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THERAPEUTIC EFFICACY OF FLORFENICOL AGAINST *Streptoccoccus agalactiae* INFECTION IN NILE TILAPIA (*Oreochromis niloticus*)

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Belo Horizonte - MG
Escola de Veterinária-UFMG
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THERAPEUTIC EFFICACY OF FLORFENICOL AGAINST *Streptoccoccus agalactiae* INFECTION IN NILE TILAPIA (*Oreochromis niloticus*)

Dissertação apresentada à Universidade Federal de Minas Gerais, Escola de Veterinária, como requisito parcial para a obtenção do grau de Mestre em Ciência Animal.

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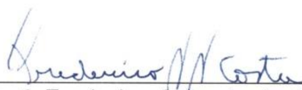

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"“‘... Valeu a pena?
Tudo vale a pena, se a alma não é pequena.
Quem quer passar além do Bojador
“Tem que passar além da dor...”.

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“Sou do tamanho daquilo que vejo e não do tamanho da minha altura”.

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ABBREVIATION LIST

BHI	Brain heart infusion broth
CAMHB	Mueller - Hinton cation adjusted broth
ºC	Celsius degrees
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
FAO	Food and Agriculture Organization
FLO	Florfenicol
g	gram
h	Hour
IBGE	Brazilian Institute of Geography and Statistics
Kg	Kilogram
L	Liter
LW	Live weight
MAPA	Brazilian Ministry of Agriculture Livestock and Food Supply
mL	mililiter
MIC	Minimum Inhibitory Concentration
µL	microliter
mg	milligram
OTC	Oxytetracycline
OD	Optical Density
PBS	Phosphate buffered saline
rpm	Rotation per minute
RR	Relative risk
RRR	Relative risk reduction
SA	*Streptococcus agalactiae*

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RESUMO

Streptococcus agalactiae (SA) é um dos mais importantes patógenos que afetam a tilapicultura mundial. A antibioticoterapia oral é a principal medida terapêutica aplicada em casos de surtos da doença. Diversos antimicrobianos têm sido empregados para o tratamento da estreptococose, sendo o florfenicol (FLO) uma das drogas mais utilizadas. No Brasil existem relatos de falhas terapêuticas e recidivas dos casos de estreptococose em lotes tratados com FLO na dose padrão de 10 mg/Kg de peso vivo. Apesar de licenciado em diferentes países, não existem estudos sobre a eficiência terapêutica e dose ideal do FLO para o tratamento de infecções por SA em tilápia do Nilo (*Oreochromis niloticus* L). O objetivo deste trabalho foi avaliar a eficiência terapêutica e dose ideal de FLO para o tratamento da infecção por SA em tilápia do Nilo (*Oreochromis niloticus*). A amostra de *S. agalactiae* SA 95-10 previamente isolada de tilápia doente no Brasil foi utilizada no presente estudo. A concentração inibitória mínima (CIM) do FLO para a amostra SA 95-10 foi determinada de acordo com manual VET04-A2 do CLSI. Para avaliação da eficiência terapêutica, juvenis de tilápia foram infectados experimentalmente com a SA 95-10 e medicados por via oral com FLO nas doses de 10, 20, 40 e 60mg/Kg por 10 dias consecutivos e observados por um período de 20 dias pós-tratamento. A CIM do FLO para SA 95-10 foi de 1µg/mL. As doses de 20 e 40mg/Kg foram eficientes nos controle das mortalidades durante o período de tratamento, porém, mortalidades de 90% e 60% foram observadas no período de acompanhamento pós-tratamento. Após o fim do período experimental 44,4% dos peixes continuavam portadores da bactéria. A SA 95-10 foi submetida a teste de avaliação *in vitro* do fenômeno persistência induzida pelo FLO, sendo exposta a 100 vezes a concentração do CIM para FLO. Essa se manteve viva e viável após 12 horas de exposição ao FLO, com uma redução na concentração de 10^8 para 10^6 UFC/mL. Para avaliar a capacidade de transmissão da bactéria de animais portadores para saudáveis, foi realizado um ensaio de coabitação. Dois grupos de peixes foram desafiados com a SA 95-10, medicados com FLO nas doses de 20 e 40 mg/Kg de PV por 10 dias consecutivos e posteriormente transferidos para aquários contendo animais saudáveis. Mortalidades de 40 e 50% foram observadas nos animais saudáveis coabitados, denotando a manutenção da capacidade de transmissão e virulência da bactéria nos portadores induzidos pela terapia. O FLO é ineficaz no controle da infecção por *S. agalactiae* em tilápia do Nilo. Esse diminui a mortalidade durante o tratamento, porém, mortalidades elevadas e quadros de portador ocorrem imediatamente após o fim do tratamento. Esse é o primeiro relato do fenômeno de persistência induzida por antibióticos em *S. agalactiae* patogênico para peixes. Futuros estudos devem ser realizados para determinar os mecanismos fisiológicos e moleculares que possibilitam a bactéria resistir ao antibiótico.

Palavras-chave: antibioticoterapia, estreptococose, florfenicol, portador assintomático, persistência, surto.

ABSTRACT

Streptococcus agalactiae is one of the most important pathogens affecting farm-raised tilapia worldwide. The oral therapy with antibiotics is the main therapeutic measure applied in outbreaks. Several antimicrobial agents have been used for the treatment of streptococcosis, being the florfenicol one of the most widely used drug. In Brazil, reports of treatment failures and recurrent mortality in herds treated with FLO have been done with the recommended dose of 10 mg/Kg of LW. Despite to being licensed in different countries, there aren't studies about the therapeutic efficacy and optimal dosage of FLO for the treatment of SA infections in Nile tilapia (*Oreochromis niloticus* L.). The aim of this work was to evaluate the therapeutic efficiency and optimal dose of FLO for the treatment of SA infection in Nile tilapia (*Oreochromis niloticus*). The *S. agalactiae* strain SA 95-10, previously isolated from diseased tilapia in Brazil was used in this study. The minimum inhibitory concentration (MIC) of FLO for the SA 95-10 was determined according to VET04-A2 guidelines of CLSI. For the evaluation of therapeutic efficiency, Nile tilapia juveniles were experimentally infected with SA 95-10 and orally medicated with FLO in doses of 10, 20, 40 and 60mg/Kg of LW for 10 consecutive days and observed for a period of 20 days post-treatment. The MIC of FLO for SA 95-10 was 1 μg/mL. The doses of 20 and 40mg/Kg were effective in the control of mortalities during the treatment period, however, mortality rates of 90% and 60% were observed at the observation period. After the end of experimental period 44.4% of the fish were shown to be carriers of bacteria. SA 95-10 was submitted to an, *in vitro* evaluation of persistence behavior induced by FLO, being exposed to 100 times the MIC value for FLO. The strain remained alive and viable after 12 hours of exposition, decreasing the bacterial counting from 10^8 CFU/mL to 10^6 CFU/mL. To evaluate the transmission of bacterium from carriers to healthy animals, a cohabitation assay was performed. Two groups of fish were challenged with the SA 95-10, medicated with FLO at 20 and 40 mg/Kg of LW for 10 consecutive days, and subsequently transferred to aquaria containing healthy animals. Mortalities of 40 to 50% and were observed in cohabitated animals. This might indicate that the ability of transmission and virulence is kept in FLO induced persistent *S. agalactiae cells* in carrier fish. The FLO was shown unable to control SA infection in Nile tilapia. It controls the disease during the treatment, however, mortalities are immediately verified when the administration ends. This is the first report of the persistence phenomenon induced by antibiotics in fish pathogenic *S. agalactiae*. Future studies should be carried out to determine the physiological and molecular mechanisms that allow the bacterium to resist to FLO.

