UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

ATIVIDADE ANTIMICROBIANA *IN VITRO* E *IN VIVO* DO PEPTÍDEO LL-37 CONTRA *Streptococcus agalactiae* EM TILÁPIA-DO-NILO

Elielma Lima de Sousa

Bióloga

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Discente: Elielma Lima de Sousa

Orientadora: Profa. Dra. Fabiana Pilarski

Dissertação apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Mestre em Microbiologia Agropecuária**.**

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TÍTULO DA DISSERTAÇÃO: ATIVIDADE ANTIMICROBIANA *in vitro* E *in vivo* DO PEPTÍDEO LL-37 CONTRA *Streptococcus agalactiae* EM TILÁPIA-DO-NILO

AUTORA: ELIELMA LIMA DE SOUSA
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Jaboticabal, 24 de julho de 2020

DADOS CURRICULARES DO AUTOR

Elielma Lima de Sousa – nascida em São Luis, Estado do Maranhão, em 05 de agosto de 1992. Ingressou no Curso de Ciências Biológicas (Licenciatura e Bacharelado) em 2011 na Universidade Estadual do Maranhão– Câmpus Paulo VI em São Luis, obtendo o título de bióloga em 2017. Durante a Graduação, realizou graduação sanduiche de um ano e quatro meses (2013-2014) na Universidade de Alberta, Canadá. Ainda como estudante de graduação, realizou outro intercâmbio, desta vez na Argentina. A carreira cientifica como pesquisadora teve início no segundo ano de graduação (2012), quando desenvolveu o primeiro trabalho de Iniciação cientifica (2012-2013) Após a realização deste trabalho, tornou-se bolsista de Incitação cientifica por mais dois anos (2015-2017). Os trabalhos de pesquisa desenvolvidos resultaram em artigos e resumos apresentados e publicados em anais de eventos científicos locais e internacionais. Realizou três estágios extracurriculares. Ingressou no curso de mestrado em março de 2018, no programa de Pós-graduação em Microbiologia Agropecuária, na Universidade Estadual Paulista “Júlio de Mesquita Filho” – FCAV – Faculdade de Ciências Agrárias e Veterinárias (Câmpus Jaboticabal).

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"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Atividade *in vitro* e *in vivo* do peptídeo LL-37 contra *Streptococcus agalactiae* em tilápia-do-nilo"**, protocolo nº 011794/19, 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 13 de setembro de 2019.

Vigência do Projeto	20/09/2019 a 15/02/2020
Espécie / Linhagem	*Oreochromis niloticus* (Tilápia do Nilo)
Nº de animais	204
Peso / Idade	100 gramas
Sexo	Machos
Origem	Piscicultura Tuiuiu, SP

Jaboticabal, 13 de setembro de 2019.

Prof. Dr. Everlon Cid Rigobelo
Vice-Coordenador – CEUA

Faculdade de Ciências Agrárias e Veterinárias
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RESUMO - A estreptococose é uma doença bacteriana que causa grandes perdas econômicas na produção de tilápia. O *Streptococcus agalactiae* é um dos principais patógenos causadores de infecção em tilápia, em várias regiões do mundo. Atualmente, no caso de infecções microbianas em peixes, existem apenas duas moléculas licenciadas para uso na aquicultura. Os peptídeos com potencial antimicrobiano são compostos promissores no desenvolvimento de uma nova classe de antimicrobianos. O peptídeo LL-37, uma catelicidina humana caracterizada como um antibiótico de amplo espectro possui propriedades quimiotáticas e imunomoduladoras, as quais inibem a colonização de patógenos. Desta forma, neste trabalho objetivou-se avaliar a atividade antimicrobiana do peptídeo LL-37 em tilápia-do-Nilo infectadas com *S. agalactiae.* Foi determinada a concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) do LL-37 para *S. agalactiae*. Adicionalmente, avaliou-se a atividade hemolítica desse peptídeo e sua eficácia no tratamento da estreptococose em tilápia-do-Nilo*.* Os resultados demonstrados neste estudo mostraram que a concentração inibitória mínima e a concentração bactericida mínima do LL-37 para *S. agalactiae* foi 31,25 µg/mL. Os parâmetros hematológicos, bioquímicos e imunológicos apresentaram valores sem diferenças significativas entre os grupos. Nossos resultados sugerem que o peptídeo LL-37 é um potente antimicrobiano contra *S. agalactiae in vitro* e não afeta a viabilidade celular dos peixes. Não foi possível, com a dose 15 mg/kg controlar a mortalidade da tilápias infectadas com a bactéria, contudo, foi possível eliminar o patógeno dos peixes sobreviventes, o que pode ser positivo no controle de estreptococose recorrente.

