sigma-Aldrich[®], Brazil). Ethyl acetate and sodium chloride were

purchased from Synth[®] (Brazil), and the Primary Secondary Amine (PSA) from Agilent Technologies (USA). All reagents and solvents were analytical grade and HPLC grade, respectively. Ultra-pure deionized water was obtained through a Milli-Q Direct-Q[®] 3 water purification system.

2.2. Standard solutions

Methanol was used as a solvent and diluent to prepare 10 µg mL⁻¹ antimicrobial (CAP, TAP and FFC) stock solutions. Standard stock solutions were stored in tightly closed, dark vials at -20 °C for further tests and analyses (maximum storage period = 1 month). The antimicrobial working solution was prepared daily, as needed.

The stability of standard stock solutions stored at -20 °C was evaluated by comparing the responses obtained with the stock solutions and solutions prepared at the time of analysis. Analyzes were performed periodically for 5 months.

2.3. Fish and study design

After an acclimation period of 14 days, 100 healthy juvenile Nile tilapia (103.96 ± 27.01g body weight (bw)) were distributed randomly and equally in 10 tanks (140 L) with constant aeration and open water flow (same water source for all tanks). The tanks were divided randomly into two experimental groups as follows: G1 (6 tanks): medicated with TAP combined with FFC (TAP + FFC: 5 + 3.75 mg kg⁻¹ bw), and G2 (4 tanks): not medicated (control).

During acclimation and the experimental period (12 days), fish were fed *ad libitum* with the same commercial diet, specific for omnivorous (Neovia Nutrição e Saúde Animal LTDA), twice a day. The water quality parameters were measured every day, using a U-52G probe (Horiba[®], Japan), and values were within the normal range and similar between the tanks

(average: temperature - 29.68 ± 0.32 °C, pH - 6.61 ± 0.46 , dissolved oxygen - 7.04 ± 4.42 mg L⁻¹, oxidation-reduction potential - 276.91 ± 53.32 mV, electrical conductivity - 0.17 ± 0.04 , total dissolved solids - 0.11 ± 0.03 g L⁻¹ and salinity - $0.01 \pm 0\%$).

The experimental design and conditions were approved by the Comissão de Ética no Uso de Animais (CEUA) of the School of Agricultural Science and Veterinary Medicine of São Paulo State University, Jaboticabal, São Paulo, Brazil, under protocol number 3772/20. This approval was consistent with the ethical principles proposed by the Brazilian College of Animal Experimentation.

2.4. Antimicrobial administration

Antimicrobial (TAP and FFC) analytical standards were dissolved in ethanol 95% and diluted in phosphate-buffered saline (PBS, pH = 7.4) to prepare 1,000 mg L⁻¹ TAP and 750 mg L⁻¹ FFC stock solutions. Stock solutions were mixed to prepare the antimicrobial working solution (TAP + FFC: 500 + 375 µg mL⁻¹). The antimicrobial working solution (G1), or PBS (G2) was then administered through gavage (1 mL per 100 g bw), using a syringe connected to a catheter.

All fish were anaesthetized in 100 mg L⁻¹ benzocaine (Sigma-Aldrich®, Brazil) solution before antimicrobial or PBS administration.

2.5. Sample collection and storage

The sampling times were 0.5, 1, 2, 4, 6, 8, 12, 16, 24, 48, 96, 168, 216, and 264 hours after antimicrobial administration. At each sampling time, ten fish (6 from G1 and 4 from G2) samples of blood and muscle plus skin (in natural proportions) were collected.

Blood was sampled from the caudal vein, dispensed into a sterile heparinized polypropylene tube (2 mL), and gently mixed immediately. Cells were removed from the plasma by

centrifugation of the blood at 1,500 *g* for 10 minutes using a refrigerated centrifuge (Himac CF-5RX, Hitachi Koki Co. Ltd., Japan) at 4 °C. The plasma of each fish was apportioned into 500 µL aliquots and immediately frozen and stored at –80 °C until analysed.

The muscle plus skin samples were packed in plastic bags, identified, and immediately frozen and stored at –80 °C until analysed.

2.6. Sample preparation and extraction procedure

2.6.1. Plasma

Antimicrobials were extracted from fish plasma using a simple liquid extraction procedure. The detailed procedure was as follows: 1,000 µL of ethyl acetate was added to a polypropylene tube (15 mL) containing 400 µL of plasma. The tube was homogenized in a vortex mixer for 60 seconds and centrifuged at 2,900 *g* for 15 minutes using a refrigerated centrifuge (Himac CF-5RX, Hitachi Koki Co. Ltd., Japan) at 20 °C. From the supernatant, 300 µL were carefully transferred to a polypropylene tube and taken to the concentrator (Centrivap[®] Concentrator 78100 series, Labconco, US) for solvent evaporation (20 minutes, 30 °C). After complete evaporation of the solvent, the sample was resuspended in 150 µL of methanol, followed by 1-minute vortexing. 150 µL of ultrapure water was added to the tube and the tube was vortexed again for 1-minute. Then, the sample was filtered through a 0.20 µm regenerated cellulose pore membrane filter to insert into an appropriate vial for injection into the analytical system.

The extraction efficiency was evaluated by comparing the responses obtained by the solutions of the analyte in spiked extract and the analyte in the spiked matrix, at three concentration levels: 100, 500 and 2,500 ng mL⁻¹ of TAP and FFC, with six replicates of each level.

2.6.2. Muscle plus skin

Extraction and cleanup of the extract were conducted using a modified QuEChERS approach. Briefly, muscle plus skin samples were thawed, ground in an impact mill (IKA), and placed (5.0 g) into a 50 mL polypropylene tube. 100 μ L of internal standard solution (500 ng mL⁻¹ CAP) was added to the tube. Samples were kept at rest, protected from light, for stabilization and interaction with the matrix. Then, 5 mL of acetonitrile was added and homogenized in a vortex mixer for 60 seconds. After homogenization, 2.0 g of anhydrous magnesium sulfate (MgSO₄) and 0.500 g of sodium chloride (NaCl) were added to the tube, homogenized by vortexing for 60 seconds, and the tube was centrifuged at 2,900 *g* for 15 minutes using a refrigerated centrifuge (Himac CF-5RX, Hitachi Koki Co. Ltd., Japan) at 4 °C. From the supernatant, 1 mL was carefully transferred to a polypropylene tube containing 0.025 g PSA, homogenized by vortexing for 60 seconds, and the tube centrifuged at 2,900 *g* for 10 minutes using a refrigerated centrifuge (Himac CF-5RX, Hitachi Koki Co. Ltd., Japan) at 4 °C. After centrifugation, the supernatant was filtered through a syringe filter with a regenerated cellulose membrane (0.20 μ m pore) and transferred to an appropriate vial for injection in the LC-MS/MS system.

For the extraction tests and the preparation of the analytical matrix curves, the blank matrix samples were spiked with CAP (internal standard), TAP and FFC before the extraction procedures. For sample analysis, samples were only spiked with the internal standard before the extraction procedure.

2.7. Validation

The analytical methods for simultaneous determination of TAP and FFC in Nile tilapia plasma and muscle plus skin were validated based on the parameters and criteria established by the Brazilian Health Regulatory Agency (Anvisa, 2017, 2012), the U.S. Department of Health and Human Services (FDA, 2018) and the VICH GL49 (VICH, 2015). The following

parameters were evaluated: selectivity, residual effect, matrix effect, calibration curve, linearity, precision, accuracy, stability, robustness, limits of detection (LOD) and quantification (LOQ).

2.7.1. Selectivity

The chromatographic selectivity was evaluated by comparing the chromatograms obtained in triplicate of the blank matrix without the addition of antimicrobial standards and the blank matrix spiked with the target analytes and internal standard at the lower limit of quantification (LLOQ) (TAP and FFC at 100 ng mL⁻¹ and CAP at 2 µg mL⁻¹). The analyzed samples of the biological matrix were obtained from 6 different sources, using four normal, two lipemic and two hemolyzed samples (for plasma samples).

Two transitions were monitored for each analyte and the internal standard, one for quantification and the other for confirmation of substance identity.

2.7.2. Residual effect

To evaluate the residual effect, three injections of the same blank sample were performed, one before and two immediately after the injection of one or more samples processed with the highest concentration level of the calibration curve.

2.7.3. Matrix effect

For muscle plus skin samples, three analytical curves were prepared (extract spiked with the target analytes and solvent curves). The matrix effect was determined by the comparison between the angular coefficients of the analytical curves prepared in a solvent with the angular coefficients of the curves prepared in extract spiked with the target analytes (Anvisa, 2017). The parallelism of the lines was used to evaluate the presence of interference between the two

constituents of the matrix during the inspections of the graph generated from the data. The F-distribution (Fisher-Snedecor distribution) was used to evaluate each concentration level, and the matrix effect was calculated using the following equation: $ME (\%) = \frac{\text{angular coefficient (extract spiked with the target analytes)} - \text{angular coefficient (solvent)}}{\text{angular coefficient (solvent)}} \times 100$.

For plasma samples, processed biological matrices were analyzed. Subsequently, the analytes and internal standard solution, and antimicrobial solutions (at the same concentrations as the matrix sample with the analyte three times higher than the lower limit of quantification, and between 75% and 85% of the highest concentration of the calibration curve) were added.

Eight samples, from different sources, were analyzed (four normal, two lipemic and two hemolyzed). The internal standard-normalized matrix factor (IS-normalized MF) was calculated for each sample, using the following formula: $MF = \frac{\text{analyte response in matrix} \div \text{internal standard response in matrix}}{\text{analyte response in solution} \div \text{internal standard response in solution}}$.

The coefficient of variation of MF (lower than 15%) of each analyte was used to evaluate the matrix effect.

2.7.4. Calibration curve

For simultaneous detection of TAP and FFC in plasma, analytical curves were prepared daily from the dilution of the antimicrobial stock solution, in six concentrations (curve 1: 800; 1,440; 2,080; 2,720; 3,360 and 4,000 ng mL⁻¹; curve 2: 4,000; 7,200; 10,400; 13,600; 16,800 and 20,000 ng mL⁻¹; curve 3: 20,000; 36,000; 52,000; 68,000; 84,000 and 100,000 ng mL⁻¹). The concentration of the internal standard (CAP) was maintained at 16,000 ng mL⁻¹. To prepare each point of the analytical curve, the matrix was spiked with the prepared solutions (50 µL).

To prepare the analytical curves in the matrix, the matrices were spiked with the target analytes before the extraction procedure to obtain concentrations equivalent to the respective curves in the solution.

For the preparation of the analytical curves in extract, the blank matrix samples were submitted to the complete extraction process and then spiked with the target analytes.

Analytical curves for simultaneous detection of TAP and FFC in plasma were prepared daily at the following concentrations (final concentrations): curve 1: 100; 180; 260; 340; 430 and 500 ng mL⁻¹; curve 2: 500; 900; 1,300; 1,700; 2,100 and 2,500 ng mL⁻¹; curve 3: 2,500; 4,500; 6,500; 8,500; 10,500 and 12,500 ng mL⁻¹. The concentration of the internal standard was maintained at 2,000 ng mL⁻¹.

To prepare the analytical curves for simultaneous detection of TAP and FFC in tilapia muscle plus skin samples, the blank samples (matrices) were spiked with the target analytes before conducting the extraction procedure. The curves were prepared daily in a matrix spiked with the target analyte at six concentrations (20; 60; 100; 140; 180 and 220 µg kg⁻¹). CAP concentration (internal standard) was maintained at 10 µg kg⁻¹ (10 ng mL⁻¹). For matrix fortification, an intermediate solution (100 µg mL⁻¹) was prepared from the stock standard solution. Another six solutions were prepared by diluting the intermediate solution in methanol (2,000; 6,000; 10,000; 14,000; 18,000 and 22,000 ng mL⁻¹). To prepare each point of the analytical curve, the matrix was spiked with the target analyte solution (50 µL). An internal standard (CAP) solution at 500 ng mL⁻¹ was prepared and 100 µL was added to the matrix to obtain the target concentration of 10 µg kg⁻¹. The spiked matrix was kept at rest for 15 minutes, protected from light, for stabilization and analyte interaction with the matrix. Then, the sample preparation procedure was conducted.

2.7.5. Linearity

Plasma

The linearity of the calibration curves was evaluated through three independent analytical curves, in six levels of concentration of TAP and FFC, obtained from extracted blank matrix spiked with the target analytes in each concentration. The concentrations used in the curves were determined based on previous studies. The concentrations of antimicrobial used are shown in Table 1. The concentration of the internal standard (CAP) was maintained at 2,000 ng mL⁻¹.

Table 10. Thiamphenicol (TAP) and florfenicol (FFC) concentrations used in the calibration curves.

Concentration range 1	Concentration range 2	Concentration range 3
TAP and FFC	TAP and FFC	TAP and FFC
concentration (ng mL ⁻¹)	concentration (ng mL ⁻¹)	concentration (ng mL ⁻¹)
100	500	2,500
180	900	4,500
260	1,300	6,500
340	1,700	8,500
430	2,100	10,500
500	2,500	12,500

The quality of the analytical curve and its linearity were evaluated through linear regression analysis. Determination and correlation coefficients and the residuals were calculated, and the residual graph was used for visual inspection. Instrument response repeatability standard deviations were analyzed for each concentration level. The homoscedasticity or

heteroscedasticity of the standard deviations was evaluated using the Fischer-Snedecor F test and the Cochran C test ($p = 0.05$).