Key-words: antibiotic therapy, carrier, florfenicol, outbreaks, persistence, streptococcosis.

1. Introduction

Aquaculture is the sector of animal production that showed the fastest grown during the last five decades in the world, with an annual increase of 3.2% (FAO, 2014). According to FAO in the year of 2014 aquaculture reached a total production of 97.2 million tons, generating an amount equivalent to, 57 billions of dollars (FAO, 2015). In Brazil, the production for the same aforementioned year was equivalent to 474.3 thousand tons, resulting in an amount of 3.87 billions of reais (IBGE, 2015). Nile tilapia (*Oreochromis niloticus*) is the main produced species worldwide, accounting for 41.9% of the total fish production in the Brazil (IBGE, 2015; FAO, 2015).

The continuous growing of aquaculture production faces several barriers, being infectious diseases appointed as one of the major obstacles to its development (FAO, 2012). In the same way, the occurrence of outbreaks of infectious diseases has been characterized as a significant adversity for the tilapia farming worldwide. There is no scientific or statistics data about the main pathogens and the economic impact of outbreaks to farm-raised tilapia. However, the bacterial diseases seem to be the principal health issue to this fish culture. Among bacterial pathogens, *Streptococcus agalactiae* is one of the most important for tilapia farming, being responsible for major economic losses annually in the producer countries.

Outbreaks caused by *Streptococcus agalactiae* in farmed and wild aquatic animals have been described in the five continents. The disease in farm-raised fish was reported in several countries, including China, Vietnam, Thailand, Kuwait, Iran, Israel, Egypt, Australia, Malaysia, Indonesia, Philippines, Ireland, USA, Honduras, Costa Rica, Colombia and Brazil (Amal et al., 2012; Bowater et al., 2012; Eldar et al., 1995; Evans et al., 2002; Evans et al., 2008; Hernandez et al., 2009; Mian et al., 2009; Plumb et al., 1974; Suanyuk et al., 2005; Ye et al., 2011; Ruane et al., 2013).

Twenty different fish species were shown to be susceptible to *S. agalactiae* infection in natural conditions, among them, Nile tilapia (*Oreochromis niloticus*) (Itsaro et al., 2012; Mian et al., 2009; Godoy et al., 2013). The disease causes septicemia and meningoencephalitis with the main clinical signs characterized by anorexia, lethargy, melanosis, exophthalmia, ascites, loss of equilibrium in the water column, corneal opacity, buccal paralysis, erratic swimming, and C-shape body.

During outbreaks, mortalities can reach up to 90% of the herd (Figueiredo et al., 2005; Mian et al., 2009; Figueiredo and Leal, 2012; Faria et al., 2013; Tavares et al., 2015). Increase in water temperature (> 27°C), high stocking density, intensive husbandry, and poor water quality (mainly acute hypoxia) are considered the principal predisposing factors for the outbreaks (Evans et al., 2002; Figueiredo et al., 2006; Tavares et al., 2015).

The treatment of *S. agalactiae* outbreaks in Nile tilapia farms is based on the oral administration of antibiotics, such as amoxilin, enrofloxacin, oxytetracycline and florfenicol (Aisyhah et al., 2015). In Brazil the antimicrobials OTC and FLO are licensed by the Ministry of Agriculture Livestock and Food Supply (MAPA) to treat cases of bacterial infections in Nile tilapia farms. The low number of drugs available limits the therapeutical choices to control streptococcosis outbreaks.

Florfenicol (FLO) is one of most used antimicrobial agents in the treatment of streptococcosis in several fish producer countries. It is currently approved to use in aquaculture USA, Norway, United Kingdom, Colombia, Costa Rica, Malaysia, Scotland, Ireland, Spain, France, Israel, Thailand, Indonesia, Ecuador, Honduras, Philippines, Malaysia, Vietnam, Venezuela, Japan, South Korea, Canada, Brazil, Chile, and China (Darwish et al., 2007; Gaikowski et al., 2013). It is a monofluorinated drug derivatives from thiamphenicol and analogous to chloramphenicol. It has bacteriostatic action, and broad-spectrum activity. It inhibits the protein synthesis by the binding to 50S subunit of the bacterial ribosome, avoiding the enzymatic action of peptidyl-transferase, thereby preventing the transfer of amino acids for formation of peptide chains (Plumb, 2004). It has been widely used in aquaculture because is heat-stable, resisting to the extrusion process of the feed, present few reports of resistance, and it is for being a drug with exclusive veterinary use (Feng et al., 2009; Gaikowski et al., 2013); in theory, it would reduce the possibility of development and transmission of resistant genes and bacterial populations through the food chain.

In Brazil, florfenicol has been used to treat infected tilapia herds since 2007 (Figueiredo et al., 2007b). In the last years, several informal reports of FLO failure have been conducted by field veterinarians and farmers. They described the inefficiency of this antibiotic in standard dosages of 10 mg/Kg of LW to control the mortalities during *S. agalactiae* outbreaks in Nile tilapia farms. In addition, they reported the recurrence of mortality immediately after finishing the therapy or in few days later. The therapeutic problem and recurrence of disease could be due to several factors such as inadequate dosage, distribution of drug in the tissues, or resistance to antimicrobials.

The FLO is recommended for treatment of several bacterial diseases (Darwish et al., 2007; Gaunt and Gao et al., 2010; Gaunt and Endris et al., 2010; Bowker et al., 2010; Roiha et al., 2011; Gaunt et al., 2015; Soto et al., 2013). It has been shown effective to control *S. iniae* infection in Nile tilapia, however, the doses employed are higher than the recommended in the literature (10 mg/Kg of LW) (Darwish et al., 2007; Gaunt and Endris et al., 2010). Similarly, the treatment of infection by *Francisella noatunensis* subsp. *orientalis* (FNO) is currently treated with FLO, but, with higher dose (15 mg/Kg of LW) and longer periods (15 days) (Soto et al., 2013). Although licensed in many countries and being effective in infection by *S. iniae* and FNO, there are no studies about the therapeutic efficiency and optimal dosage of FLO for the treatment of infections caused by *S. agalactiae* in Nile tilapia (*Oreochromis niloticus*).

The pharmacokinetics and pharmacodynamics of FLO in the Nile tilapia have been previously studied. This antibiotic shows a rapid absorption when administered orally, and has a broad distribution in the tissues, reaching a maximum serum concentration after 12 hours. The metabolization of the drug varies with the temperature, showing fast elimination in high temperatures (Feng et al., 2008; Feng et al., 2009). A point of concern about florfenicol therapy is the absence of data about the capacity of this drug to pass the blood-brain barrier, and the concentration reached in the brain tissue. Streptococosis classically affect the brain of Nile tilapia, therefore, to be efficient a drug should reach this tissue.

The antibiotic resistance in samples of *S. agalactiae* from fish has been previously studied. Few studies found resistant isolates to gentamycin, kanamycin, trimethoprim, nitrofurantoin, ampicillin, spiramycin, oleandomycin, sulphamethoxazole, oxolinic acid, penicillin, erythromycin, and oxytetracyline (Duremdez et al., 2004; Evans et al., 2002; Faria et al., 2013; Musa et al., 2009; Godoy , 2006; Soto et al., 2015). There is not data in the literature describing resistance to florfenicol in fish isolates of *S. agalactiae*. Recently, the MIC values of FLO for

100 Brazilian strains of *S. agalactiae* isolated from Nile tilapia were evaluated (data not published). The normalized resistance interpretation (NIR) methodology, was utilized to determine the epidemiological cut off value, enabling it to be calculated with one laboratory data, and being able to detect samples with low resistance level. All strains were shown wild-type or sensitive based on it.