Palavras-chave: peptídeo, ação antimicrobiana, tratamento

ANTIMICROBIAL ACTIVITY *IN VITRO* AND *IN VIVO* OF LL-37 PEPTIDE AGAINST *Streptococcus agalactiae* IN NILE TILAPIA

ABSTRACT - Streptococcosis is a bacterial disease. It causes elevate economic savings in tilapia production. *Streptococcus agalactiae* is a pathogen wich cause motality of red tilapia in many regions of the world. Currently, microbial infections in fish are treated only with two molecules which one are licensed for use in aquaculture. Peptides with antimicrobial potential are promising compounds in the development of a new class of antimicrobials. LL-37 peptide has broad spectrum bacterial activity. In the present study, we aimed to evaluate antimicrobial activity of peptide LL37 in Nile tilapia infected with *S. agalactiae*. The minimum inhibitory concentration (MIC) and the concentration bacterial resistance (CBR) from LL-37 to *S. agalactiae* were analyzed. Additionally, hemolytic activity of this peptide was tested. As well, we evaluated efficacy of LL-37 in the treatment of streptococcosis in Nile tilapia. As the results, the minimum inhibitory concentration and the minimum bacterial concentration of LL-37 for *S. agalactiae* were 31.25 µg/mL. LL-37 in the treatment of infectious diseases by *S. agalactiae* was not effective at the dose of 15 mg/kg. Hematological, biochemical and immunological parameters include values within the range for healthy fish. Changes in blood cells and tissue of brain, kidney, liver and splen were found. Our results suggest LL-37 is a potent antimicrobial against *S. agalactia* in vitro and it does not affect fish cell viability. We conclude, It was not possible to control the mortality of streptococcosis with the dose of 15 mg/kg of LL-37, however, it eliminated either pathogen in surviving fish.
Keywords: peptide, antimicrobials, treatment

CAPÍTULO 1 – Considerações gerais

1 INTRODUÇÃO

A ausência de um manejo adequado nos viveiros e tanques-rede utilizados nos diferentes sistemas de produção pode levar a deterioração da qualidade da água, com consequências para todo o sistema aquático e também para a criação de organismos aquáticos (Meneghine, 2013).

Assim, faz se necessário o monitoramento dessa atividade, sobretudo nos lugares onde há rápida expansão e concentrada densidade de organismos nestes sistemas de criação (Dias e Sipaúba-Tavares, 2012).

Alterações físicas e químicas do meio aquático são críticas para desenvolvimento de doenças parasitárias e bacterianas em peixes. A combinação de um patógeno presente na água, um hospedeiro suscetível e um ambiente com baixa qualidade define a patogênese de muitas doenças infecciosas em peixes (Plumb e Hanson, 2011).

Nos últimos anos, a criação de tilápia tem sido prejudicada economicamente por elevada taxa de mortalidade decorrente principalmente de doenças bacterianas, e dentre elas, destaca-se a estreptococose. De acordo com Figueiredo et al. (2006), infecções causadas por *Streptococcus* spp. aumentaram nos últimos anos por elevação na ocorrência de casos clínicos, principalmente, associados a *Streptococcus agalactiae (S. agalactiae)* com perdas na tilapicultura mundial.