Muscle plus skin

Three independent analytical curves in a matrix were prepared and analyzed. The matrices were spiked with the target analytes at six concentration levels (20, 60, 100, 140, 180 and 220 $\mu\text{g kg}^{-1}$) and processed as described in section 2.6.2.. The internal standard was maintained at 10 $\mu\text{g kg}^{-1}$. The line equations were obtained by plotting the response graphs as a function of the concentration of each analyte and the internal standard. The linear association between the variables was evaluated through the correlation coefficient and the significance of the angular coefficient. Linear correlation coefficient (r) above 0.990, and angular coefficient significantly different from zero were used as criteria to evaluate the linearity. Also, the criteria established for samples in the precision test were used to evaluate variations in responses between points on the curve. The homoscedasticity of the data was also investigated, for which the residual plots and the Cochran test were considered (Anvisa, 2017).

2.7.6. Precision and accuracy

To assess accuracy and precision, six independent samples were prepared, three samples on the same day (intra-day precision or repeatability) and three samples on different days (inter-days or intermediate precision). This procedure was performed for each concentration used in the analytical curves.

2.7.7. Stability

Stability studies were carried out according to the storage, preparation, and analysis conditions of the samples. Thus, a set of biological matrix samples spiked with the target

analyte and the same anticoagulant used in the sample collection (for plasma samples) were kept in a freezer at -20°C until the moment of analysis and spiked with the internal standard.

Stability in the matrix was evaluated using blank samples spiked with the target analyte at the lowest and highest concentrations of the analytical curve, corresponding to 100 and 100,500 ng g^{-1} for plasma, and 20 and 220 ng g^{-1} for muscle plus skin samples, respectively. The spiked samples were analyzed periodically, in triplicate, up to 14 days (plasma) and 25 days (muscle plus skin) after storage.

Matrix stability was considered acceptable if the average concentration obtained at a given point agreed with the initial concentration, according to the established criteria for accuracy. The stability of the processed matrix (samples after the extraction procedure) was also evaluated. Processed samples were compared with recently prepared samples (prepared on the day of analysis). Stability was considered adequate if there was no deviation greater than 15% of the average concentrations obtained when compared to the nominal value.

2.7.8. Robustness

Four parameters were evaluated, two related to the chromatographic method and two related to the extraction procedure, being: the composition of the mobile phase (52 and 57% acetonitrile), the column temperature of the HPLC system (35 and 45°C), the amount of MgSO_4 (1.8 and 2.2 g) and the centrifuge temperature (3 and 5°C). The results obtained with the challenge conditions were compared with the normal condition, established by the method. Relative errors (%) were calculated and the same criteria applied to the accuracy test were adopted for acceptance.

2.7.9. Limits of detection and quantification

Limits of detection (LOD) were estimated after verifying the existence of a linear relationship between analyte concentration and instrumental response. Three analytical curves in solution were prepared and analyzed (analyte concentrations: 20, 60, 100, 140, 180 and 220 ng mL⁻¹). The results were plotted in graphs and the line equations were obtained. The limits were calculated using the following equation: $LOD = \frac{3.3 \times \sigma}{S}$, where σ and S represent the standard deviation of y-intercept and slope of the calibration curve, respectively (Anvisa, 2017).

After estimating the initial LOD value for each analyte, the concentration expressed in ng mL⁻¹ (solution) was converted to $\mu\text{g kg}^{-1}$ (matrix). The limit of quantification (LOQ) was defined as the lowest concentration used in the analytical curve, whose precision and accuracy were evaluated.

2.8. Equipment

Chromatographic analysis was performed using a high-performance liquid chromatography system (Shimadzu, Japan) composed of three pumps, a controller, a column oven, and an automatic injector. Chromatographic separation of analytes was carried out on a reversed-phase Waters Xterra MS C₁₈ column (100 mm × 3.9 mm, 3.5 μm) and a Supelco guard column with the same composition (20 mm × 3.9 mm, 3.5 μm), using an isocratic mobile phase composed of acetonitrile in ultrapure water (55:45, by volume) at a flow rate of 0.30 mL min⁻¹. The column temperature was maintained at 25°C, the sample injection volume was 10 μL , and the duration of each analysis was 7 minutes.

Mass spectrophotometric analysis was carried out using a triple-quadrupole analyzer Micromass Quattro LC (Micromass Co., Manchester, UK) with electrospray ionization (ESI) operated in the negative ionization mode (ESI⁻) (temperature of 100 °C and a desolvation gas temperature of 350 °C) for all analytes. Nitrogen was used as desolvation gas at a flow rate of

440 L h⁻¹ and as cone gas at 90 L h⁻¹. The capillary voltage was set to 1 kV and argon gas was used in the collision chamber under a pressure of 1.72×10^{-3} mbar.

Two transitions (one for quantification and the other for confirmation of analyte identity) were monitored through the multiple reaction monitoring (MRM) mode of the ions of the deprotonated molecules [M-H]⁻ and their respective fragments, for both analytes (TAP and CAP) and the internal standard (CAP). The specific MS/MS parameters for each target analyte are shown in Table 2. Data acquisition and processing were performed using Micromass MassLynx software version 4.1 (Waters Co., USA).

Table 11. Mass spectrometer settings for TFC and FFC analysis.

Analyte	Precursor ion (m/z)	Fragment ion (m/z)	Cone voltage (V)	Collision energy (eV)
TAP	354	290 ^a	28	20
	354	227 ^b	28	12
FFC	335	185 ^a	29	15
	335	79 ^b	29	20
CAP	321	152 ^a	29	15
	321	257 ^b	29	12

^a transition selected for analyte quantification.

^b transition selected to confirm analyte identity.

2.9. Pharmacokinetic analysis

Pharmacokinetic parameters were estimated by a non-compartmental model (linear up/log down) using PKSolver 2.0 software (China Pharmaceutical University, Nanjing, China) (Zhang et al., 2010). The following parameters were estimated: half-life ($t_{1/2}$); peak time ($T_{\max}$); peak concentration ($C_{\max}$); area under the plasma and muscle concentration-time curve (AUC) up to the last measurable concentration (AUC_{0-t}), and to infinite time ($AUC_{0-\infty}$); mean residence time (MRT) up to the last measurable concentration (MRT_{0-t}), and to infinite time ($MRT_{0-\infty}$); the apparent volume of distribution during terminal phase (V_z/F), and oral clearance (Cl/F) of TAP and FFC in plasma and muscle plus skin.

Thiamphenicol and FFC concentrations versus time in plasma and muscle plus skin in natural proportions were expressed as arithmetic mean $\pm$ standard deviation (SD) and examined for normality (Shapiro-Wilk) and homogeneity of variance (Leven's), and through analysis of variance (One-Way ANOVA) followed by Holm-Sidak, Games-Howell or Dwass-Steel-Critchlow-Fligner post-hoc tests at a significance level of 0.05.

3. Results and discussion

3.1. Analytical method for simultaneous determination of thiamphenicol (TAP) and florfenicol (FFC) in Nile tilapia muscle plus skin samples

The original and modified QuEChERS procedure was evaluated for use in the determination of thiamphenicol (TAP) and florfenicol (FFC) in tilapia muscle plus skin samples, since this approach is often applied in the analysis of organic compounds present in complex matrices (Jung et al., 2022), such as fish muscle. In the original procedure, 10 g of sample and 10 mL of acetonitrile (to which 4.0 g of $MgSO_4$ and 1.0 g of NaCl are added) are used in the sample preparation step (Anastassiades et al., 2003). In this study, these amounts were proportionally reduced to 5 g of sample, 5 mL of acetonitrile (solvent), 2.0 g of $MgSO_4$ and 0.5 g of NaCl.

The reduction in the use of solvents and other reagents also reduces the cost of analysis and contributes to less waste generation and environmental pollution.

In the purification step of the original QuEChERS, 0.150 g of MgSO_4 and 0.025 g of primary-secondary amine (PSA) is used for 1 mL of extract. In the developed method, the amounts of sorbent and drying salt were varied and evaluated, and the results obtained with cleaning only with 0.025 g of PSA showed more adequate analytical responses, with an increase in the areas corresponding to the peaks of the analytes.

The use of C_{18} sorbent was also evaluated, however, there was a decrease in response, which may be a result of analyte interactions with this sorbent. Other tests with different solvents and salts were performed, such as the use of a solution of methanol and acetonitrile (1:1) in the extraction step and the addition of 1.0 g of sodium acetate in the partition step.

Due to the solubility of the analytes in ethyl extract, and the low extraction of interferences from the matrix using this solvent, a simple extraction with 1.0 g of sample and different volumes of ethyl acetate was evaluated. However, the QuEChERS procedure provided results with a higher analytical signal and samples with an efficient cleaning of the extract, without the need to evaporate the solvent for injection in the system used.

The extraction procedure developed is easy to perform, provides an extract suitable for injection into the analytical system used and has adequate extraction efficiency, according to the purpose of the method.

3.1.1. Validation

Linearity

The three matrix-prepared analytical curves were analysed, and responses were acquired as a function of the analyte concentration (Figures S1 and S2). The values obtained for the linear correlation coefficients (or Pearson's coefficient, r) for the three matrix analytical curves met

the established criterion ($r \geq 0.990$), suggesting a linear association between the variables. The line equations and the respective correlation coefficients obtained for each analyte in each of the curves are presented in Table 3.

Table 12. Line equations and correlation coefficients (r) of thiamphenicol (TAP) and florfenicol (FFC) linearity evaluation performed with three different analytical curves prepared in a matrix.

Analyte	Line equation	r
TAP	$y = 241.64x - 310.4$	0.9983
	$y = 256.16x - 1417.7$	0.9984
	$y = 257.33x + 540.49$	0.9985
FFC	$y = 319.75x - 2222$	0.9974
	$y = 297.55x + 312.58$	0.9987
	$y = 337.12x - 346.67$	0.9994

Each point of the three analytical curves was analyzed once ($n = 3$). The values obtained as instrumental responses were used to calculate the mean, standard deviation, coefficient of variation and variance, as shown in Table 4.

Table 13. Mean response, standard deviation, coefficients of variation, and variances of each analyte, for each concentration studied in the analytical curve (n = 3).

Analyte	Concentration ($\mu\text{g kg}^{-1}$)	Response (mean)	Standard deviation	Coefficient of variation (%)	Variance
TAP	20	5,369.98	387.78	7.22	150,377.1
	60	13,192.06	1,106.87	8.39	1,225,161
	100	25,597.87	2,086.37	8.15	4,352,942
	140	34,736.4	1,787.38	5.14	3,194,723
	180	45,058.66	1,680.33	3.73	2,823,503
	220	54,901.22	2,438.87	4.44	5,948,087
FFC	20	5,710.11	91.05	1.59	8,290.00
	60	18,360.78	2,207.12	12.02	4,871,364
	100	30,483.2	2,714.78	8.91	7,370,036
	140	43,490.86	2,419.02	5.56	5,851,674
	180	58,249.53	3,185.05	5.47	10,144,523
	220	68,255.81	4,578.93	6.71	20,966,576

The criteria recommended by the VICH guide GL 49 (VICH, 2015), for the concentrations studied, were met. Regarding the homoscedasticity of the variances, evaluated by the Cochran test, the results indicate that the method can be considered homoscedastic for both analytes. For the TAP, the $C_{\text{calculated}}$ value was 0.3361, while for the FFC the $C_{\text{calculated}}$ value was 0.4260.

The $C_{critical}$ value is tabulated, and in this case, considering six concentration levels ($k = 6$) and three replicates of each concentration ($n = 3$), with a significance level of 5%, the tabulated value is 0.6161. Thus, as the $C_{calculated}$ values are smaller than the $C_{critical}$ value, the variances can be considered statistically not different at a 95% confidence level. This result enables the use of the ordinary least squares method for linear regression, as indicated by the Brazilian Health Regulatory Agency (Anvisa, 2017). In addition to the Cochran test, a visual inspection was also carried out on the residual graphs generated by the regression for each analyte (Figures S3 and S4).

By inspecting the residuals graphs, it is possible to identify that the points are close and randomly distributed on the x-axis, without showing any regular behaviour or trend. This result, added to the result of Cochran's C statistical test, for each analyte, indicates that the data has homoscedastic distribution.

Precision and accuracy

According to the analytical guide VICH GL49 (VICH, 2015), for precision, at concentrations between 10 and 100 $\mu\text{g kg}^{-1}$, the coefficient of variation (CV) must be below 15% for repeatability and below 23% for intermediate precision, while for concentrations above 100 $\mu\text{g kg}^{-1}$, acceptable CV values are 10 and 16%, respectively. For accuracy, in the range of 10 to 100 $\mu\text{g kg}^{-1}$, values of relative error between -30% to $+10\%$ are acceptable, while for concentrations above 100 $\mu\text{g kg}^{-1}$, values between -20% to $+10\%$. Based on these criteria, the developed method met the precision and accuracy criteria for the TAP and FFC analytes at the concentrations studied (Table 5 e 6).

Table 14. Thiamphenicol (TAP) and florfenicol (FFC) concentrations obtained in precision and accuracy tests.

Analyte	Added concentration ($\mu\text{g kg}^{-1}$)	Concentration obtained ($\mu\text{g kg}^{-1}$)			
		Precision – Repeatability			Intermediate precision
		Day 1	Day 2	Day 3	
TAP	20	19.23	21.44	21.95	24.27
					20.50
					22.73
FFC		20.08	22.44	21.60	20.28
					23.01
					23.66
TAP	60	55.74	52.01	54.31	58.80
					54.04
					52.40
FFC		60.43	60.46	63.09	54.61
					53.99
					67.55
TAP	100	112.51	108.99	104.94	99.85
					106.72

					102.76
FFC		97.96	102.54	91.98	89.75
					95.63
					106.01
TAP	140	142.12	139.69	134.93	135.28
					140.50
					143.33
FFC		138.34	144.91	124.20	146.20
					125.47
					149.94
TAP	180	179.07	178.42	178.37	173.34
					180.19
					181.82
FFC		169.53	187.79	163.34	174.92
					165.74
					196.12
TAP	220	216.32	219.46	223.51	228.46
					218.06
					216.96

FFC	212.51	241.90	215.68	198.89
				213.55
				236.40

Table 15. Coefficient of variation (CV) and relative error (%) of precision and accuracy test of the method developed for the TAP and FFC determination in Nile tilapia, *Oreochromis niloticus*, muscle plus skin samples.