Recently, a phenomenon known as persistence has been associated as one of the forms of bacterial resistance to antibiotics. This differs from "conventional resistance" to antimicrobials, since there is no genetic alteration in these resistant cells, being genetically equal to sensitive cells of the same population (Fauvart et al., 2011). The persistence may occur by several factors in response to environmental stimuli received by bacteria, working as a survival strategy; can occurs when a population is exposed to antibiotics, starving, temperature and pH alterations. In those critical conditions, a subpopulation switch to a dormant state, presenting different phenotypes from the sensitive cells. The dormancy allows the persistent subpopulation to maintain themselves alive and to be able to grow again when the treatment cease, besides this state might responsible for the antimicrobial tolerance showed for this cells (Willenborg et al., 2014; Zhang et al., 2014; Fauvart et al., 2011; Lewis, 2010).

That phenomenon has been associated with treatment failures in several diseases of humans and animal, such as tuberculosis (caused by *Mycobacterium tuberculosis*), cystic fibrosis pneumonia (infection by *Pseudomonas aeruginosa*), urinary infection caused by *E.coli*, and pneumonia caused by *Streptococcus suis* (Fauvart et al., 2011; Willenborg et al., 2014).

In this context, the knowledge about optimal dose and therapeutic efficiency of florfenicol to treat *S. agalactiae* infection in Nile tilapia is essential.

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2. Objectives

The aims of this study were to evaluate the therapeutic efficiency of florfenicol to treat *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) and the occurrence of persistent *S. agalactiae* cells induced by this antibiotic.

3. Chapter 1

Florfenicol therapy fails to control *Streptoccoccus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*)

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3.1 Introduction

The Nile tilapia (*Oreochromis niloticus*) is the main cultivated fish species in Brazil and worldwide (FAO, 2014). In the country, it accounts for 41.9% of the total fish farming produced in 2014 (IBGE, 2015).

The occurrence of outbreaks of infectious diseases has been characterized as a significant adversity for the tilapia farming worldwide, and the bacterial diseases seem to be the principal health issue to this fish (FAO, 2012).

Among bacterial pathogens, *Streptococcus agalactiae* is one of the most important for tilapia farming, being responsible for major economic losses annually. During outbreaks, mortalities can reach up to 90% of the herd (Figueiredo et al., 2005; Mian et al., 2009; Figueiredo and Leal, 2012; Faria et al., 2013; Tavares et al., 2015). Diseased animals showed an acute septicemia and meningoencephalitis; the main clinical signs observed are anorexia, lethargy, melanosis, exophthalmia, corneal opacity, buccal paralysis, erratic swimming, and C-shape body (Evans et al., 2002; Pasnik et al., 2005 Figueiredo et al., 2007a; Mian et al., 2009). Main predisposing factors for the outbreaks are, increase in water temperature (> 27°C), high stocking density, intensive husbandry and poor water quality (mainly acute hypoxia)(Evans et al., 2002; Figueiredo et al., 2006; Tavares et al., 2015).

The main treatment applied in *S. agalactiae* outbreaks in Nile tilapia farms is the oral antibiotic therapy of the herd (Faria et al., 2013). For the treatment of streptococcosis, florfenicol (FLO) is one of the most widely used antimicrobial. This drug is a broad-spectrum antibiotic that acts by inhibiting protein synthesis (Plumb, 2004). In Brazil, several informal reports have been done by field veterinarians and farmers describing the inefficiency of this antibiotic in the standard dose of 10 mg/Kg LW to control the mortalities during *S. agalactiae* outbreaks; besides the recurrence of the disease has been frequently observed after the treatment period in Nile tilapia farms.

The treatment problem and recurrence of disease could be due to different factors such as incorrect antibiotic dosage, inadequate drug distribution in tissues, or resistance to antimicrobials. FLO in the Nile tilapia shows fast absorption (maximum serum concentration after 12 hours) after oral administration, and broad distribution in host tissues. Metabolization of the drug is temperature dependent, being faster in high water temperatures (Feng et al., 2008; Feng et al., 2009). There are no reports in the literature of strains *S. agalactiae,* from fish that possess mobile genetic elements or constitutive resistance, although, some fish strains of *S. agalactiae* showed to be resistant to gentamycin, kanamycin, trimethoprim, nitrofurantoin, ampicillin, spiramycin, oleandomycin, sulphamethoxazole, oxolinic acid, penicillin, erythromycin, and oxytetracyline (Duremdez et al., 2004; Evans et al., 2002; Faria et al., 2013; Musa et al., 2009; Soto et al., 2015). However, there is no data about florfenicol resistance in fish isolates of *S. agalactiae*.

The FLO is widely used to treat bacterial infections in farmed fish, for example those caused by *Flavobacterium columnare* and *Edwardsiella ictaluri* in catfish (Gaunt and Gao et al., 2010; Gaunt et al., 2015), *Vibrio anguillarum* in Atlantic halibut (Roiha et al., 2011), and *Streptococcus iniae* and *Francisella noatunensis* subsp. *orientalis* in Nile tilapia (Gaunt et al., 2010; Darwish et al., 2007; Bowker et al., 2010; Soto et al., 2013). Despite to be licensed in different countries and used in many treatment protocols, there are no studies about the therapeutic efficacy and optimal dosage of FLO for the treatment of *Streptococcus agalactiae* infections in Nile tilapia (*Oreochromis niloticus* L.).

The aims of this study were to evaluate the therapeutic efficiency of florfenicol to treat *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) and the occurrence of persistent *S. agalactiae* cells induced by this antibiotic.

3.2 Material and Methods

3.2.1 Bacterial strains

The strain SA 95-10 (serotype Ib; ST-260) of *Streptococcus agalactiae* was used in the assays of therapeutic efficiency, persistence *in vitro*, and transmission. The strain was previously isolated from diseased Nile tilapia with typical clinical signs of streptococcosis, and identified by phenotypic and molecular methods (Godoy et al., 2013). The isolate belongs to AQUAVET-Aquatic Animal Diseases Laboratory (Veterinary School, Federal University of Minas Gerais-/UFMG) culture collection and was stored at -80ºC until use.

3.2.2 Minimum Inhibitory Concentration (MIC)

MIC value was determined according to the guidelines "VET04-A2 Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals" of Clinical and Laboratory Standards Institute (CLSI, 2014). The strain was thawed, streaked onto 5% sheep blood agar, and incubated at 28°C for 48 h. After, a colony was selected, inoculated in brain heart infusion

broth (BHI), and incubated at 28°C under low agitation (140 rpm) until reaching the bacteria concentration required for each assay.

The bacterial suspension was prepared in saline solution (0.85%), and the inoculum was standardized to bacterial concentration of 1–2 x 10^8 CFU/mL based on corresponding absorbance (OD_{600}) of 0.08–0.130. Afterwards, the bacterial suspension was diluted 1:9 (v:v) in cation adjusted Mueller–Hinton medium (CAMHB) (Difco,USA) supplemented with 2.5% lysed horse blood. The MIC assays were performed in sterile dry-form 96-well microplates. 100 µL of CAMHB with 2.5% horse blood were added in each well. In the first well of each line, 100 µL of FLO (at a concentration of 128 µg/mL) was inoculated and serially 2-fold diluted. Tested concentrations ranged from 64µg/mL to 0.06µg/mL. After antibiotic dilution, 5µL of freshly prepared bacterial inoculum was inoculated in each well. The microplates were shaken, sealed and incubated at 28°C for 24 h, and then the results were read. *Escherichia coli* ATCC 25922 was used as a quality control for the plates.

