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CENTRO DE AQUICULTURA DA UNESP

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

Inácio Mateus Assane

Jaboticabal, SP

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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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Orientadora: Profa. Dra. Fabiana Pilarski

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DEDICO

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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 bacterina 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 controlo 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 ºC (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 (Samuelsen, 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, Thongpiam, 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.

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 ± 0.32ºC. 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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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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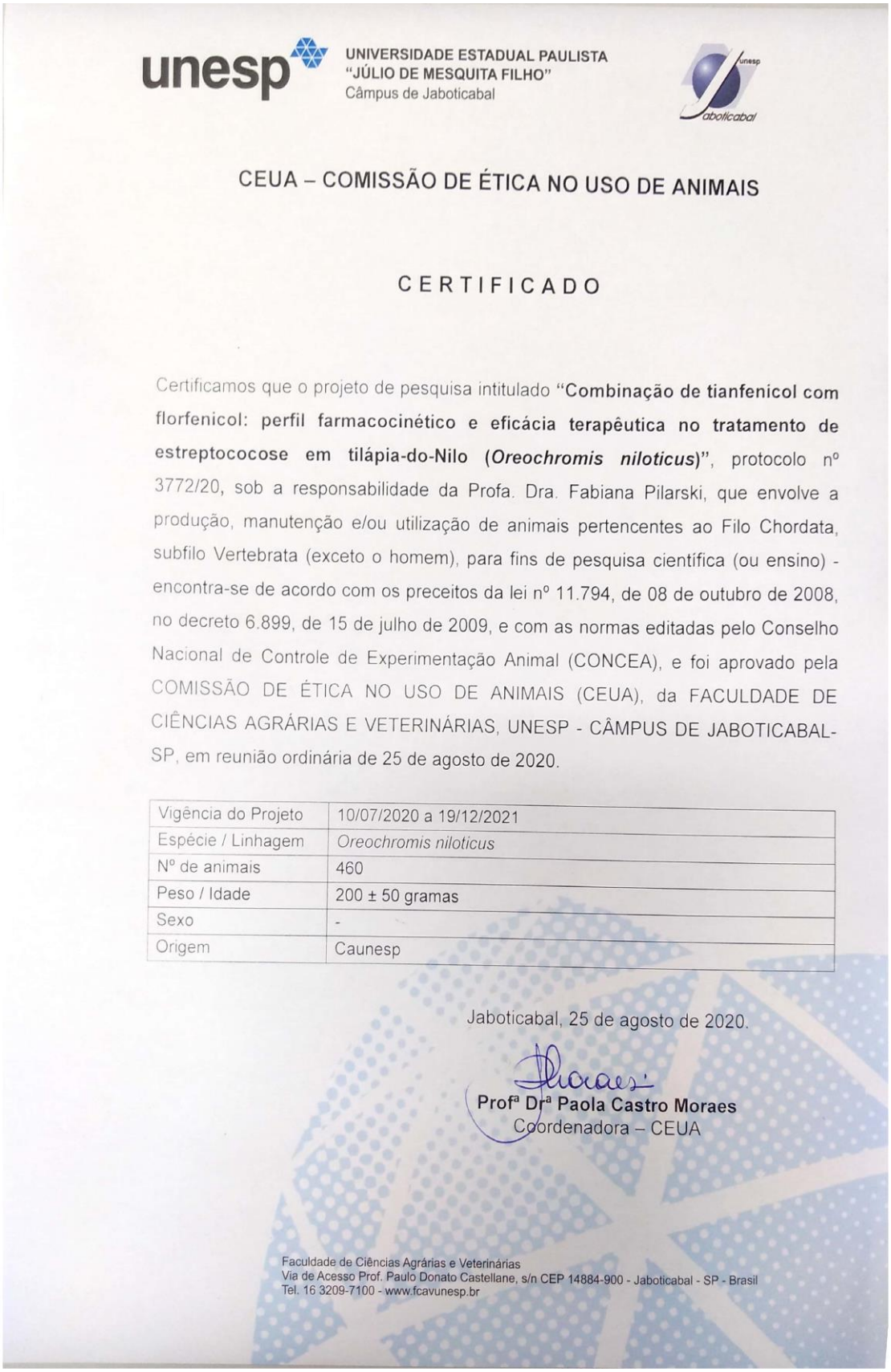
Anexos

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	*Oreochromis niloticus*
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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