A bactéria *S. agalactiae* além das mortalidades que provoca, também apresenta potencial de infectar mamíferos, humanos e aves, constituindo-se em um risco para a saúde pública. Em neonatos humanos, o *S. agalactiae* é o principal agente etiológico de septicemia, com evolução para outros quadros clínicos graves (Domelier et al., 2008).

Atualmente, no caso de infecções microbianas em peixes, existe apenas dois antimicrobianos licenciados para uso na aquicultura, o florfenicol (específico para o tratamento da estreptococose) e a oxitetraciclina. A ineficácia desses medicamentos

pode levar ao uso irregular de outros antimicrobianos e aplicação de elevadas doses favorecendo a resistência bacteriana, o que pode gerar impacto ambiental e acúmulo de resíduos no produto destinado ao consumo humano (Hölmstrom et al., 2003).

A resistência de patógenos aos antimicrobianos licenciados tem se tornado um problema mundial de interesse na produção aquícola. Vários microrganismos multirresistentes vêm surgindo atualmente e, em sua grande parte, são bactérias causadoras de infecções em peixes (Costa et al., 2008; Amarante et al., 2018).

A presença de bactérias resistentes aumenta tanto a mortalidade como também o custo do tratamento, a evolução do quadro infeccioso e a duração da doença (Fernandes et al., 2014). Para solucionar essa problemática, diversas estratégias vêm emergindo. Uma delas é através de estudos com peptídeos antimicrobianos (PAMs), os quais são componentes do sistema imune de vertebrados e invertebrados e a primeira linha de defesa contra a invasão de microrganismos (Brogden, 2005; Brown e Hancock, 2006).

Os PAMs podem ser utilizados em combinação ou não com outros fármacos e são compostos promissores no desenvolvimento de uma nova classe de antimicrobianos, pois apresentam características como a capacidade de eliminar bactérias Gram positivas e negativas, e atividade contra patógenos já multirresistentes aos fármacos comerciais (Da Silva et al., 2013; Krelin et al., 2016; Santos-Filho et al., 2017).

Os PAMs possuem atividade antimicrobiana já comprovada contra diversos patógenos (Kim et al., 2002; Rahnamaeian, 2011; Bahar e Ren, 2013 Santos-Filho et al., 2017; Kim et al., 2017; Liu et al., 2018). O tratamento com peptídeos se torna uma alternativa ao uso de antimicrobianos, representando uma possibilidade interessante no controle de surtos de doenças.

Entre os peptídeos mais utilizados em estudos científicos destaca-se o peptídeo LL-37, um peptídeo catiônico pertecente a família das catelicinas e presente nos humanos, com amplo espectro de ação, predominantemente encontrado e secretado em vários tecidos e fluidos (De Smet e Contreras, 2005; Cederlund et al., 2011). E é um peptídeo com capacidade de neutralizar a parede das bactérias (LPS) e interromper o seu crescimento (Cederlund et al., 2011).

Por ser um peptídeo com atividade antimicrobiana promissora (Mookherjee e Hancock, 2007; Overhage et al., 2008; Molhoek et al., 2009; Hell et al., 2010, Kanthawong et al., 2010; Thennarasu et al., 2010; Kanthawong et al., 2012) o LL-37 foi selecionado para ser usado neste trabalho, uma vez que não foram encontradas informações na literatura sobre o seu uso no tratamento de infecções causadas por *S. agalactiae* em tilápia-do-nilo.

Desta forma, neste estudo objetivo-se avaliar a atividade antimicrobiana *in vitro* e *in vivo* do peptídeo LL-37 em tilápia-do-Nilo infectadas com *S. agalactiae*; a atividade citotóxica do peptídeo nas células sanguíneas e alerações nos parâmetros hematológicos, imunológicos e bioquimicos de juvenis de tilápia.