The MIC was defined as the lowest concentration of antibiotic that could prevent visible bacterial growth. The strain was classified as wild-type (WT) or non-wild-type (NWT) based on the *in house* provisional epidemiological cut off value calculated using normalized resistance interpretation (NIR) (Kronvall, 2003; Smith et al., 2007) for 100 Brazilian *S. agalactiae* isolates (data not shown).

3.2.3 Fish

Two hundred and twenty Nile tilapia juveniles with average weight 27.34 ± 4.04 g were acquired from commercial tilapia hatchery. 10 fish were randomly selected and submitted to bacteriological analysis to ensure they were free of bacterial infections. The fish were acclimatized for a period of 10 days in 57 L aquarium with supplementary aeration system and flow-through water 0.5L/h. The temperature was maintained at 28°C. The animals were fed twice a day until satiation (Laguna-Ocialis, Brazil- 36% of crude protein). The Ethics Committee for Animal Experiments of the Federal University of Minas Gerais approved all the protocols used in this study (CEUA-UFMG, protocol n° 330/2015).

3.2.4 Safety assay

To address possible side effects and alterations caused by higher doses of florfenicol the safety of this antibiotic to Nile tilapia juveniles was evaluated.

The medicated feed was produced by incorporation of analytical standard grade florfenicol (Sigma-Aldrich, USA) to the commercial extruded feed (Laguna-Ocialis, Brazil) for a feeding rate of 2% LW, in a proportion corresponding to different doses of FLO. The antibiotic was added to the feed and vigorously homogenized. Afterwards 10 ml of soybean oil was administered to the feed to avoid the antibiotic loss by hydrosolubilization during feeding. In the positive control group (fed with non-medicated feed) 10 ml of soybean oil was added in the

same concentration and homogenized with the feed. Medicated and non-medicated feed were daily prepared and stored at 2-8°C until use.

Each experimental group was composed of 10 fish. The experimental groups were: Group 1-treated with FLO at 20mg/Kg of LW; Group 2-treated with FLO at 40mg/Kg of LW; Group 3-treated with FLO at 60mg/Kg of LW; and Group 4- with non-medicated feed. They were fed three times a day and received the medicated feed for 10 consecutive days. The fish were monitored four times a day and the following parameters were recorded: water temperature; dissolved oxygen; feeding behavior; swimming behavior; and clinical signs of physiological alterations and diseases. At the end of the experimental period, fish were euthanized by overdose of benzocaine (250mg/L) (Sigma-Aldrich,USA) and submitted to necropsy, bacteriology and tissues were collected for histopathological evaluation.

3.2.5 Pathological evaluation

The pathological evaluation was conducted in all treated fish. Samples of the fish organs (liver, kidney, and spleen) were collected and fixed in Bouin's fixative for 24 h. Samples of spleen and liver were transferred to 70% ethanol until processing. Kidney samples were collected attached to the fish's spinal cord and were decalcified in 0.5 M EDTA for 10 days. Samples were processed in a histo-processor (TP 1020, Leica Biosystems, Germany) using standards protocols for fish histology. Fish tissues were dehydrated in graded ethanol series and xylene. Embedding and blocking were performed in a Leica EG-1150 (Nussloch, Germany) modular tissue embedding center and sectioned in paraffin blocks at 5 µm using a Leica RM 2135 microtome (Nussloch, Germany). Sections were stained with Harry's hematoxylin and eosin (Mumford et al., 2007).

3.2.6 Therapeutic Efficiency of Florfenicol

The therapeutic efficiency of florfenicol was evaluated through the experimental challenge of Nile tilapia juveniles with SA 95-10 followed by the treatment with different FLO doses. Each experimental group was composed of 10 Nile tilapia juveniles, according to the different antibiotic dosages and experimental groups: Group 1- challenged and treated with FLO at 10 mg/Kg of LW, Group 2- challenged and treated with FLO at 20 mg/Kg of LW, Group 3-challenged and treated with FLO at 40 mg/Kg of LW, Group 4- challenged and treated with FLO at 60 mg/Kg of LW, Group 5- challenged and feed with non-medicated feed (Positive control group); and Group 6- not challenged and feed with non-medicated feed (Negative control group).

For experimental infection, the SA 95-10 was thawed, streaked onto 5% sheep blood agar, and incubated at 28°C for 48 h. After, a colony was selected, inoculated in brain heart infusion broth (BHI), and incubated at 28°C under low agitation (140 rpm) until reaching 0.05 (OD_{600}) equivalent a concentration of 10^7 CFU/mL. Fish were anesthetized in a water solution containing 10 mg/L of benzocaine (Sigma-Aldrich, USA). The challenged groups were injected intraperitoneally with 0.1 mL of *S. agalactiae* inoculum, corresponding to 10^6 CFU/fish. The negative control group was injected with 0.1 mL of sterile BHI broth.

Challenged fish were monitored three times a day. The following parameters were evaluated: water temperature; dissolved oxygen; feeding behavior; clinical signs of diseases; and mortalities. The medicated feed was provided one hour post-challenge. The FLO treated groups were medicated for 10 consecutive days, followed by 20 days of observation, being the overall experimental period of 30 days.

During the observation period fish were fed with commercial feed. Samples of brain and kidney were aseptically collected from all animals that died during experimental period or still alive until the end. The samples were inoculated onto 5% sheep blood agar, and incubated at 28°C for 48 hours.

The isolates were characterized serologically by Slidex Latex kit Agglutination (BioMerieux, France) and confirmed by PCR species-specific (Mata et al., 2004).

3.2.7 Persistence assay

The capacity of FLO to induce persistent *S. agalactiae* cells were evaluated according to the methodology proposed by Willenborg et al. (2014), with some modifications. The persistence behavior was tested in bacterium under two different growth stages: 1- exponential growth, and 2- stationary phase. The SA 95-10 was thawed, streaked onto 5% sheep blood agar, and incubated at 28°C for 48 h. After, a colony was selected, inoculated into CAMHB supplemented with 2.5% lysed horse blood, and incubated at 28°C under low agitation (140 rpm). For exponential growth, the strain was incubated until reach OD_{600} of 0.166 (middle of exponential phase) with approximately 28 hours of incubation. For stationary phase, the bacterium was considered in this stage when presented three consecutive stable OD_{600} measures (variation lower than 0.02), in an interval of two hours between each evaluation. The bacterial cultures were aliquoted in 10 ml and submitted to centrifugation at 4.000 x *g* for 10 minutes at 4°C. The pellets were washed three times with sterile PBS and resuspended in CAMHB broth supplemented with 2.5% lysed horse blood.

For both phases, bacterial suspensions were adjusted to 10^8 CFU/mL. The test medium were prepared by adding 100 µg/ml (100 x the MIC value of FLO for SA 95-10) of florfenicol sterilized by filtration to bacterial suspension resuspended in CAMHB broth supplemented with 2.5% lysed horse blood. The flasks were incubated at 28°C under low agitation (140 rpm). Two individual flasks were used for exponential and stationary phases. Samples of test medium inoculated bacterial suspensions were taken for bacterial counting at 1, 2, 4, 8, and 12 hours post-inoculation. The bacterial counting was performed in triplicate for each growth phase

3.2.8 Cohabitation Assay

To evaluate the ability of transmission of SA 95-10 from the florfenicol treated group to healthy fish a cohabitation assay was performed. Six groups of 10 Nile tilapia juveniles were used. They were kept in the same conditions as described in the item 3.2.3. Groups A and B were challenged (as described in the item 3.2.4) with SA 95-10 and treated with florfenicol at doses of 20 and 40 mg/Kg of LW for 10 consecutive days.