Analyte	Concentration ($\mu\text{g kg}^{-1}$)	Accuracy	Precision CV (%)	
		Relative error (%)	Intra-day	Inter-days
TAP	20	8.43	10.94	8.42
FFC		9.22	5.60	8.04
TAP	60	9.08	3.45	6.04
FFC		-0.04	2.49	13.04
TAP	100	5.96	3.48	3.43
FFC		-2.70	5.43	8.48
TAP	140	0.50	2.64	2.92
FFC		1.30	7.79	9.38
TAP	180	0.81	0.22	2.52
FFC		2.09	7.32	8.71
TAP	220	-0.21	1.64	2.87

FFC	0.08	7.22	8.74

Limits of detection and quantification

The concentrations corresponding to the limits of detection (LOD) were estimated based on the parameters obtained with the analytical curves of the analytes in solution, as shown in Table 7.

Table 16. Parameters of the analytical curves in thiamphenicol (TAP) and florfenicol (FFC) solution used to calculate the detection limits estimate.

Analyte	Intercept	Intercept standard deviation (σ)	Slope
TAP	102.26	1,097.34	303.82
	2,047.6		363.1
	1,954.8		337.9
FFC	1368	1,546.47	297.11
	1,253.1		462.08
	4,589.6		505.87

The calculated LOD values were 9.83 ng mL⁻¹ for TAP and 11.00 ng mL⁻¹ for FFC, corresponding to approximately 10 µg kg⁻¹ and 11 µg kg⁻¹, respectively. The limits of quantification (LOQ) were defined as the concentration of the first point of the analytical curve, corresponding to 20 µg kg⁻¹ for both antimicrobials. Considering that these values are below the florfenicol maximum residue level (MRL) in fish muscle and skin in natural proportions

(1,000 $\mu\text{g kg}^{-1}$), established by many regulatory agencies (Anvisa, 2019; EMA, 2000; FAO, 2016), and the epidemiological cut of values of FFC against most non-wild-type fish bacterial pathogens (≥ 4) (CLSI, 2020b), the developed method can be used for monitoring FFC residues in Nile tilapia muscle and skin in natural proportions samples, estimate the pharmacokinetics (PK) and pharmacodynamics (PD) in Nile tilapia reared in different conditions, and evaluate the potential therapeutic effect of the antimicrobial against pathogens that affect the fish skin and muscle, like *Flavobacterium* (de Abreu Reis Ferreira et al., 2022).

Selectivity

The analyzes of the matrices of the six sources evaluated, without the addition of the analytes, did not present a signal (instrumental response) in the transitions monitored by the mass spectrometer during the runs (Figure S5). The characteristic chromatogram of the matrix spiked with the analytes (TAP, FFC and CAP/internal standard) is shown in Figure 1.

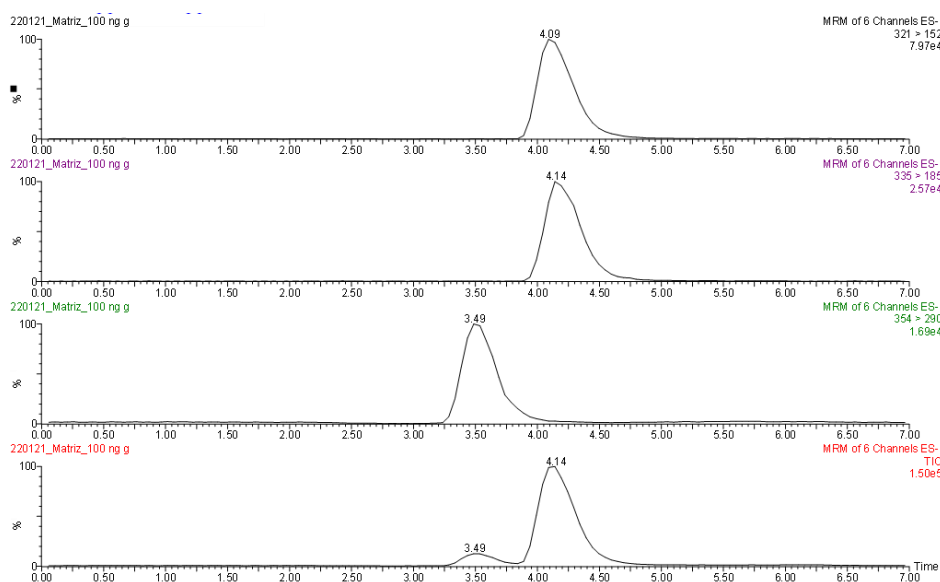


Figure 1. Characteristic chromatogram resulting from the analysis of matrix spiked with thiamphenicol (TAP) and florfenicol (FFC) at 100 ng g^{-1} and chloramphenicol (CAP - internal standard) at 10 ng g^{-1} , in the transitions referring to CAP, FFC, TAP and the sum of the ionic current.

Matrix effect

The graphs in which the analytical curves of TAP and FFC were plotted in solution and extract spiked with analyte as a function of the response (area) are shown in Figures S6 and S7. For the comparison of variances, at each level of fortification, the F test was applied Fisher-Snedecor test, as shown in Table 8.

Table 17. Results observed in Student's t-tests considering equivalent variances.

Analyte	Spike level ($\mu\text{g kg}^{-1}$)	$t_{\text{calculated}}$	$t_{\text{critic bi-caudal}}$
TAP	20	1.99	2.78
	60	0.29	2.78
	100	0.35	2.78
	140	0.99	2.78
	180	0.88	2.78
	220	1.64	2.78
FFC	20	-0.04	2.78
	60	0.16	2.78
	100	-0.18	2.78
	140	-0.31	2.78
	180	-0.16	2.78
	220	0.03	2.78

Because the $t_{\text{calculated}}$ values are smaller than the t_{critical} values, there is evidence to accept that the means are equivalent to each other. Although the tests indicate that the variances and means are equivalent and that it is possible to suggest a non-significant matrix effect, the value of this effect was calculated according to the equation shown above. For TAP, the matrix effect had a value of -16.28 , indicating a suppression of the TAP signal, while for the FFC it was 1.09% , indicating an increase in the analytical signal of the FFC.

Stability

The results obtained from the stability tests suggest that the sample remains viable after 25 days stored at -20°C (Table 9).

The study of processed matrix stability evaluated the stability of the solution obtained after the extraction process, stored in a freezer at -20°C . The solution was analyzed initially, on the day of preparation, and stored until the next analysis, periodically. The analysis results indicated that the processed matrix solution does not undergo significant degradation within 3 days after preparation if stored in a freezer at -20°C . However, regarding the samples analyzed in this study, they were prepared and analyzed on the same day, and it was not necessary to analyze solutions from previously processed matrices.

Table 18. Results of the matrix stability test of samples spiked with thiamphenicol (TAP) and florfenicol (FFC) at 20 and 220 ng g^{-1} and stored in a freezer at -20°C for different periods ($n = 18$).

Period of evaluation	TAP concentration ($\mu\text{g kg}^{-1}$)	Relative error (%)	FFC concentration ($\mu\text{g kg}^{-1}$)	Relative error (%)
1 day	20.34	-1.68	21.78	1.69

	221.64	-1.75	220.12	-0.23
7 days	20.03	+4.62	18.56	16.19
	208.65	+6.47	200.90	8.52
25 days	19.70	+5.79	19.17	14.81
	226.85	-13.74	220.19	-0.26

Robustness

The values obtained for the relative errors are included in the range established for the concentration studied, between -30% and +10%. However, factors such as the amount of MgSO₄ and the centrifuge temperature must be well controlled during the process so that greater variations do not occur that could interfere with the analytical response.

With the evaluations of the validation parameters recommended by the VICH GL49 guide (VICH, 2015) and the fulfilment of its acceptance criteria (Table 10), it can be considered that the method developed is adequate and reliable for quantification of FFC and TAP in Nile tilapia muscle plus skin samples.

Table 19. Results obtained for the average concentration ($\mu\text{g kg}^{-1}$), average recovery factor (%) and relative error (%) for thiamphenicol (TAP) and florfenicol (FFC) in the robustness test of the analytical method.

Factor	TAP			FFC		
	Concentration	Recovery Factor	Relative error	Concentration	Recovery Factor	Relative error
Normal conditions						
	19.59	97.96	−2.04	18.15	90.76	−9.24
HPLC system						
35 °C	20.22	101.09	1.09	18.74	93.69	−6.31
45 °C	20.12	100.59	0.59	19.09	95.45	−4.55
Mobile phase						
52 % acetonitrile	21.15	105.77	5.77	18.96	94.78	−5.22
57 % acetonitrile	20.44	102.22	2.22	18.54	92.70	−7.30
MgSO ₄						

1.8 g	19.38	96.89	−3.11	17.08	85.38	−14.62
2.2 g	17.81	89.04	−10.96	16.76	83.81	−16.19
Centrifuge temperature						
3 °C	18.59	92.93	−7.07	17.68	88.39	−11.61
5 °C	16.14	80.71	−19.29	16.53	82.66	−17.34

3.2. Analytical method for simultaneous determination of TAP and FFC in Nile tilapia plasma

3.2.1. Validation

Selectivity

The analyzes of the blank matrices from six sources did not present a signal (instrumental response) in the transitions monitored by the mass spectrometer during the runs (Figures S8). The characteristic chromatogram of the matrix spiked with the analytes (TAP, FFC and CAP/internal standard) is shown in Figure 2.

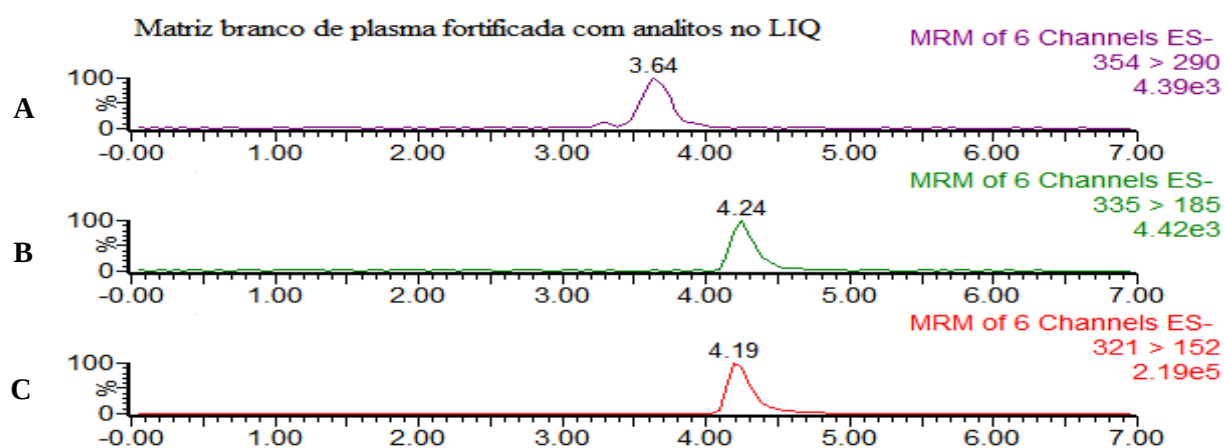


Figure 2. Chromatograms resulting from the analysis of blank plasma matrix spiked with thiamphenicol (TAP) and florfenicol (FFC) at 100 ng g⁻¹ (A, B and C) and chloramphenicol (CAP - internal standard) at 2000 ng g⁻¹.

Residual Effect

As shown in Figure 3, there was no signal (instrumental response) in the transitions monitored by the mass spectrometer during the runs before and after the injection of the plasma sample spiked with the highest concentration level of the calibration curve (Figure 3).