2 REVISÃO DE LITERATURA

2.1 Piscicultura no Brasil

A produção de peixes continentais vem aumentando a cada ano. De acordo com dados da FAO (2019), em 2017 a produção aquícola no Brasil representou 45,8% da produção total de pescado nacional. Nesse cenário, em 2018 a piscicultura brasileira produziu 579,3 mil toneladas de pescado (FAO, 2019).

Desde 2014, a cadeia produtiva acumula uma expansão de 24,83% (PeixeBr, 2019). A tilápia (*Oreochromis* sp.) esta entre os principais organismos mais produzidos no mundo, sendo que, o Brasil foi o quinto maior produtor de tilápia do mundo em 2017 (FAO, 2019), sendo que os estados brasileiros com maior produção de tilápia são Paraná, São Paulo e Santa Catarina (PeixeBr, 2019).

Com a intensificação da produção, também aumentaram o surgimento de doenças. A multiplicação dos microrganismos no ambiente está relacionada ao manejo e a degradação da qualidade da água devido ao aumento da quantidade de matéria orgânica. Nessas condições, a queda na concentração de oxigênio

dissolvido na água propicia o aumento de estresse nos peixes, predispondo os animais as doenças infecciosas e parasitárias, que acarretam prejuízos econômicos aos produtores (Ranzani-Paiva, 1997; Carraschi et al., 2011).

Dentre as doenças de origem bacteriana, as do gênero *Streptococcus* são hoje os principais patógenos de peixes tropicais produzidos em todo o mundo nas últimas cinco décadas. No Brasil, a ocorrência de surtos de estreptococose na criação de tilápia é um dos principais desafios para a viabilidade produtiva da atividade (Salvador et al. 2005; Figueiredo et al. 2006).

2.2 Estreptococose: um desafio para tilapicultura mundial

A estreptococose é uma doença causada por bactérias do gênero *Streptococcus* (Plumb e Hanson, 2011; Shoemaker e Klesius, 1997). O *Streptococcus* spp. é um dos patógenos mais encontrados na piscicultura, causando grandes perdas na produção de peixes marinhos e de água doce em regiões quentes. Embora sua identificação inicial tenha corrido em 1976, a septicemia causada por *Streptococcus* spp. ainda é um problema sanitário em sistemas de criação intensiva de tilápia em todo o mundo (Wendy Agnew e Barnes, 2007).

O gênero *Streptococcus* spp. é formado por cocos Gram-positivos, variando entre 0,5-0,2 µm de diâmetro, ocorrendo em pares ou cadeias quando crescem em meio líquido. São imóveis, não formam esporos e são anaeróbios facultativos (Holt et al., 1994).

Além disso, os estreptococos são bactérias patogênicas com aderência às superfícies epiteliais, invasão as células do epitélio e do endotélio e causam injúria direta aos tecidos, características que contribuem para sua virulência (Nizet e Rubens, 2000). Kitao et al. (1981) observaram que esses patógenos podem sobreviver mesmo em condições ambientais adversas, uma vez que estreptococos foram encontradas na água e em sedimentos de pisciculturas em diferentes estações do ano.

As principais espécies responsáveis pela estreptococose são *S. iniae, S. agalactiae* e *S. dysgalactiae*, provocando sinais clínicos muito semelhantes em seus hospedeiros (Evans et al., 2002; Shelby et al., 2002). A bactéria infecta peixes marinhos como o *Notemigonus crysoleucas*, *Pampus argenteus, Sparus auratus* e *Liza klunzingeri* (Robinson e Meyer, 1966; Evans et al., 2002; Duremdez et al., 2004) e peixes de água doce como *Oreochromis niloticus, Oreochromis aura X O. niloticus* e *Oncorhynchus mykiss* (Salvador et al., 2003; Figueiredo et al., 2006; Mian et al., 2009; Pourgholam et al., 2011).

Wu (1970) relatou o primeiro isolamento de *Streptococcus* spp. em tilápia e desde então, este gênero tem sido isolado e identificado como responsável por elevados prejuízos na piscicultura em diversos países.