After the treatment period, fish from the groups A and B were captured, anesthetized by immersion in a benzocaine water solution (10 mg/L), and identified using rubber tags. The animals of these groups were divided in sub groups of five animals and transferred to new aquaria with 10 healthy fish each. Group C received fish from group A (treated with florfenicol at 20 mg/Kg of LW), and group D fish from group B (treated with florfenicol at 40 mg/Kg of LW). This part of the assay, was made in duplicate.

The fish were fed to satiation twice a day. They were monitored four times a day and when the following parameters were evaluated: water temperature; dissolved oxygen; feeding behavior; clinical signs of diseases; and mortalities. The fish were observed for 30 days after cohabitation.

Samples of brain and kidney were aseptically collected from all animals that died during experimental period or still alive until the end of this phase. The samples were inoculated onto 5% sheep blood agar, and incubated at 28°C for 48 hours. The isolates were characterized serologically by Slidex Latex kit Agglutination (BioMerieux, France) and confirmed by PCR species-specific (Mata et al., 2004).

3.2.9 Statistical analysis

The mortality data from FLO treated and control groups were compared by Fisher's exact test. *P* values < 0.05 were considered significant. The Mantel-Haenszel methods and 95% CI were used to calculate relative risk (RR). All analysis was performed using MedCalc Statistical Software Version 15.11.4 (MedCalc Statistical Software version 12.7.8, 2014).

3.3 Results

3.3.1 MIC

The MIC value of FLO for SA 95-10 was 1.0 µg/mL. Based on the *in house* epidemiological cut off value of 6.6 µg/mL calculated by normalized resistance interpretation (Kronvall, 2003) (data not shown), the sample SA 95-10 was classified as wild-type or sensitive to FLO.

3.3.2 Safety Assay and Pathological Evaluation

During the safety trial, physiological alterations, clinical signs of disease, and mortalities were not verified in the negative control and in the FLO treated groups at all dosages. There was no alteration in feeding behavior or in any other observed parameters.

The histopathological evaluation of kidney reveal dose-dependend alterations of tissue injures in the dosages of 40 e 60mg/Kg LW FLO. It was possible to observe gills with lamellar hyperplasia, necrosis in cranial kidney, tubular epithelial degeneration and mineralization in the posterior kidneys.
The histopathological analisys of spleen and liver did not reveal alterations at all dosages of FLO used. In the liver of fish from all groups was verified abnormal glycogen deposit. Since that alteration was verified in all groups in similar degrees, including negative control one, it seems not to be associated with florfenicol therapy.

3.3.3 Therapeutic Efficiency of Florfenicol

Neither mortalities nor clinical signs of disease were observed in the negative control group during the experimental period. At the end of experiment, all fish of control group were euthanized by benzocaine overdose and submitted to bacteriological exam. They presented negative results for bacterial pathogens at bacteriology. The positive control group (challenged with SA 95-10 and not treated with florfenicol) showed cumulative mortality of 100% during the treatment period. The main clinical signs verified were anorexia, lethargy, melanosis, exophthalmia, and corneal opacity.

The standard dose of 10 mg/Kg of LW was unable to control the mortality caused by *S. agalactiae* infection, showing mortalities of 90% during treatment period (Fig. 1). This treatment dose presented a relative risk of 90% (*P* =0.1818) (Fig. 1; Tab.1). All dead fish of 10 mg/Kg group presented positive results for bacteriology. In contrast, the doses of 20 and 40mg/Kg of LW were efficient to control the mortalities caused by *S. agalactiae* infection during the treatment period. They promoted a relative risk reduction (RRR) of 95.3% of death by *S. agalactiae* in the first 10 days post-challenge (Tab. 1). The dose of 60 mg/Kg of LW was also efficient; however, lower RRR was verified (90%). The doses of 20, 40, and 60 mg/Kg of LW were statistically superior to standard dose (10 mg/Kg of LW) to control the mortalities caused by *S. agalactiae* infection in Nile tilapia (Tab. 2). Compared to this dose, they promoted a RRR of death by streptococcosis in the first 10 days after challenge of 94.8% for 20 and 40 mg/Kg of LW, and 89% for 60 mg/Kg of LW.

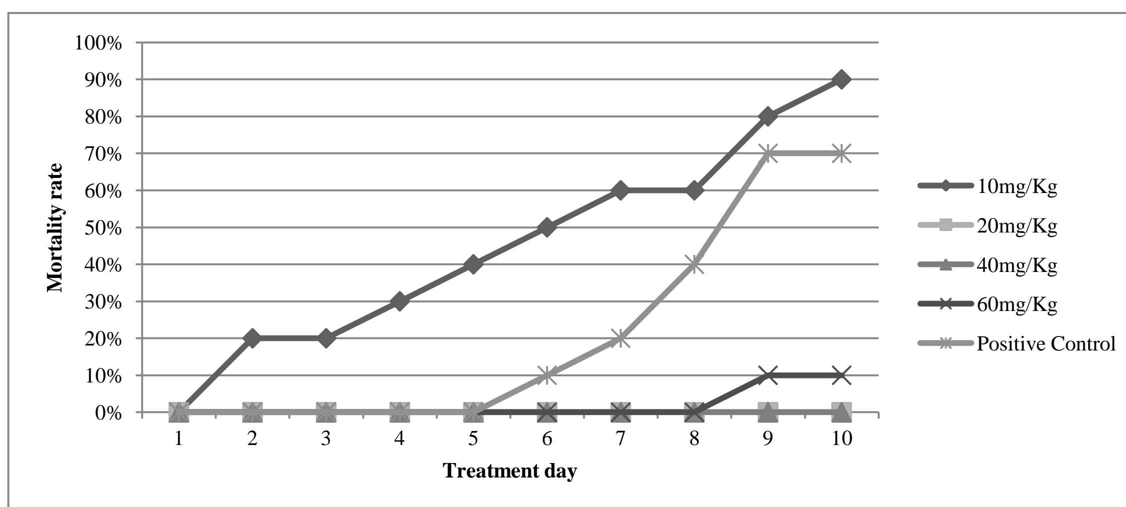

Figure 1: Cumulative mortality of the experimental groups challenged with *Streptococcus agalactiae,* and treated with different doses of FLO during the experimental period.

Table 1: Mortality data and statistical analyses of therapeutic efficacy of FLO, during the treatment period, compared to positive control group.

	Mortality									
	Yes		No		Total					
Treatment	n	%	n	%	N	%	RR	$IC_{95\%}$ [a]	RRR % (Efficacy)[b]	*P* value[c]
10mg/Kg	9	90	1	10	10	100	0.900	0.723-0.106	10	0.1818
20mg/Kg	0	0	10	100	10	100	0.047	0.003-0.716	95.3	0.000011
40mg/Kg	0	0	10	100	10	100	0.047	0.003-0.716	95.3	0.000011
60mg/Kg	1	10	9	90	10	100	0.100	0.015-0.642	90	0.000119
Positive control	10	100	0	0	10	100	--	--	--	--

RR. Relative risk
[a]IC 95%: confidence interval of 95%
[b]RRR: reduction relative risk
[c]*P* value: Fisher's exact test

Table 2: Mortality data and statistical analyses of therapeutic efficacy of FLO, during the treatment period, compared to group treated with FLO at dose of 10 mg/Kg of LW.