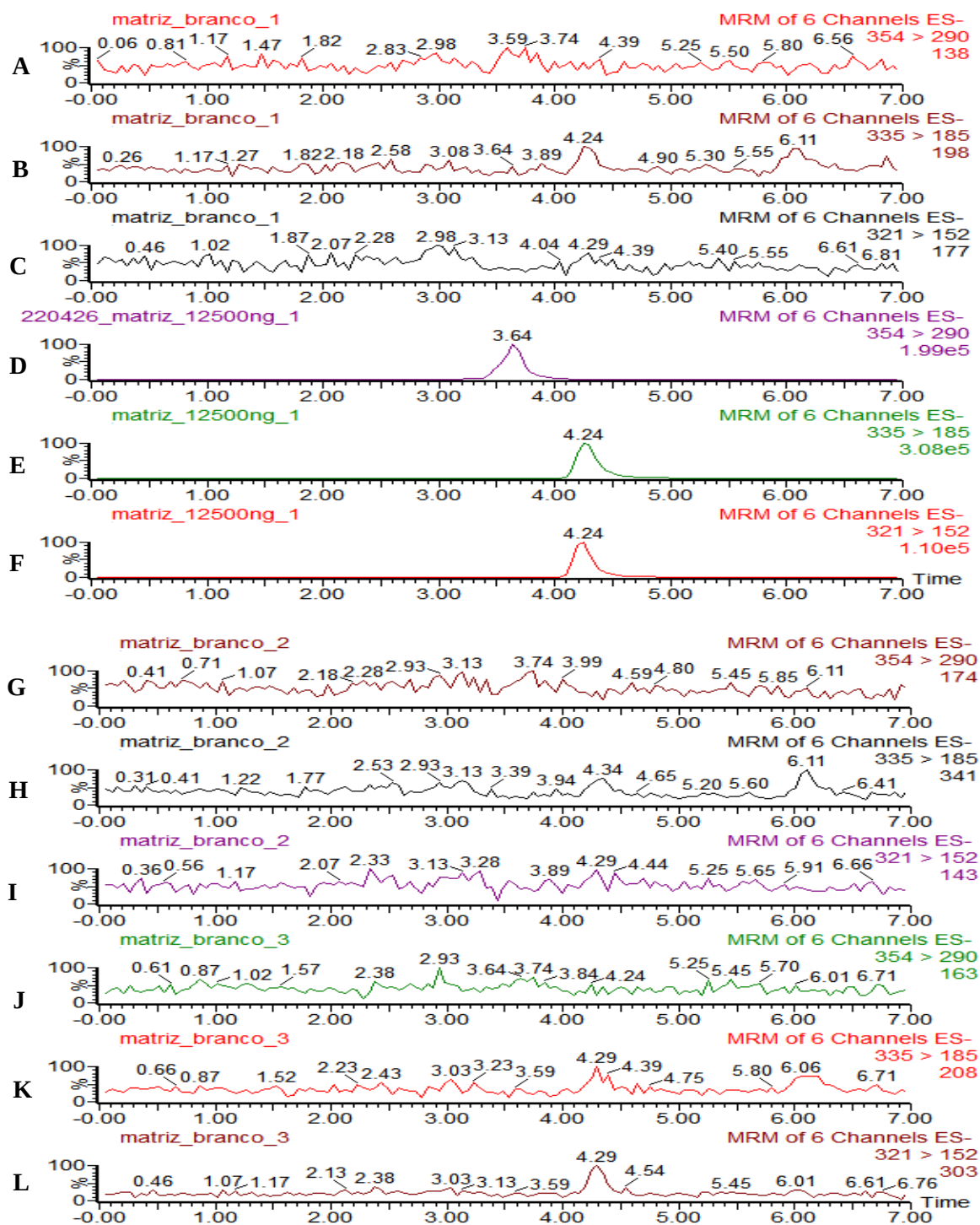


Figure 3. Chromatograms resulting from the analysis of blank plasma matrix before (A-C) and after (G-L) injection of a plasma sample spiked with the highest concentration level of the calibration curve of thiamphenicol (TAP, D) and florfenicol (FFC, E) at 12,500 ng g⁻¹ and chloramphenicol (CAP - internal standard, F) at 2000 ng g⁻¹.

Matrix Effect

As shown in Tabel 11, the results of the matrix effect assessment indicate that a non-significant matrix effect was observed using the developed method.

Table 20. Standard deviation (SD) and coefficient of variation (CV) of the internal standard - normalized matrix factor (IS-normalized MF) of eight samples of Nile tilapia plasma from different sources (four normal, two lipemic and two hemolyzed).

Analyte	Concentration (ng g ⁻¹)	Average IS-normalized MF	SD	CV
TAP	100	1.1424	0.0970	8.49
	420	1.1458	0.0162	1.41
	2,100	0.8724	0.0466	5.34
	10,500	1.0261	0.0492	4.79
FFC	100	0.9194	0.1186	12.90
	420	1.0334	0.0151	1.46
	2,100	1.0050	0.0421	4.18
	10,500	0.8104	0.0749	9.23

Linearity

The three matrix-prepared analytical curves were analysed, and responses were acquired as a function of the analyte concentration (Figures S9). The values obtained for the linear correlation coefficients (or Pearson's coefficient, r) for the three matrix analytical curves met the established criterion ($r \geq 0.990$), suggesting a linear association between the variables. The line equations and the respective correlation coefficients obtained for each analyte in each of the curves are presented in Table 12.

Table 21. Line equations and correlation coefficients (r) of thiamphenicol (TAP) and florfenicol (FFC) linearity evaluation performed with three different analytical curves prepared in a matrix.

Analyte	Line equation	r
TAP	$y = 0.4245x + 17.977$	0.9965
	$y = 0.466x + 10.45$	0.9981
	$y = 0.4652x + 8.3092$	0.9983
FFC	$y = 0.5239x - 19.598$	0.9937
	$y = 0.5287x - 12.591$	0.9956
	$y = 0.4864x - 14.48$	0.9946
TAP	$y = 0.3067x + 29.480$	0.9979
	$y = 0.3063x + 27.450$	0.9920
	$y = 0.3339x + 7.1051$	0.9990
FFC	$y = 0.5685x + 19.064$	0.9998
	$y = 0.4558x + 146.57$	0.9964
	$y = 0.5988x - 37.573$	0.9955
TAP	$y = 0.2488x + 75.209$	0.9960
	$y = 0.2683x - 35.972$	0.9982
	$y = 0.2395x + 167.26$	0.9960
FFC	$y = 0.4126x + 356.45$	0.9989

$y = 0.4752x + 145.88$	0.9973
$y = 0.4295x + 168.67$	0.9968

Regarding the homoscedasticity of the variances, evaluated by the Cochran test, the results indicate that the method can be considered homoscedastic for both analytes. The $C_{critical}$ value is tabulated, and in this case, considering six concentration levels ($k = 6$) and three replicates of each concentration ($n = 3$), with a significance level of 5%, the tabulated value is 0.6161. Thus, as the $C_{calculated}$ values are smaller than the $C_{critical}$ value, the variances can be considered statistically not different at a 95% confidence level (Table 13). This result enables the use of the ordinary least squares method for linear regression, as indicated by the Brazilian Health Regulatory Agency (Anvisa, 2017). In addition to the Cochran test, a visual inspection was also carried out on the residual graphs generated by the regression for each analyte (Figures S10).

Table 22. Results of the evaluation of the homoscedasticity of variances by the Cochran method.

Concentration level	Analyte	$C_{calculated}$	C_{critic}
1	TAP	0.3561	0.6161
	FFC	0.5385	0.6161
2	TAP	0.3237	0.6161
	FFC	0.2715	0.6161

3	TAP	0.3218	0.6161
	FFC	0.5088	0.6161

By inspecting the residuals graphs, it is possible to identify that the points are close and randomly distributed on the x-axis, without showing any regular behaviour or trend. This result, added to the result of Cochran's C statistical test, for each analyte, indicates that the data has homoscedastic distribution.

Precision and Accuracy

According to the Brazilian Health Regulatory Agency (Anvisa, 2012), the standard deviation of the precision and accuracy must be below 15% for each concentration level, except for the LOQ level which must be below 20%. Based on these criteria, the developed method can be considered precise and accurate for TAP and FFC quantification at the concentrations studied (Table 14).

Table 23. Thiamphenicol (TAP) and florfenicol (FFC) concentrations obtained in precision and accuracy tests.

Analyte	Concentration level	Nominal concentration (ng g ⁻¹)	Curve			Average concentration (ng g ⁻¹)	Precision	Accuracy
			1	2	3			
TAP	1	Intraday						
		100	97.70918	95.05334	97.5891	96.7838	1.55	-3.22
		180	176.1149	179.8033	172.5482	176.1554	2.06	-2.14
		260	275.8691	275.6797	276.3897	275.9795	0.13	6.15
		340	318.5749	330.9884	333.9553	327.8395	2.49	-3.58
		420	441.9455	423.6781	424.57273	430.0654	2.39	2.40
		500	489.6171	497.6977	494.8233	494.0460	0.83	-1.19
		Interday						
		100	101.5367	97.58911	99.2727	99.4662	1.99	-0.53
		180	174.4912	172.5483	170.6993	172.5796	1.10	-4.12
		260	254.3364	276.3898	260.6609	263.7957	4.30	1.46
		340	329.3237	333.9554	363.9326	342.4038	5.49	0.71
		420	422.1102	424.5727	411.2227	419.3018	1.69	-0.17

	500	501.2737	494.8233	494.3435	496.8135	0.78	−0.64
2	Intraday						
	500	499.6779	512.7321	498.0738	503.4947	1.60	0.70
	900	942.9515	873.8924	878.8152	898.5531	4.29	−0.16
	1,300	1,237.3933	1,328.067	1,350.328	1,305.263	4.58	0.40
	1,700	1741.8231	1,661.6406	1,659.799	1,687.754	2.77	−0.72
	2,100	2,033.6422	2,132.1744	2,122.13	2,095.982	2.59	−0.19
	2,500	2,544.3171	2,490.8476	2,489.891	2,508.352	1.24	0.33
	Interday						
	500	500.29420	430.3844	481.0998	470.5928	7.68	−5.88
	900	907.3836	883.2373	1,031.508	940.7097	8.46	4.52
	1,300	1,321.1046	1,469.5547	1,218.656	1,336.438	9.44	2.80
	1,700	1,695.1299	1,689.5363	1,574.23	1,652.966	4.13	−2.77
	2,100	2,016.3992	2,028.8563	2,163.041	2,069.432	3.93	−1.46
	2,500	2,560.2525	2,499.2790	2,530.885	2,530.139	1.21	1.21
3	Intraday						

		2,500	2,498.1235	2,642.5287	2,802.5873	2,647.747	5.75	5.91
		4,500	4,391.4770	4,450.6157	4,408.3453	4,416.813	0.69	−1.85
		6,500	6,844.3911	6,274.2719	5,934.2213	6,350.961	7.24	−2.29
		8,500	8,909.5677	8,781.9638	8,599.5952	8,763.709	1.78	3.10
		10,500	9,088.8797	10,081.4062	10,836.4907	10,002.26	8.76	−4.74
		12,500	13,266.1998	12,759.4783	12,409.8837	12,811.85	3.36	2.49
		Interday						
		2,500	2,972.2686	2,702.4302	2,726.2148	2,800.305	5.34	12.01
		4,500	4,161.8089	4,413.8132	4,559.1777	4,378.267	4.59	−2.71
		6,500	6,219.7583	6,116.3903	5,955.2154	6,097.121	2.19	−6.20
		8,500	8,271.6797	8,718.1109	8,639.5646	8,543.118	2.79	0.51
		10,500	10,787.8282	10,533.1132	10,475.3711	10,598.77	1.57	0.94
		12,500	12,586.0973	12,507.9596	12,637.4467	12,577.17	0.52	0.62
FFC	1	Intraday						
		100	98.4736	110.4147	116.1344	108.3409	8.32	8.34
		180	166.8632	179.8571	174.5788	173.7664	3.76	−3.46

2	260	275.2537	252.4715	253.6274	260.4509	4.93	0.17
	340	343.6307	336.7862	329.4036	336.6069	2.11	−1.00
	420	427.3228	397.6810	401.3780	408.794	3.95	−2.67
	500	488.5523	522.8890	524.9096	512.117	3.99	2.42
	Interday						
	100	113.7299	110.4147	116.8316	113.6588	2.82	13.66
	180	174.6737	179.8571	178.7193	177.7501	1.53	−1.25
	260	253.7056	252.4715	240.1729	248.7834	3.01	−4.31
	340	328.0381	336.7862	329.7306	331.5184	1.40	−2.49
	420	415.4667	397.6810	421.1591	411.4357	2.98	−2.04
	500	514.3865	522.8890	513.4867	516.9208	1.00	3.38
	Intraday						
	500	544.3687	505.3640	475.3201	508.351	6.81	1.67
	900	883.2650	890.3779	895.9406	889.8612	0.71	−1.13
	1,300	1,223.8614	1,302.2549	1,303.161	1,276.426	3.57	−1.81
	1,700	1,753.4185	1,721.4854	1,741.58	1,738.828	0.93	2.28

3	2,100	2,065.9981	2,062.5079	2,147.591	2,092.032	2.30	−0.38
	2,500	2,528.6935	2,518.3454	2,436.754	2,494.598	2.02	−0.22
	Interday						
	500	561.4593	495.1929	540.8012	532.4845	6.37	6.50
	900	1,025.4207	973.2948	903.5634	967.4263	6.32	7.49
	1,300	1,465.4363	1,281.6711	1,275.672	1,340.926	8.04	3.15
	1,700	1,959.7870	1,600.1680	1,580.35	1,713.435	12.46	0.79
	2,100	2,391.9549	2,085.8410	2,192.795	2,223.53	6.99	5.88
	2,500	2,825.0624	2,564.0179	2,506.102	2,631.728	6.46	5.27
	Intraday						
	2,500	2,812.6995	2,454.7773	2,448.641	2,572.039	8.10	2.88
	4,500	4,416.2472	4,844.4968	4,867.671	4,709.472	5.40	4.65
	6,500	6,114.5797	5,807.3439	5,591.734	5,837.886	4.50	−10.19
	8,500	9,014.8026	9,560.6499	9,667.247	9,414.233	3.72	10.76
	10,500	9,397.5634	9,313.3479	9,680.509	9,463.807	2.03	−9.87
	12,500	13,243.7373	13,024.3488	12,746.58	13,004.89	1.92	4.04

Interday							
2,500	2,400.6031	2,460.7941	2,989.451	2,616.95	12.38	4.68	
4,500	4,053.578047	4,802.0405	4,660.474	4,505.364	8.83	0.12	
6,500	5,957.882669	6,235.9117	5,739.895	5,977.896	4.16	−8.03	
8,500	7,667.750632	8,563.6198	8,034.507	8,088.626	5.57	−4.84	
10,500	9,934.049804	10,145.7768	10,628.3	10,236.04	3.48	−2.51	
12,500	11,421.25749	12,787.7394	12,950.93	12,386.64	6.78	−0.91	

Stability

The results obtained from the stability tests suggest that the sample remains viable after 14 days stored at -20°C (Table 15). However, regarding the samples analyzed in this study, they were prepared and analyzed on the same day, and it was not necessary to analyze solutions from previously processed matrices.

Table 24. Results of the matrix stability test of samples spiked with thiamphenicol (TAP) and florfenicol (FFC) at 100 and 10,500 ng g^{-1} and stored in a freezer at -20°C for different periods ($n = 24$).