O primeiro isolamento de *S. agalactiae* causando doença em peixe foi descrito em 1966, nos Estados Unidos, por Robinson e Meyer (1966). Nos anos seguintes, novos isolamentos de *S. agalactiae* foram descritos em peixes nos Estados Unidos, Kuwait, Israel, Brasil, Bélgica, Honduras, Tailândia, Malásia e Iran (Plumb et al., 1974; Evans et al., 2002; Salvador et al., 2003, 2005; Duremdez et al., 2004; Figueiredo et al., 2006; Mian et al., 2009; Zamri-Saad, Amal e Siti-Zahrah, 2010).

No Brasil, o primeiro isolamento de *Streptococcus agalactiae* foi realizado por Salvador et al. (2005), em tilápia-do-Nilo com sinais neurológicos (natação errática e em movimentos circulares). Após esse primeiro isolamento, o patógeno tem sido isolado de animais homeotermos terrestres ou heterotermos aquáticos, apresentando assim ampla abrangência de hospedeiros em todo o mundo (Evans et al., 2002).

Mian et al. (2009) ao investigar aspectos da epidemiologia, transmissão e virulência das infecções por *S. agalactiae* em produções de tilápia no Brasil, observou variação no peso dos peixes infectados e elevada taxa de mortalidade.

Salvador et al. (2003) descreveram como principais sinais clínicos e lesões macroscópicas nos peixes com *Streptococcus* sp.: hepatomegalia, esplenomegalia, lesão na pele e na base das nadadeiras e exoftalmia com opacidade de córnea. No estudo de Figueiredo et al. (2006), outras características importantes foram encontradas, como escurecimento dos peixes, pequenas lesões na pele com perda

de escamas e petéquias na base das nadadeiras ventrais, natação em movimentos circulares e alta mortalidade.

A bactéria *S. agalactiae* ocasiona altas taxas de mortalidade, que pode atingir até 90% do plantel (Mian et al., 2009). Alguns autores sugeriram que isolados de tilápias-do-Nilo com *S. agalactiae* de diferentes hospedeiros podem compartilhar fatores de virulência envolvidos na patogênese das infecções. Em infecções em humanos, a sorotipagem do *S. agalactiae* é a principal ferramenta epidemiológica utilizada. Dez tipos capsulares já foram descritos (Slotved et al., 2007; Maisey et al., 2008; Rajagopal, 2009).

A patogênese de *S. agalactiae* envolve septicemia e colonização de órgãos como cérebro, rins e intestinos (Evans et al., 2002; Pasnik et al., 2005; Figueiredo et al., 2006; Mian et al., 2009; Zamri Saad et al. 2010). A transmissão do *S. agalactiae* ocorre de forma horizontal, através do contato entre peixes portadores assintomáticos ou peixes infectados (Evans et al., 2002; Mian et al., 2009).

Dada a gravidade da infecção causada por *S. agalactiae* em humanos, muitos fatores de virulência e os mecanismos envolvidos na patogênese da doença em mamíferos têm sido descritos e caracterizados (Rajagopal, 2009). Em peixes, pouco ainda se sabe sobre a patogênese dos processos infecciosos causados por essa bactéria. Em estudos experimentais, isolados de *S. agalactiae* oriundos de humanos foram capazes de infectar tilápias-do-Nilo (Evans et al., 2002; Pereira et al., 2010). Esses achados implicam que fatores de virulência e mecanismos de invasão são compartilhados entre isolados de diferentes hospedeiros.

2.3 Tratamento da estreptococose na piscicultura

O tratamento da estreptococose é realizado com a utilização do antimicrobiano florfenicol. Todavia, o uso inadequado desse antimicrobiano ou de qualquer outro produto químico não licenciado pode levar a degradação do meio ambiente, a seleção de cepas resistentes e risco à saúde do consumidor (Grisez e Ollevier, 1995; Swain et al., 2002; Klesius et al., 2004; Serrano, 2005).