	Mortality									
	Yes		No		Total					
Treatment	n	%	n	%	N	%	RR	$IC_{95\%}$ [a]	RRR % (Efficacy)[b]	*P* value[c]
20mg/Kg	0	0	10	100	10	100	0.052	0.0034-0.797	94.8	0.000119
40mg/Kg	0	0	10	100	10	100	0.047	0.0034-0.797	94.8	0.000119
60mg/Kg	1	10	9	90	10	100	0.111	0.0170-0.721	89	0.000119
10mg/Kg	9	90	1	10	10	100	--	--	--	--

RR: Relative risk
[a]IC 95%: confidence interval of 95%
[b]RRR: reduction relative risk
[c]*P* value: Fisher's exact test

In spite of the promising results during the treatment period, the efficiency of florfenicol to control the mortalities reduced significantly during the overall experimental period (30 days) (Fig. 2). The doses of 20 and 60 mg/Kg of LW were not able to control the disease after the end of antibiotic administration, showing mortality rates of 90% in the observation period (Fig. 2). The dose of 40 mg/Kg of LW promoted relative risk reduction of 40% in the mortality caused by *S. agalactiae*. However, it was not statistically significant (*P*= 0.2507).

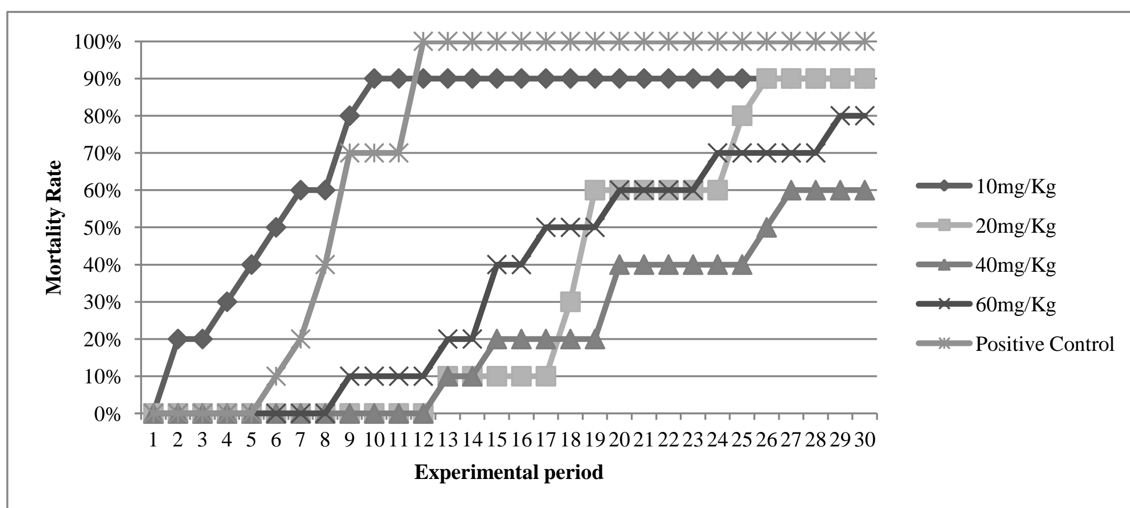

Figure 2: Cumulative mortality of the experimental groups challenged with *Streptococcus agalactiae* and treated with different doses of FLO during the period of observation.

In addition, at the end of the trial period, 25% and 100% of survival fish treated with the doses of 40 and 60 mg/Kg showed positive results in the brain and kidney bacteriology to *S. agalactiae*, characterizing a carrier state (Tab. 3).

Table 3: Mortality data and statistical analyses of therapeutic efficacy of FLO, during the whole experimental period.

	Mortality											
	Yes		No		Total		Carrier					
Treatment	n	%	n	%	N	%	S %	N %	RR	$IC_{95\%}$[a]	RRR % (Efficacy)[b]	*P* value[c]
20mg/Kg	9	90	1	100	10	100	0	10	0.9	0.732-1.106	10	1.0
40mg/Kg	6	60	4	40	10	100	25	75	0.4	0.361-0.995	40	0.2507
60mg/Kg	9	90	1	10	10	100	10	0	--	0.732-1.106	10	1.0
10mg/Kg	9	90	1	10	10	100	0	10	--	--	--	--

RR: Relative risk
[a]IC 95%: confidence interval of 95%
[b]RRR: reduction relative risk
[c]*P* value: Fisher's exact test

3.3.4 Persistence Assay

An *in vitro* persistence assay was carried out to evaluate the occurrence of this particular phenomenon in fish pathogenic *S. agalactiae*. The strain SA 95-10 was exposed to 100-fold its MIC value for florfenicol. The bacterium was able to survive at this concentration and persistent cell subpopulation was triggered. In exponential growth, an initial mortality was verified after the exposition of bacterial suspension to antibiotic, decreasing the bacterial counting from 1.84 x 10^8 to 1.44 x 10^6 CFU/mL. This bacterial concentration was kept approximately constant until 12 hours of exposition (1.44 x 10^6 CFU/mL) (Fig. 3). Similar results were verified to stationary phase bacterial suspension. The bacterial concentration decreased from 5 x 10^8 CFU/mL to 6.8 x 10^6 CFU/mL and maintained similar concentration until 12 hours of exposition (2.8 x 10^6 CFU/mL) (Fig. 4).

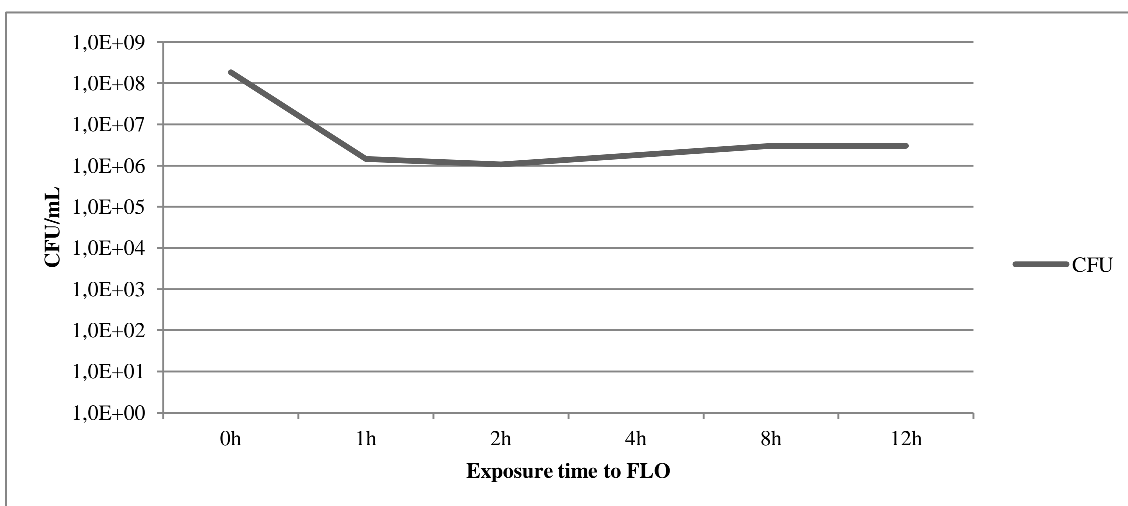

Figure 3: Exponential grown SA 95-10 strain, treated with 100-fold MIC of FLO over the time.