Period of evaluation	TAP (ng g^{-1})	Relative error (%)	FFC (ng g^{-1})	Relative error (%)
1 hour	96.5	-3.5	98.09	-1.90
	10,710.92	2.0	10,318.40	-1.72
1 day	93.91	-6.08	98.47	-1.52
	10,652.59	1.45	10,611.86	1.06
7 days	103.5	3.5	106.86	6.86
	10,477.63	-0.22	10,455.16	-0.42
14 days	103,.8	3.8	104.63	4.63
	10,263.13	-2.25	10,170.66	-3.13

3.4. Pharmacokinetic analysis

Fish remained in good general health during the entire experimental period. The developed and validated analytical method for simultaneous detection and quantification of TAP and FFC in tilapia plasma and muscle samples was successfully applied to analyze the plasma and muscle samples in the pharmacokinetic (PK) study. A total of 200 samples of healthy juvenile Nile tilapia plasma (100 samples) and muscle plus skin in natural proportions (100 samples) were analyzed in order to determine the PK of TAP and FFC in freshwater tilapia at $30 \pm 0.32^{\circ}\text{C}$ after a single oral dose of 5 mg kg^{-1} TAP + 3.75 mg kg^{-1} FFC. The mean TAP and FFC concentrations versus time in plasma and muscle plus skin in natural proportions, and a summary of the PK parameters estimated are shown in Tables 16 and 17, and Figures 4 and 5.

The highest concentration of TAP and FFC was detected at 0.5 h (6.34 and $3.34 \mu\text{g mL}^{-1}$) and 16h (8.20 and $11.37 \mu\text{g mL}^{-1}$) post administration in plasma and muscle, respectively (Table 16). Mean $\pm$ SD plasma concentration of TAP was only higher than that from muscle plus skin at 1 h after antimicrobial administration ($P < 0.05$). On the other hand, the mean $\pm$ SD muscle plus skin concentration at 24 h after administration was higher than that of plasma ($P < 0.05$). In some sampling time points (6, 16 and 24 h), the mean $\pm$ SD concentration of FFC in muscle plus skin was higher than that in plasma ($P < 0.05$).

There were no secondary peaks in plasma antimicrobial concentrations (Figure 4). However, consistent secondary peaks of TAP and FFC concentrations in muscle were detected between 4 and 12 h (Figure 5). This multiple peaking phenomenon in TAP and FFC PK was also reported by Castells et al. (2000), Feng and Jia (2009), Sun et al. (2010), and Feng et al. (2016) in different tissues, including plasma, after administration of a single oral dose of TAP or FFC in the sea-bass (*Dicentrarchus labrax*), tilapia (*Oreochromis niloticus* $\times$ *O. aureus*), crucian carp (*Carassius auratus cuvieri*), and

orange-spotted grouper (*Epinephelus coioides*), respectively. Although this phenomenon is often multifactorial and includes (but is not limited to) solubility-limited absorption, complexation, gastric emptying and variability in the intestinal transit time, site-specific absorption, gastric secretion-enteral reabsorption, and alterations from anaesthesia (Davies et al., 2010), the higher distribution and slower elimination of FFC in fish bile, reported by Feng et al. (2016), suggest that the biliary secretion followed by intestinal reabsorption (enterohepatic recycling) and site-specific absorption are the main reasons for these secondary peaks observed in our study.

The calculated PK parameters (Table 17) and the curve obtained for both antimicrobials emphasizes their fast absorption and distribution. These data are similar to those reported by other authors who detected higher concentrations of TAP and FFC in plasma and muscle, of different fish species reared at similar temperatures ($\sim 28\text{ }^{\circ}\text{C}$), between 3 and 12 h post-administration of a single oral dose of these antimicrobials (Castells et al., 2000; Feng et al., 2008; Sun et al., 2010).

There was no significant difference between the peak concentration ($C_{\max}$) and apparent volume of distribution during the terminal phase (V_z/F) of each antimicrobial in different tissues. However, the peak time ($T_{\max}$), the area under the concentration-time curve (AUC), and mean residence time (MRT) were higher in the muscle, for both antimicrobials ($P < 0.05$), suggesting delayed antibacterial activity and higher antimicrobial exposure in muscle plus skin.

Although the influence of water temperature and salinity on TAP and FFC excretion and the presence of their metabolites were not investigated in the present study, the rapid total body clearance and short half-life of both antimicrobials suggest that its withdrawal period after combination therapy in Nile tilapia reared in freshwater at $\sim 30\text{ }^{\circ}\text{C}$ might be similar to those previously described for each antimicrobial used individually, and shorter

than for other antimicrobials currently used in aquaculture. However, for designing an effective dose regimen for TAP and FFC combination in freshwater tilapia, further studies of their pharmacodynamics and antimicrobial susceptibility must be performed. These pieces of information could provide benefits to the tilapia aquaculture industry.

Table 25. Thiamphenicol (TAP) and florfenicol (FFC) concentration (arithmetic mean $\pm$ standard deviation) in freshwater tilapia (at $30 \pm 0.32^\circ\text{C}$) plasma ($\mu\text{g mL}^{-1}$) and muscle plus skin in natural proportions ($\mu\text{g g}^{-1}$) at different time points post a single oral dose of 5 mg kg^{-1} TAP + 3.75 mg kg^{-1} FFC.

Time (h)	Plasma		Muscle plus skin	
	TAP	FFC	TAP	FFC
0	<LOQ*	<LOQ*	<LOQ*	<LOQ*
0.5	6.34 ± 4.19^a	3.34 ± 2.51^b	1.50 ± 0.15^a	2.33 ± 0.19^b
1	4.27 ± 2.47^a	2.64 ± 1.78^c	0.63 ± 0.17^b	0.75 ± 0.25^c
2	3.18 ± 2.27^a	2.16 ± 1.57^b	1.63 ± 0.30^a	1.50 ± 0.18^b
4	2.48 ± 2.13^a	1.34 ± 0.76^b	1.86 ± 0.44^a	1.61 ± 0.35^b
6	1.84 ± 1.62^a	1.11 ± 0.86^b	2.68 ± 1.71^a	3.59 ± 2.39^c
8	1.50 ± 1.18^a	0.89 ± 0.77^b	3.90 ± 4.00^a	3.36 ± 3.33^b
12	1.11 ± 0.72^a	0.65 ± 0.51^b	3.91 ± 4.17^a	3.57 ± 3.96^b
16	0.69 ± 0.33^a	0.29 ± 0.10^b	8.20 ± 11.08^a	11.37 ± 11.23^c
24	0.55 ± 0.21^a	0.09 ± 0.11^c	1.66 ± 0.44^b	0.86 ± 0.41^d
48	<LOQ*	<LOQ*	0.11 ± 0.00	0.04 ± 0.02
96	<LOQ*	<LOQ*	0.15 ± 0.11	0.12 ± 0.10
168	<LOQ*	<LOQ*	0.09 ± 0.04	0.10 ± 0.04
216	<LOQ*	<LOQ*	<LOQ*	<LOQ*

264	<LOQ*	<LOQ*	<LOQ*	<LOQ*

*LOQ = limit of quantification.

Different superscripts signal significant differences in a line for each antimicrobial (TAP in plasma *versus* TAP in muscle plus skin, and FFC in plasma *versus* FFC in muscle plus skin): ^{a,b;c,d} $P < 0.05$.

Table 26. Pharmacokinetic parameters estimated for thiamphenicol (TAP) and florfenicol (FFC) in plasma and muscle plus skin (in natural proportions) of freshwater tilapia (at $30 \pm 0.32^\circ\text{C}$) following a single oral administration of 5 mg kg^{-1} TAP + 3.75 mg kg^{-1} FFC.

Parameter	Plasma		Muscle plus skin	
	TAP	FFC	TAP	FFC
$t_{1/2}$ (h)	10.43 ± 5.94^a	4.25 ± 2.14^b	45.17 ± 45.22^a	31.36 ± 16.31^c
$T_{\max}$ (h)	0.5 ± 0.22^a	0.4 ± 0.2^c	14.67 ± 7.46^b	8.07 ± 6.74^d
$C_{\max}$ ($\mu\text{g mL}^{-1}$)	6.34 ± 4.61^a	2.67 ± 2.36^b	12.55 ± 8.87^a	8.89 ± 8.83^b
AUC_{0-t} ($\mu\text{g mL}^{-1} \text{ h}$)	34.73 ± 25.65^a	14.73 ± 12.85^c	128.35 ± 57.63^b	89.02 ± 62.49^d
$\text{AUC}_{0-\infty}$ ($\mu\text{g mL}^{-1} \text{ h}$)	42.35 ± 28.03^a	15.97 ± 13.22^c	134.25 ± 58.27^b	93.90 ± 63.17^d
MRT_{0-t} (h)	7.91 ± 3.57^a	4.81 ± 2.50^c	31.88 ± 15.09^b	28.27 ± 17.27^d
$\text{MRT}_{0-\infty}$ (h)	14.78 ± 7.78^a	6.46 ± 3.25^c	44.77 ± 22.81^b	38.43 ± 24.54^d
V_z/F ((mg) ($\mu\text{g mL}^{-1}$) $^{-1}$)	2.32 ± 1.64^a	150.48 ± 102.05^b	3.22 ± 3.79^a	194.59 ± 140.6^b
Cl/F ((mg) ($\mu\text{g mL}^{-1}$) $^{-1} \text{ h}^{-1}$)	0.14 ± 0.09^a	19.67 ± 13.58^b	0.04 ± 0.02^a	3.51 ± 2.23^c

Different superscripts signal significant differences in a line for each antimicrobial (TAP in plasma *versus* TAP in muscle plus skin, and FFC in plasma *versus* FFC in muscle plus skin): $^{a,b,c,d}P < 0.05$.

$t_{1/2}$ = half-life, $T_{\max}$ = peak time, $C_{\max}$ = peak concentration, AUC_{0-t} = area under the plasma and muscle concentration-time curve up to the last measurable concentration, $AUC_{0-\infty}$ = area under the plasma and muscle concentration-time curve up to infinite time, MRT_{0-t} = mean residence time up to the last measurable concentration, $MRT_{0-\infty}$ = mean residence time up to infinite time, V_z/F = apparent volume of distribution during the terminal phase, Cl/F = oral clearance.

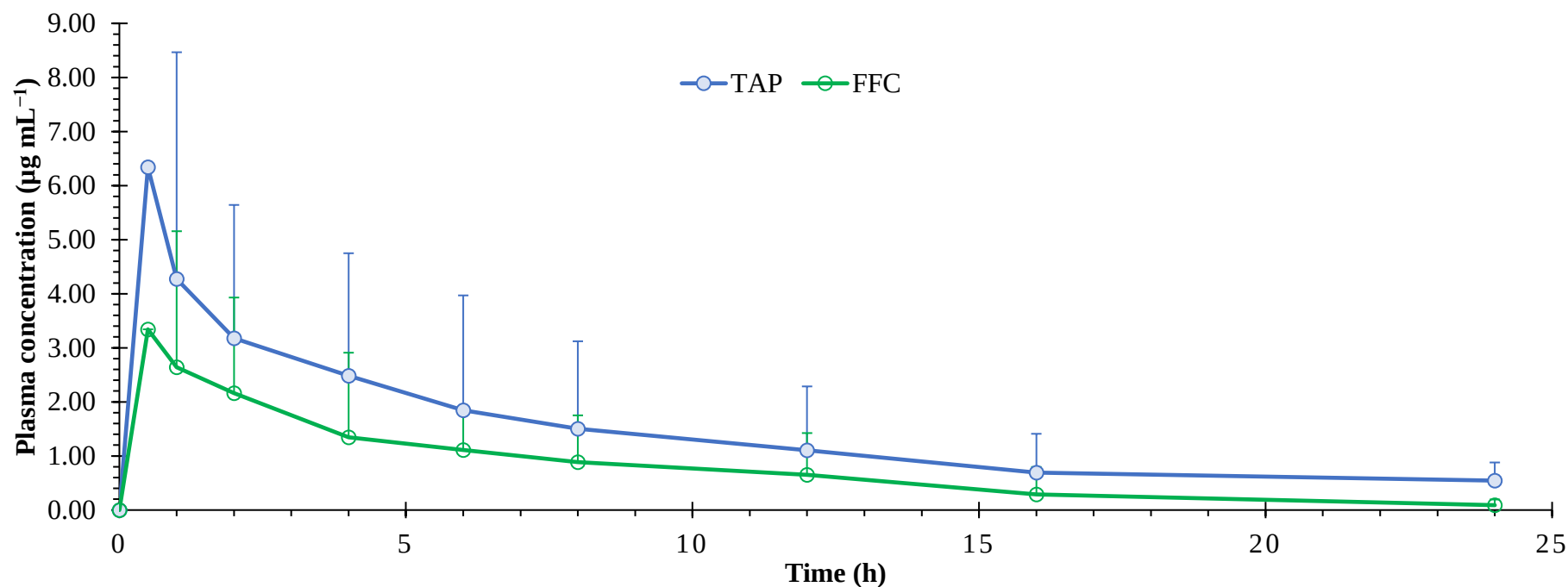


Figure 4. Mean antimicrobial concentration (+SD) in plasma of freshwater tilapia (at $30 \pm 0.32^\circ\text{C}$) versus time following a single oral administration

of 5 mg kg^{-1} TAP + 3.75 mg kg^{-1} FFC. Note that the antimicrobial concentration declined below the limit of quantification at 48 h post-administration.