A busca por tratamentos novos, que causem menores impactos ambientais, menor risco de resistência bacteriana e sejam eficazes contra bacterioses é cada vez mais evidente. A grande dificuldade no tratamento das infecções bacterianas está relacionada ao surgimento de cepas resistentes aos principais antimicrobianos existentes no mercado e a eficácia do tratamento, uma vez que a maioria dos peixes doentes são anoréxicos e não consomem a quantidade adequada do antimicrobiano.

Assim, faz se necessário o uso de tratamentos alternativos com menos impactos negativos, tanto aos animais quanto à saúde do consumidor. Neste contexto, os peptídeos antimicrobianos (PAMs) oferecem uma alternativa promissora em contraste aos antimicrobianos convencionais para o tratamento e prevenção de doenças na piscicultura.

2.4 Uso de peptídeos como antimicrobiano

Peptídeos antimicrobianos (PAMs) são componentes do sistema imune inato de vertebrados e invertebrados e possuem um amplo espectro de atividade contra diversos microorganismos (Fernandez-Lopez et al., 2001; Arrighi et al., 2002; Zasloff, 2002; Zhang et al., 2002; Gomes et al., 2005). A ação dos peptídeos antimicrobianos não é limitada a eliminar microorganismos, eles possuem vários efeitos biológicos importantes como respostas inflamatória e imunológica (Hancock e Diamond, 2000; Brown e Hancock, 2006).

O uso de peptídeos no tratamento de infecções se torna uma alternativa ao uso de antibióticos, com menor risco do surgimento de bactérias resistentes (Rodriguez-Rojas, Makarova e Rolff, 2014; Parachin e Franco, 2014; Maria-Neto et al. 2015), representando uma alternativa interessante no controle de surtos de infecções.

O mecanismo de ação dos PAMs está diretamente ligado a distruição da bicamada da membrana de células alvos (Marcotte, et al., 2003). A membrana pode ser destruída pela formação de poros através do mecanismo “barrel-stave’, ou formação de canais de íons específicos, ou de acordo com o modelo toroidal

(Brogden, 2005). Contudo, alguns peptídeos induzem a morte celular por ativação de alvos intracelulares (Brogden, 2005; Brown e Hancock, 2006).

Alguns peptídeos combinados com antimicrobianos convencionais mostraram um efeito sinérgico, potencializando a sua ação *in vivo*, possivelmente promovendo seu acesso a célula bacteriana (Park et al., 2011). Outros PAMs podem ser utilizados no controle de infecções sistêmicas, pois auxiliam na redução do dano tecidual provocado pela resposta inflamatória à infecção, enquanto estimulam ao mesmo tempo, a atividade antimicrobiana do sistema imune (Upton et al., 2012).

Peptídeos foram utilizados também para impedir a colonização e o crescimento de microrganismos em materiais poliméricos sintéticos, tais como cateteres intravenosos de uso médico (Melvin et al., 2016).

Porém, ainda são necessários estudos para o conhecimento do potencial antimicrobiano de diversos peptídeos para uso no tratamento de doenças em peixes e seus efeitos tóxicos sobre as células do hospedeiro. Quanto ao uso de peptídeos no tratamento de infecções causadas por *S. agalactiae* em tilápia-do-Nilo, não foram encontradas informações na literatura.

Os peptídeos desempenham diversas funções, como antimicrobiana, modulação do sistema imunológico, antioxidantes e fatores antitumorais (Valero et al., 2013). Alguns estudos tem demonstrado a ação antimicrobiana desses elementos contra bactérias patogênicas de peixes (Jia et al., 2000; Huang e Chen, 2013; Pan et al., 2012; Chettri et al., 2017).

2.5 Peptídeos Antimicrobianos na Aquicultura

O sistema imunológico dos peixes é robusto e atua com a primeira linha de defesa, sendo dividido em resposta imune inata e adaptativa (Rauta et al., 2012).