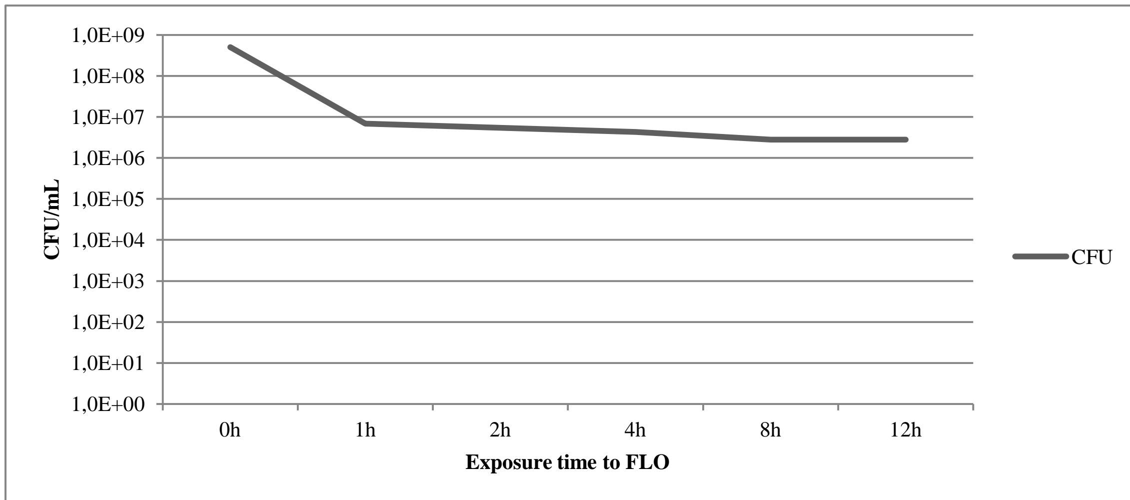

Figure 4: Stationary grown SA 95-10 strain, treated with 100-fold MIC of FLO over the time.

3.3.5 Cohabitation Assay

Neither mortalities nor clinical signs of disease were observed in the negative control group during the experimental period. At the end of experiment, they were euthanized by benzocaine overdose and submitted to bacteriological exam. All of them presented negative results for *S. agalactiae*.

All challenged fish with *S. agalactiae* and treated with 20 and 40 mg/Kg LW of FLO did not survive (Fig. 5). The first mortalities were verified 24 post-cohabitation. Five fish from the group that were transferred to group C died in the first 72 hours of cohabitation. In the other group, challenged fish reached 100% of mortality until the 13th day of cohabitation. The main clinical signs verified in challenge fish were melanosis, erratic swimming, exophthalmia, and corneal opacity. All SA challenged fish presented positive results in the bacteriology to *S. agalactiae*.

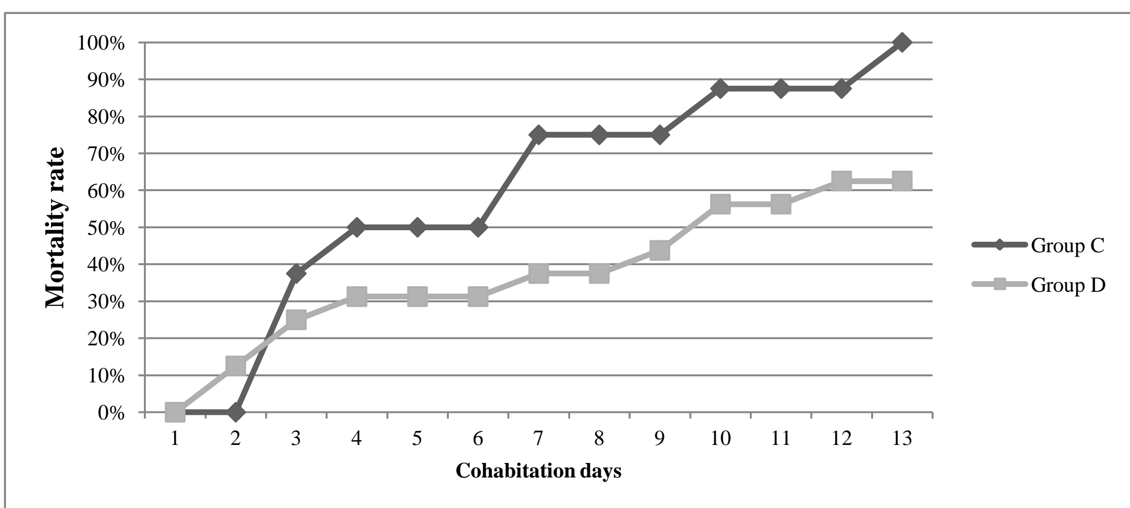

Figure 5: Cumulative mortality of fish challenged with *Streptococcus agalactiae* in cohabitation assay. The fish of Group A were treated with 20mg/Kg LW of FLO and cohabited in group C, and fish of Group B treated with 40mg/Kg LW and cohabited in group D.

The bacterium was successfully transmitted from SA challenged fish and treated with FLO to healthy fish. The healthy fish belonging to the Group C (healthy animals cohabitated with animals challenged with SA 95-10 and treated with 20mg/Kg LW of FLO) showed the first clinical signs (anorexia, lethargy, and exophthalmia) on the eighth day of cohabitation, and the first mortality, occurred in the tenth day (Fig. 6). Healthy fish belonging to group D (healthy fish cohabitated with fish challenged with SA 95-10 and treated with 40 mg/Kg LW of FLO) showed the first clinical signs (anorexia, lethargy, melanosis, exophthalmia and corneal opacity) on the eleventh day of trial and the first death occurred at the thirteenth day of assay (Fig. 6). The mortality rates in the healthy fish were 50% e 40% respectively in the groups C, D (Tab. 4).

All dead fish presented positive results in the bacteriology exam to *Streptococcus agalactiae*. Besides, at the end of experimental period, one asymptomatic fish of group D was shown positive to *S. agalactiae*, characterizing a carrier state.

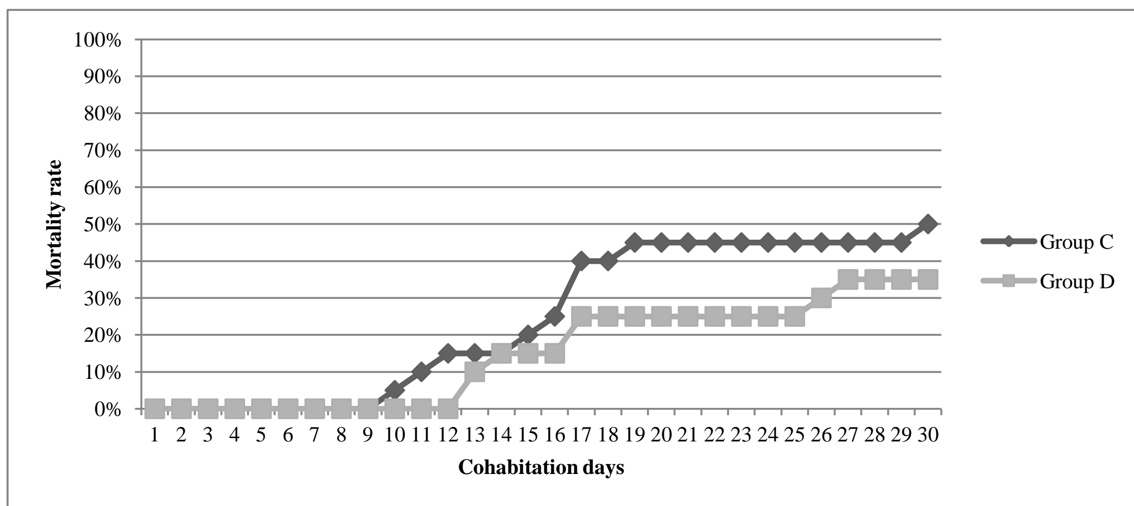

Figure 6: Cumulative mortality of healthy fish cohabitated with fish challenged with *Streptococcus agalactiae* and treated with 20mg/Kg of LW (Group C) and 40mg/Kg of LW (Group D) of FLO, during the cohabitation period.

Table 4: Mortality of healthy fish cohabitated with fish challenged with *Streptococcus agalactiae* and treated with 20mg/Kg LW (Group C) and 40mg/Kg LW (Group D) of FLO during the cohabitation period.