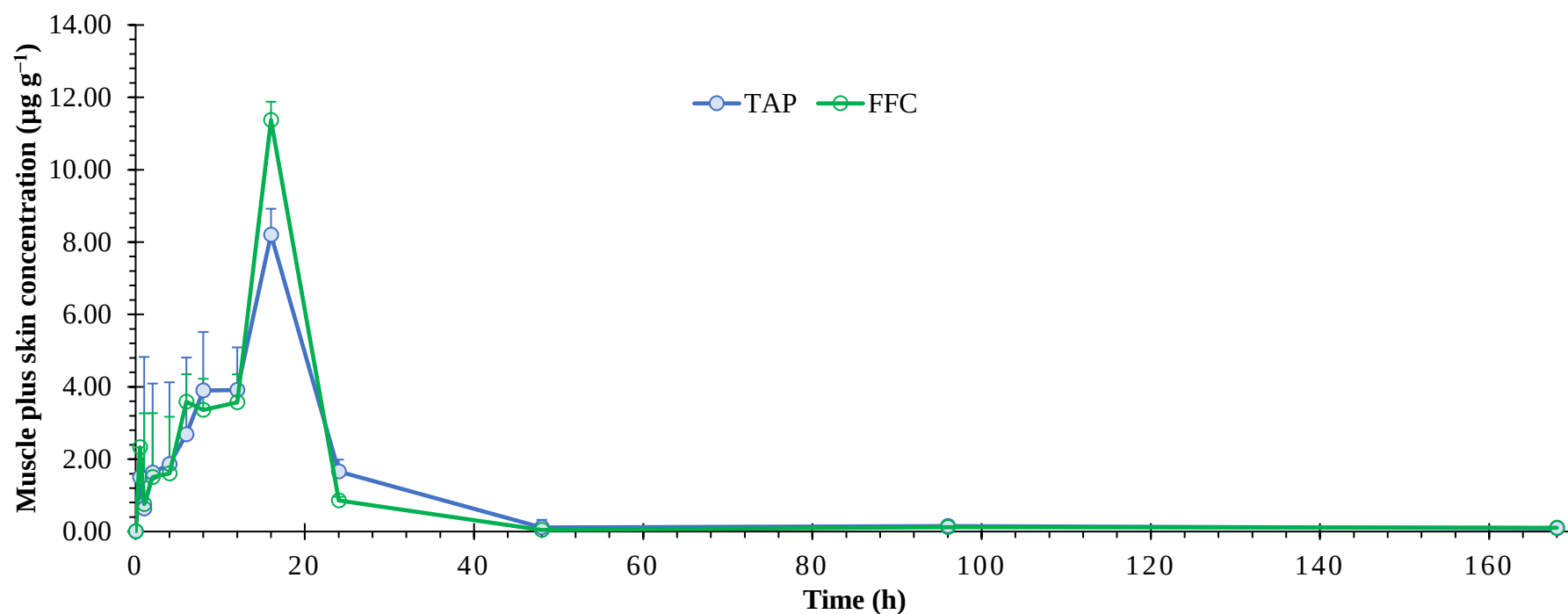


Figure 5. Mean antimicrobial concentration (+SD) in muscle plus skin (in natural proportions) of freshwater tilapia (at $30 \pm 0.32^\circ\text{C}$) *versus* time following a single oral administration of 5 mg kg^{-1} TAP + 3.75 mg kg^{-1} FFC. Note that the antimicrobial concentration declined below the limit of quantification at 216 h post-administration.

4. Conclusions

To our knowledge, this is the first study to develop and validate an analytical method for simultaneous detection and quantification of TAP and FFC in fish plasma and muscle plus skin (in natural proportions) samples and examine the PK characteristics of TAP and FFC after combination therapy. Observed results indicate that the developed and validated method is rapid, simple, precise, and sensitive for the simultaneous determination of TAP and FFC in plasma and muscle plus skin samples. TAP and FFC had fast absorption and distribution in freshwater Nile tilapia reared at $30 \pm 0.32^{\circ}\text{C}$. The results from this study could help establish antimicrobial susceptibility testing interpretative categories and design rational dosing regimens for TAP and FFC combination.

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7. Supplementary Figures

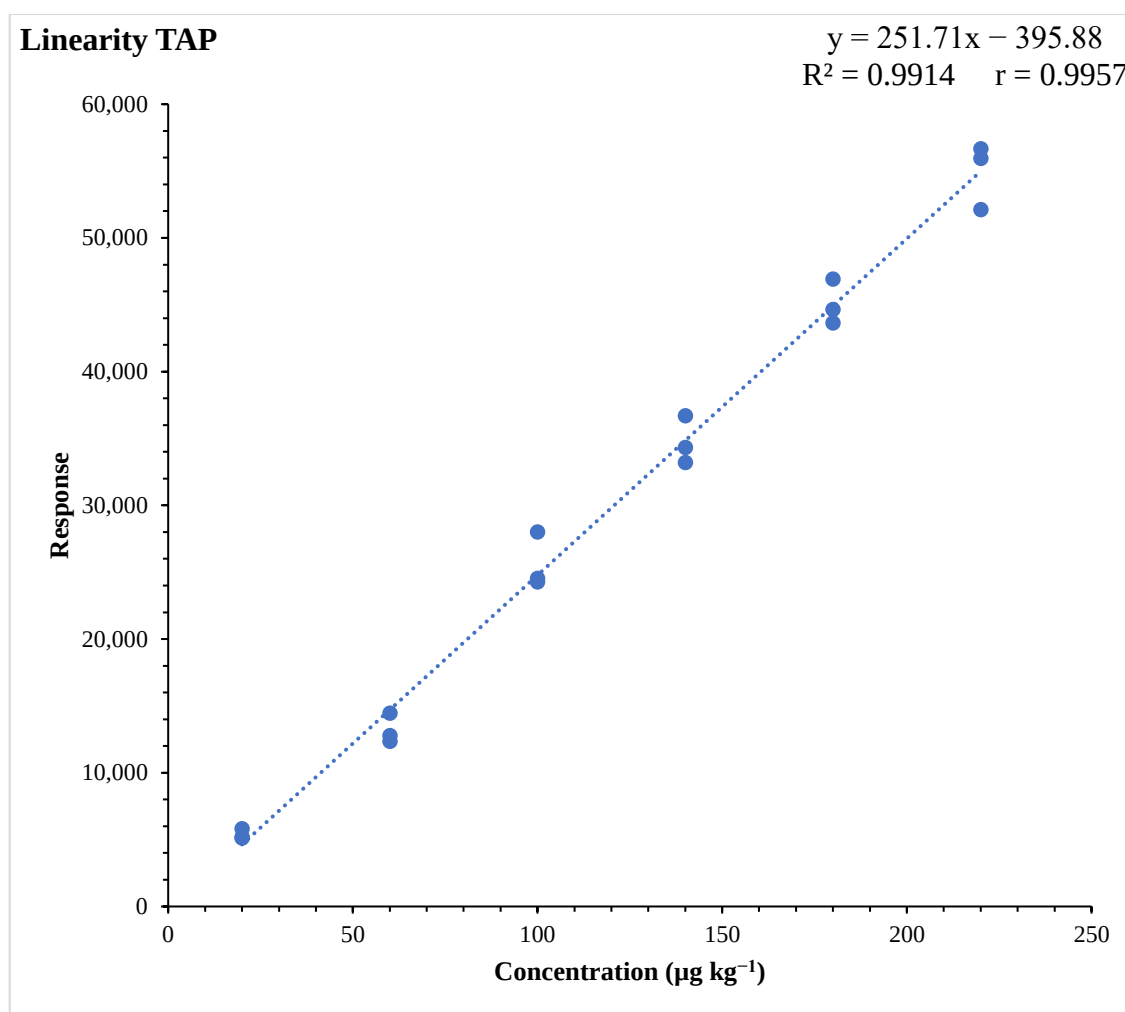


Figure S1. Linear equation (y) of the relation between responses and thiamphenicol (TAP) concentration for three analytical curves prepared using a matrix spiked with six concentrations (20, 60, 100, 140, 180 e 220 $\mu\text{g kg}^{-1}$) of TAP. R^2 , determination coefficient; r , Pearson's correlation coefficient.

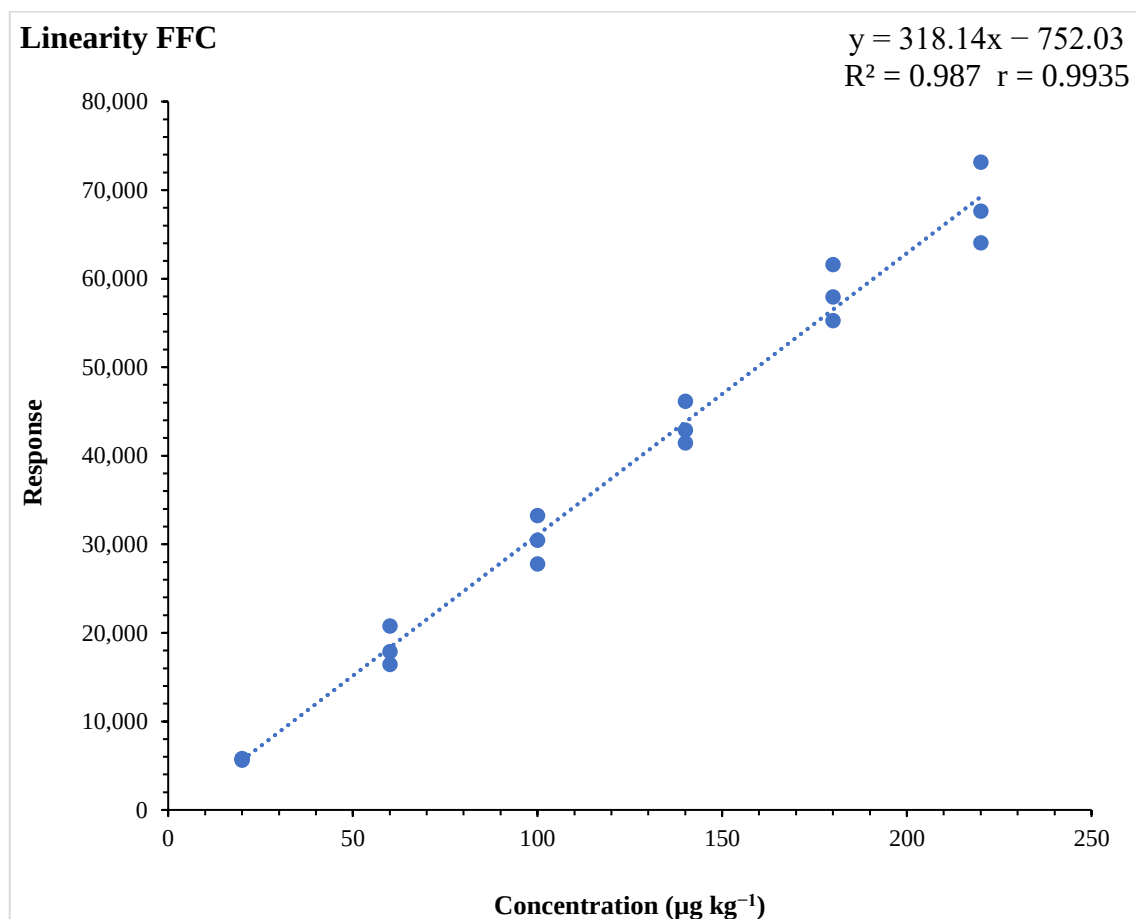


Figure S2. Linear equation (y) of the relation between responses and florfenicol (FFC) concentration for three analytical curves prepared in a matrix spiked with six concentrations (20, 60, 100, 140, 180 e 220 µg kg⁻¹) of FFC. R^2 , determination coefficient; r, Pearson's correlation coefficient.

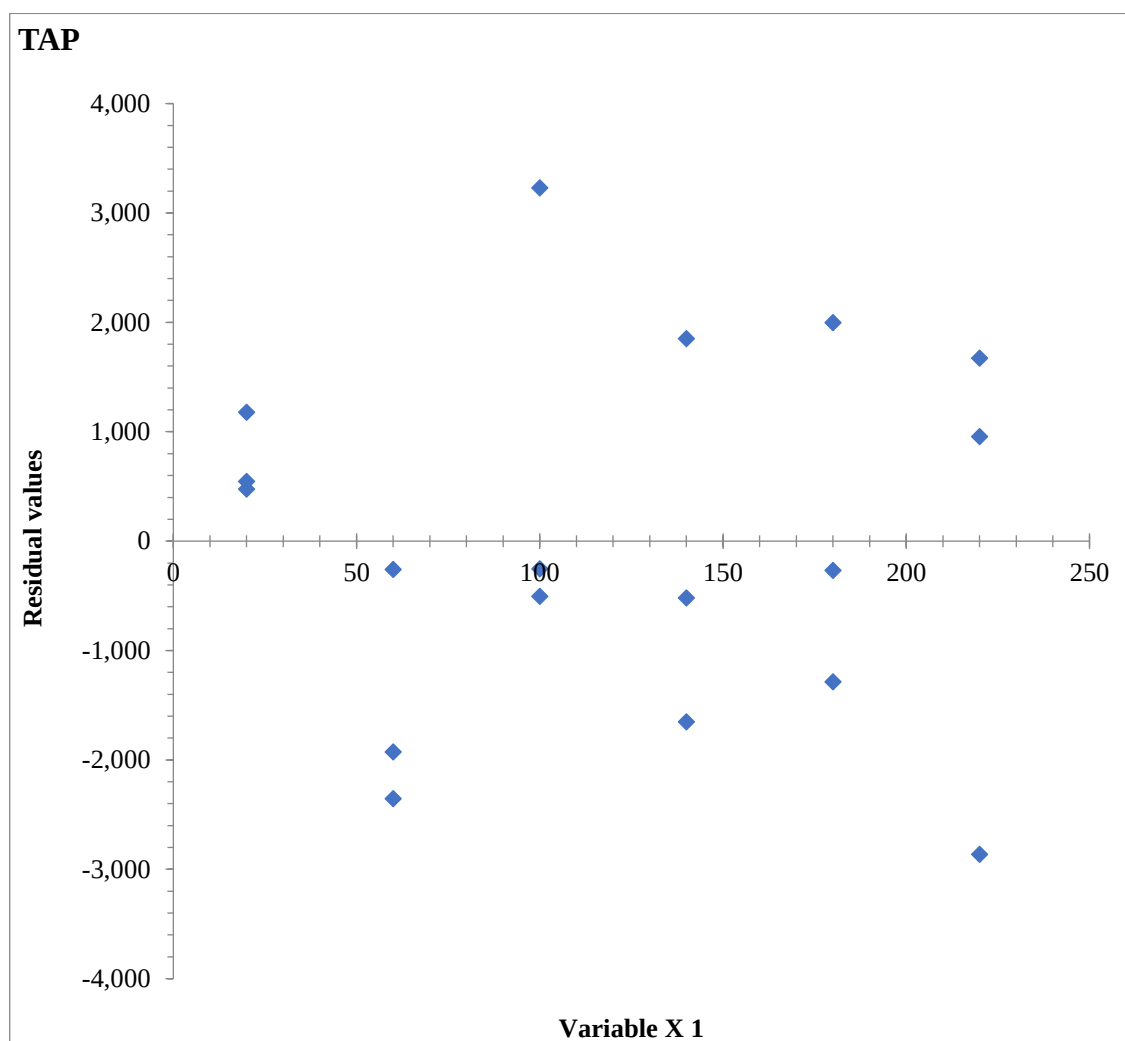


Figure S3. Residual distribution graph resulting from regression analysis with the results obtained from three analytical curves of matrix spiked with six concentrations (20, 60, 100, 140, 180 e 220 $\mu\text{g kg}^{-1}$) of thiamphenicol (TAP).

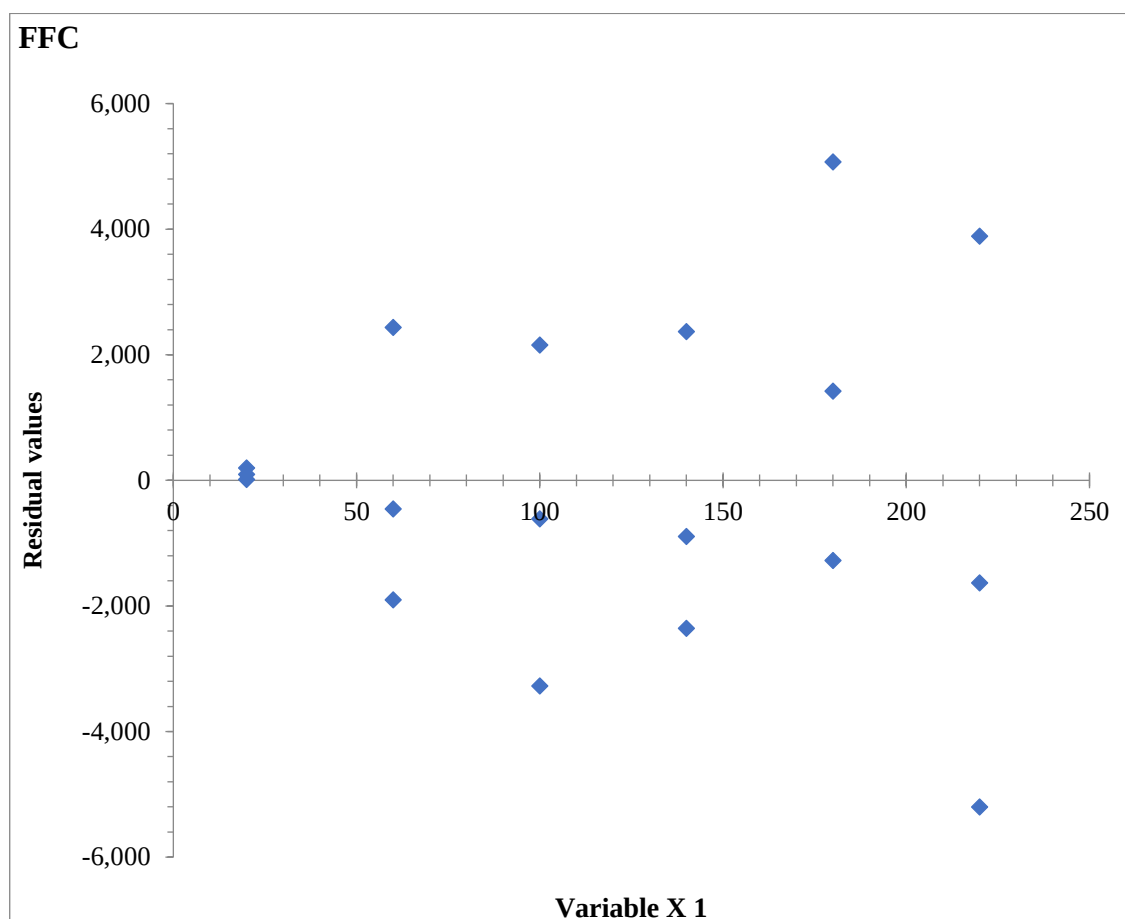


Figure S4. Residual distribution graph resulting from regression analysis with the results obtained from three analytical curves of matrix spiked with six concentrations (20, 60, 100, 140, 180 e 220 $\mu\text{g kg}^{-1}$) of florfenicol (FFC).

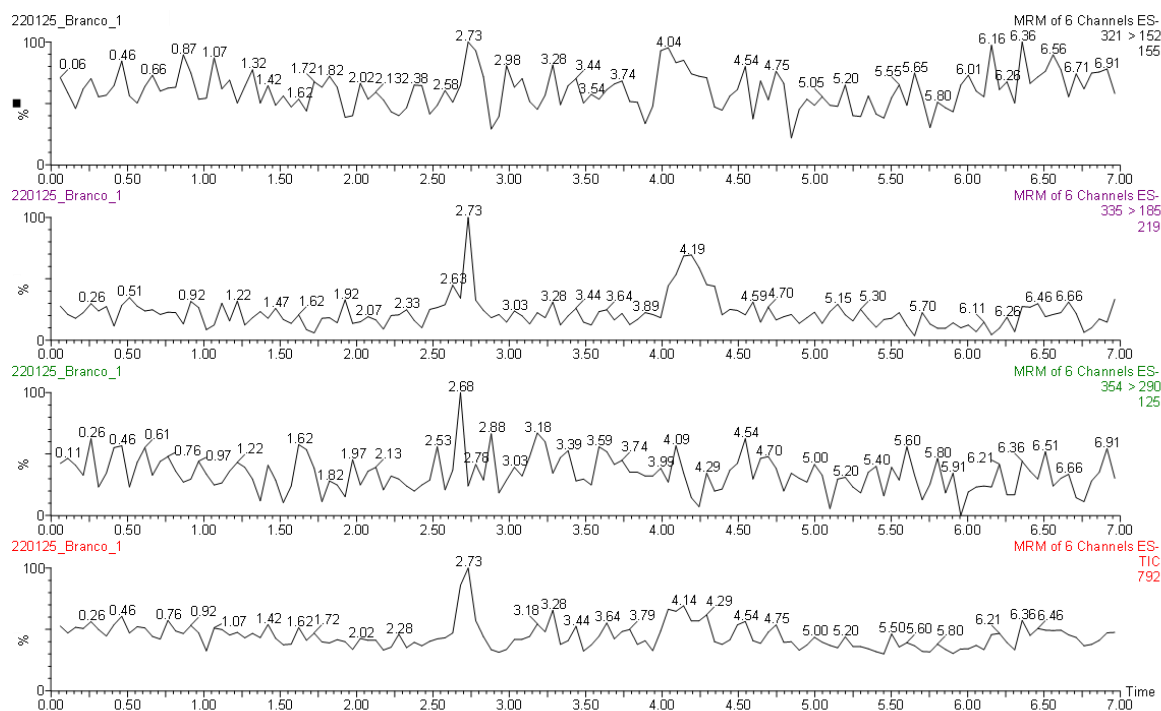


Figure S5. Chromatograms resulting from the analysis of the blank matrix, in the transitions referring to chloramphenicol (CAP), florfenicol (FFC) and thiamphenicol (TAP) and the total sum of the ionic current.

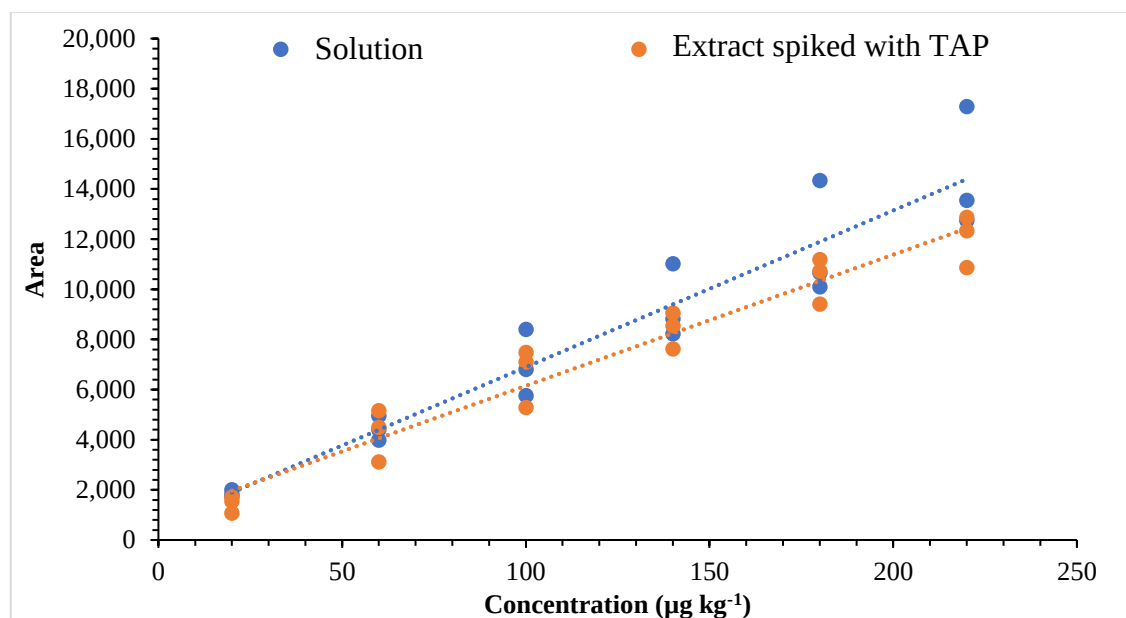


Figure S6. Graphic representation of the analytical curves in extract spiked with thiamphenicol (TAP) and in solution to evaluate the matrix effect of the method.

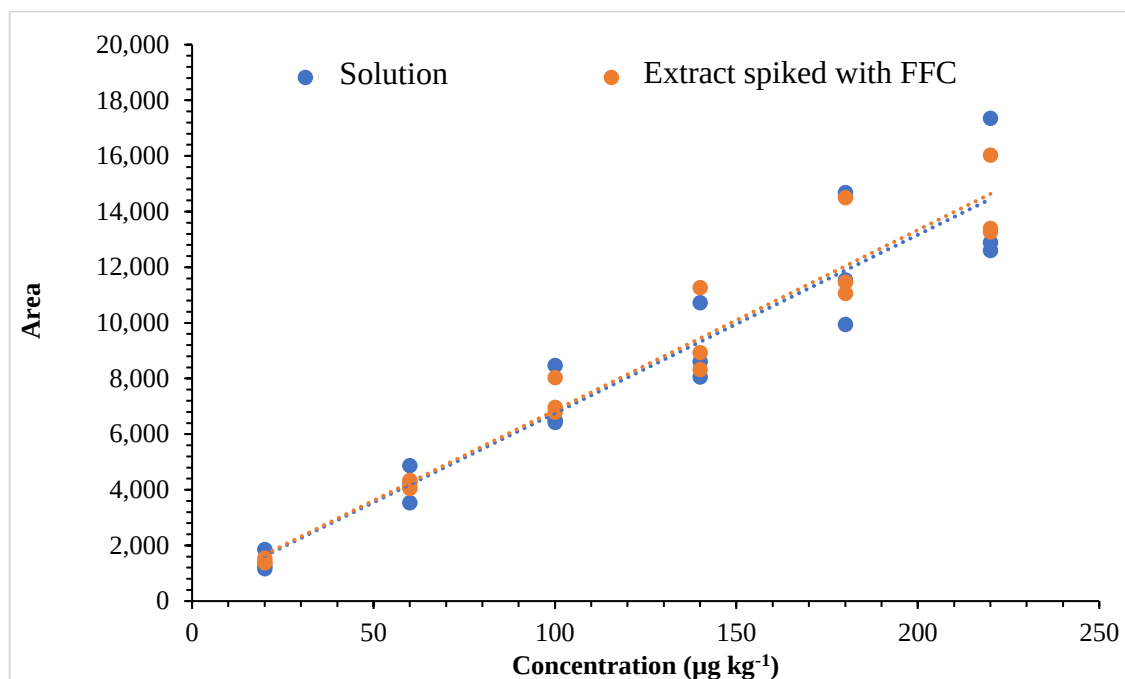


Figure S7. Graphic representation of the analytical curves in extract spiked with florfenicol (FFC) and in solution to evaluate the matrix effect of the method.