	Mortality					
	Yes		No		Total	
Cohabitation Groups	n	%	n	%	N	%
C	5	50	5	50	10	100
D	4	40	6	60	10	100
Negative control	0	00	10	100	10	100

3.4 Discussion

Firstly the safety of florfenicol therapy for Nile tilapia juveniles in higher doses than 10 mg/Kg of live weight was addressed. There was no difference in the consumption of ration in fish fed with medicated feed until the highest dose evaluated (60 mg/Kg of LW), during the treatment period of 10 consecutive days. Reiterating the results found in assays with catfish (*Ictalurus punctatus*), sunshine bass (*Morone chrysops* x *Morone saxatilis*) and Nile tilapia (*Oreochromis niloticus*) (Gaunt et al., 2010; Bowker et al., 2010; Gaunt et al., 2015; Darwish et al., 2012). Those results, contrasted with previous studies performed by Gaikowski et al. (2003) and Gaikowski et al. (2013). This author found that doses higher than 30 and 40 mg/Kg LW FLO when administered for 20 consecutive days, result in a decrease of consumption of the medicated feed.

Furthermore, tissue injures were verified in fish fed with high doses of florfenicol, with direct correlation between lesions degrees and antibiotic doses. In this study, the same pattern of dose dependent lesions was observed in animals treated with 40 e 60mg/Kg LW of FLO. However, the abnormal glycogen deposit in hepatocytes was similar in fish receiving medicated and non-medicated feed, it may not be caused by florfenicol therapy. The main difference between our and previous reports was the composition of florfenicol used. This study used analytical standard grade florfenicol, in contrast to Gaikowski et al. (2003) and Gaikowski et al. (2013) that evaluated the commercial antibiotic Aquaflor (florfenicol 50%; MSD, USA). It might suggest that there is some compound in the premix of commercial antibiotic (lactose monohydrate and povidone), which may inhibit the consumption of medicated feed, when this one is administered for longer periods than recommended, potentially causing alterations in feed behavior when doses higher than 30 and 40 mg/Kg of live weight were used.

After the safety assay, the therapeutic efficacy of FLO was tested. The florfenicol in doses from 10 to 60 mg/Kg of live weight was shown to be ineffective to control *S. agalactiae* infection in Nile tilapia. It was just able to control the mortalities during the treatment period. That result disagree with all previous studies that evaluated the florfenicol as a treatment measure against bacterial infections in fish, mainly because post-treatment period, wherein was observed the recurrence of the disease, were shorter (12 to 17 days) when compared to this study (Gaunt et al., 2010; Bowker et al., 2010; Darwish et al., 2007; Roiha et al., 2011; Gaunt et al., 2015). To the best of our knowledge it is first description of the failure of florfenicol to control a bacterial disease in animal, including fish. Doses of 20 and 40 mg/Kg of LW were able to control the mortality during the treatment period, but, they were not capable to eliminate bacteria from the host. After the ends of administration, the treated fish presented clinical signs of streptococcosis and mortalities from 60% to 90% of the groups. Similar results were verified in the treatment of infectious in the urinary tract caused by *E. coli* when quinolones, trimethoprim and aminoglycosides were administered (Pallet et al., 2010; Can et al., 2015), in pneumonia caused by *S. pneumoniae* treated with fluoroquinolones (Fuller et al., 2005) and infection by *Staphylococcus aureus* in which were administered β-lactam (Chang et al., 2003) in humans.

In addition, the florfenicol was not able to avoid the transmission of bacteria from challenged and treated to health fish as verified in the cohabitation assay. Besides not being effective in the treatment, the drug did not affect the ability to transmit of bacterial virulence. Being able to indicate the possibility of the antibiotic alter the expression of some genes, that are responsible

for the virulence factors, ranging them with drug concentrations or could function as a trigger for the release of endotoxins that interact with host cells, suggesting the pathogen's ability to modulate their response as variations of drugs and their concentrations, as with the *M. tuberculsis* and *P. aeruginosa* (Martinez et al., 2002).

The treatment failure of florfenicol to control *S. agalactiae* infection in Nile tilapia could not be explained by the antibiotic resistance of the strain, pharmacokinetic/pharmacodynamics of the drug, and inadequate dose used. The strain SA95-10 was shown to have a florfenicol MIC value of 1 µg/ml, being considered wild-type or sensitive. Also pharmacokinetics/pharmacodynamics and inadequate dosages may not be considered, since FLO when orally administered at a dose of 10 mg/Kg of LW is capable to reached higher concentrations in tissues, ten times greater than the MIC value of SA95-10 after 12 h (Feng et al., 2009).

Recently, a phenomenon known as persistence has been determined as one of the forms of bacterial resistance to antibiotics. This consists in a bistable behavior, which part of bacterial population switches to a dormant state during the presence of antibiotic. After treatment ends, that subpopulation recover their vegetative and pathogenic state (Willenborg et al., 2014; Zhang et al., 2014; Fauvart et al., 2011; Lewis, 2008). Persistent population are genetically identical to sensitive cells, which distinguishes them from "conventional resistance", when genetic alterations are verified (Fauvart et al., 2011). This kind of behavior is not well understood and some authors might be associated with epigenetic factors, that are capable of modulating gene expression through bistable form, besides to altering the gene expression of regulatory genes that are connected to persistence, and may confer several levels of persistent cells within the same population or a response to environmental factors such as nutrient depletion, temperature, pH, sos-system activation, antimicrobials (exposure time, different concentrations, different classes of drugs) (Zhang et al., 2014; Hughes et al., 2014).That persistence phenomenon have been associated with antibiotic treatment failures against human and animal diseases, such tuberculosis, cystic fibrosis and pneumonia caused by *Pseudomonas aeruginosa*, urinary infection by *E. coli*, and pneumonia by *S. suis* (Fauvart et al.,2011; Willenborg et al., 2014).

The therapeutic inefficacy of florfenicol to control *S. agalactiae* infection in Nile tilapia could be due to a persistence capacity of this bacterium. To address this hypothesis, the strain SA95-10 was submitted to persistence test assay. After exposed to 100-fold its MIC value of florfenicol, the bacterium remained viable and showed a classical persistence behavior, similar to verified to other animals pathogens (Willenborg et al., 2014). It is the first description of antibiotic failure in fish pathogen attributed to bacterial persistence. The mechanisms involved in the development and regulation for persistence are not elucidated nowadays (Fauvart et al., 2011). However, it is possible to affirm, that this characteristic results in a very difficult obstacle to control streptococcosis in Nile tilapia farming. Future studies should be conducted in order to elucidated, the physiological and molecular mechanisms that allow the *S. agalactiae* to develop the antibiotic persistence.

3.5 Conclusion

The florfenicol at dose of 10mg/Kg LW is not efficient to control the infections caused by *S. agalactiae* in Nile tilapia juveniles. Doses of 20 and 40mg/Kg LW were shown to be able to control the mortality during the treatment period. However, recurrence of the disease occurred after treatment period.

FLO is unable to eliminate the bacteria from de host in all doses tested, inducing carrier state in treated fish. This inefficiency in eliminate the bacterial agent might be associate to the antibiotic persistence phenomenon exhibited by this fish pathogenic *S. agalactiae*.

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4. Final Considerations

This study demonstrated that florfenicol in the standard dose of 10 mg/Kg of LW is not efficient for the treatment and control of infections caused by *S. agalactiae* in Nile tilapia. Higher doses (20 and 40 mg/Kg of LW) should be used during outbreaks to control the mortalities and probably more than once, since the disease can recur after treatment finished.

The antibiotic persistence is an ability exhibited by fish pathogenic *S. agalactiae*, which may interfere in the efficiency of antibiotic therapy against streptococcosis. Future studies should be performed to determine the physiological and molecular mechanisms that enable emergence of persistent *S. agalactiae* populations.