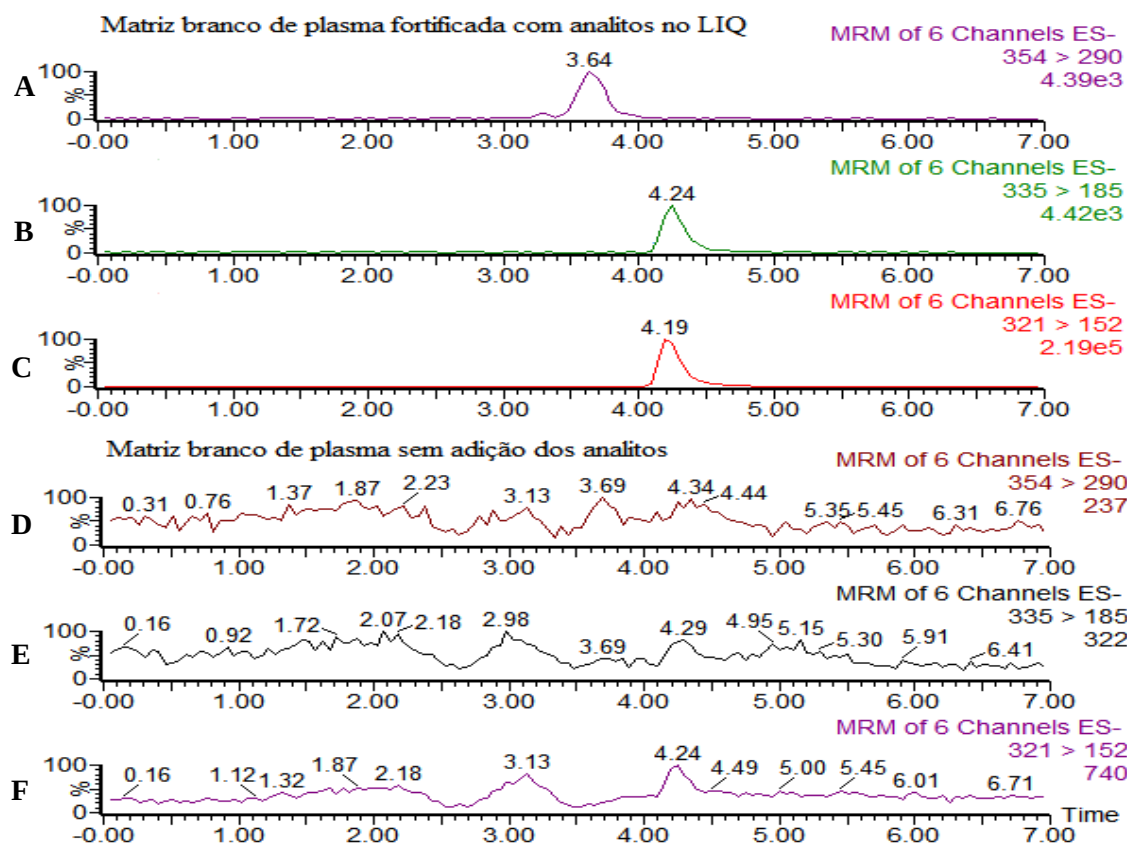


Figure S8. Chromatograms resulting from the analysis of blank plasma matrix spiked with thiamphenicol (TAP) and florfenicol (FFC) at 100 ng g⁻¹ (A, B and C) and chloramphenicol (CAP - internal standard) at 2000 ng g⁻¹, and blank plasma matrix (D, E and F).

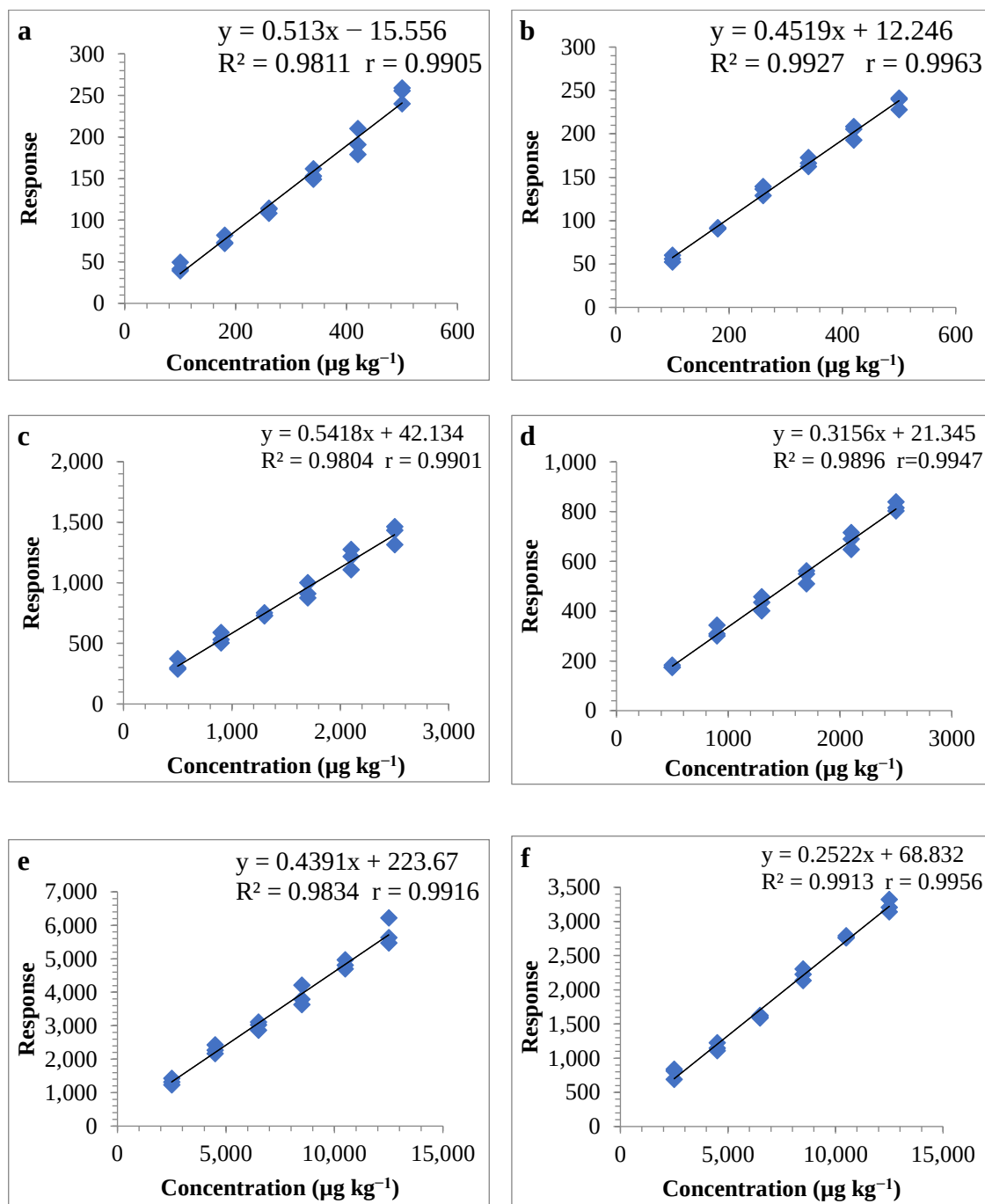


Figure S9. Linear equation (y) of the relationship between responses and florfenicol (FFC, a., c. and e.) and thiamphenicol (TAP, b., d., and f.) concentration for three analytical curves prepared using a matrix spiked with six concentrations (100, 180, 260, 340, 430, and 500 $\mu\text{g mL}^{-1}$) of FFC and TAP, respectively. R^2 , determination coefficient; r , Pearson's correlation coefficient.

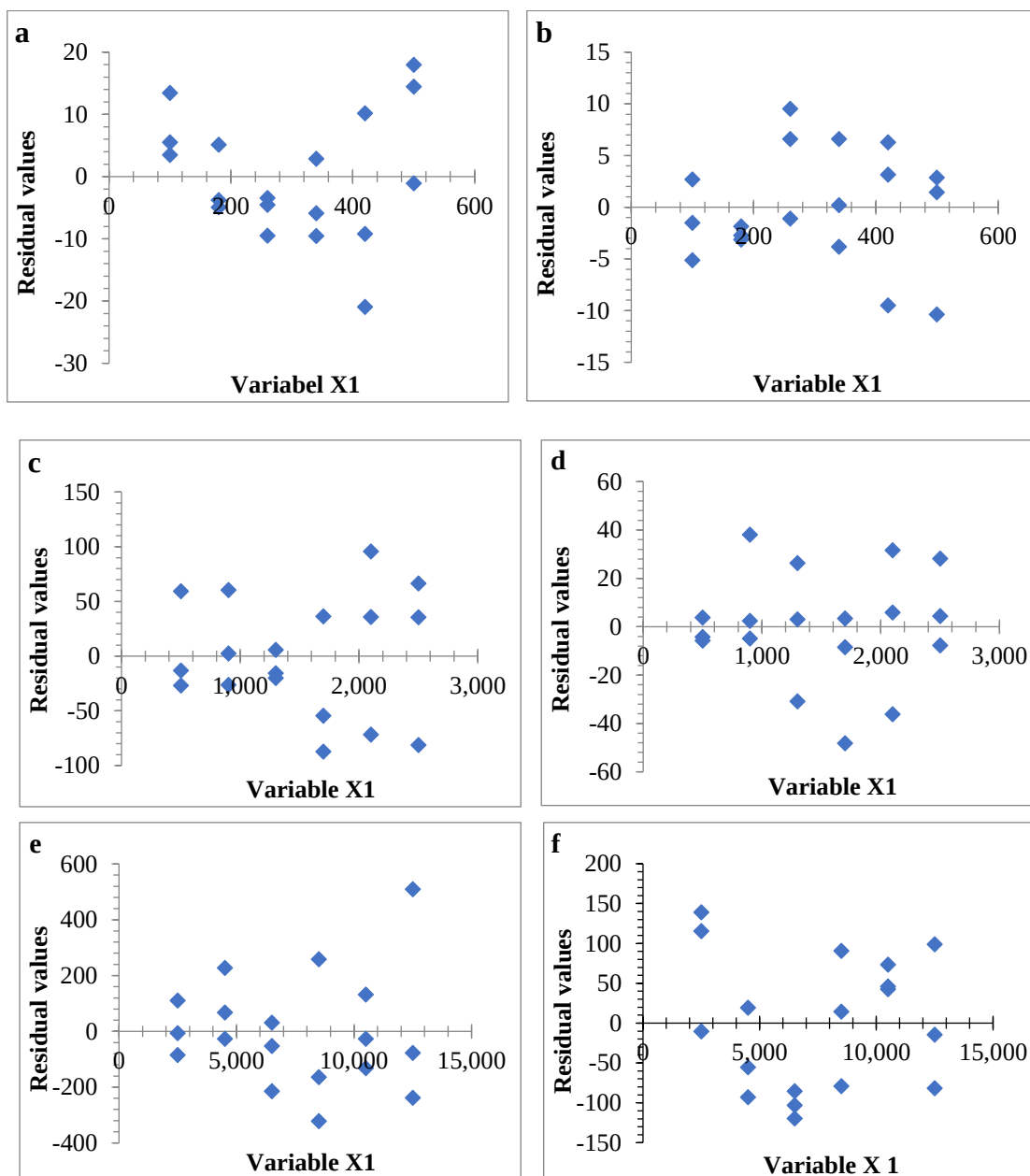


Figure S10. Residual distribution graph resulting from regression analysis with the results obtained from three analytical curves of matrix spiked with six concentrations (100, 180, 260, 340, 430, and 500 $\mu\text{g mL}^{-1}$) of florfenicol (FFC, a., c. and e.) and thiamphenicol (TAP, b., d., and f.), respectively.

General Conclusion

Microbial resistance and bacterial diseases are one of the main obstacles to the increase in tilapia production in Brazil. Since disease outbreaks in fish farming can occur at any stage of the production cycle, causing high morbidity and mortality; high economic impacts resulting from outbreaks can be registered when effective prophylaxis and therapies are not available. Therefore, the proposed research aimed to provide subsidies for sustainable development of Nile tilapia production in Brazil, through the phenotypic and genotypic characterization of the main pathogens associated with high rates of morbidity and mortality; prospection of new environmentally friendly antimicrobials, an effective form of therapy and identification of strains with potential for use in vaccine production.

Considering that the correct diagnosis and knowledge of the epidemiology of diseases is important for the establishment of effective strategies for the prevention, control and treatment of diseases, and the scarcity of this information on the main pathogens of tilapia cultivated in Brazil, it is expected that the results on the phenotypic, genotypic and geographic distribution of the pathogens described in this thesis contribute to the reduction of the impacts of diseases on Brazilian fish farming, through their correct identification and the establishment of effective prevention, control and treatment strategies in fish farming.

Currently, only florfenicol is approved for the treatment of bacterial diseases in tilapia farming in Brazil. For more antimicrobials to be approved, studies on pharmacokinetics, pharmacodynamics, residue depletion and therapeutic efficacy, under local production conditions, must be carried out. For this reason, it is expected that the results of studies of antimicrobial activity and pharmacokinetics of the evaluated antimicrobials and their combinations, will contribute to the rapid development of effective therapies against the main pathogens of tilapia. Despite the limitation of in vitro studies of antimicrobial activity, used to assess the potential of the antimicrobials and forms of therapies studied, in predicting clinical outcomes, we believe that the data presented here constitute a solid and useful basis of information for future studies in the field of drug development for the treatment of bacterial diseases in Brazilian and world aquaculture.

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Anexos

Certificado de Aprovação do Projeto pela Comissão de Ética no Uso de Animais



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal



CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado “Combinação de tianfenicol com florfenicol: perfil farmacocinético e eficácia terapêutica no tratamento de estreptococose em tilápia-do-Nilo (*Oreochromis niloticus*)”, protocolo nº 3772/20, sob a responsabilidade da Profa. Dra. Fabiana Pilarski, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 25 de agosto de 2020.

Vigência do Projeto	10/07/2020 a 19/12/2021
Espécie / Linhagem	<i>Oreochromis niloticus</i>
Nº de animais	460
Peso / Idade	200 ± 50 gramas
Sexo	-
Origem	Caunesp

Jaboticabal, 25 de agosto de 2020.



Profª Drª Paola Castro Moraes
Coordenadora – CEUA

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