Os PAMs, nos peixes pertencem ao sistema imune inato e possuem a função de defesa cujo mecanismo de ação ainda não foi elucidado (Valero et al., 2013; Rauta et., 2012). Os teleósteos expressam um grande número de peptideos, e muitos deles foram isolados mas ainda não testados (Primor 1980; Cole et al. 1997;

Park et al. 1997; Park et al.,1998; Zhang et al. 2004; Huang et al. 2007; Pan et al. 2007; Valero et al., 2020).

Peptídeos líticos sintéticos apresentaram efeitos tóxicos em patógenos bacterianos de peixes que causam perdas significativas na produção comercial (Kelly et al., 1990).

A adição de peptideos a dieta dos peixes pode ativar genes relacionados ao sistema imunológico e controlar infecções causadas por *Vibrio vulnificus* (*V. Vulnificus*). O BL-21 (recombinante de epinecidina-1) misturado à forragem reduziu significativamente a mortalidade de *Polyprion americanus* e *Danio rerio* infectado experimentalmente com *V. Vulnificus* (Pan et al., 2012).

Enquanto humanos e camundongos tem uma única catelicidina, a maioria das espécies possui mais de um membro desta família. No salmão do Atlântico, dois genes da catelicidina foram identificados, o asCATH1 e o asCATH2 (Chang et al., 2006).

Bridle et al. (2011) observaram que expressão de asCATH2 e asCATH1 é induzida na presença de infecção por *Y. ruckeri*. Além disso, sugerem que essas catelicidinas têm papel imunomodulador em peixes.

Wang et al. (2010) demonstraram que os peptídeos sintéticos epinecidina-1 e a hepcidina 1–5 têm atividades antivirais *in vivo,* em desafio viral o peixe *Polyprion americanus* apresentou redução do agravamento da infecção causada pelo vírus.

O uso de peptideos no controle de infecções em peixes mostra-se uma alternativa promissora para reduzir perdas na produção, sendo um novo caminho a ser pecorrido, com muitas lacunas a serem preenchidas sobre informações de seu uso, função e aplicação na aquicultura.

2.6 Peptídeo LL-37

Os PAMs classificados como catelicidinas são um grupo estruturalmente diverso encontrado em vários mamíferos. Na espécie humana, há uma única

proteína catelicidina identificada (LL-37) (Sorensen et al., 2001). O LL-37 tem atividade antimicrobiana contra uma ampla variedade de bactérias Gram-positivas e Gram-negativas *in vitro* (Travis et al., 2000).

O LL-37 é um peptídeo formado a partir de um pré-peptideo inativo chamado hCAP18, e é produzido por diversos tipos celulares, como neutrófilos, monócitos, linfócitos e células epiteliais (Frohm et al., 1997; Sorensen et al., 1997).

O principal interesse nesse peptídeo é por ser o único antimicrobiano humano do grupo catelicidina. Além disso, ao contrário de muitos outros peptídeos, o LL-37 tem atividade bactericida de amplo espectro em concentrações salinas elevadas, o que lhe confere uma vantagem distinta para potenciais usos terapêuticos (Mookherjee e Hancock, 2007; Thennarasu et al., 2010). É desprovido de pontes de dissulfeto, facilitando a síntese química, sendo assim, menos onerosa**.** LL-37 também é capaz de neutralizar a atividade lipopolissacarídica através do bloqueio da ligação do LPS bacteriano à seu receptor de membrana celular (Rosenfeld, Papo e Shai, 2005).

Recentes estudos demonstraram que LL-37 desempenha potente atividade bactericida estando envolvido na resposta imunológica inata (quimiotaxia de monócitos, linfócitos T e neutrófilos) e na modulação da expressão de citocinas, quimiocinas de macrófagos e células dendríticas (Kanda et al., 2010).

Kanthawong et al. (2010) e Kanthawong et al. (2012) confirmaram a atividade antibacteriana e citoxócica do LL-37 contra *Burkholderia thailandensis e Burkholderia pseudomallei,* e observaram irregularidade na membrana celular bacteriana e o extravazamento de componentes celulares, sugerindo que a hidrofobicidade do peptideo teve papel importante no rompimento da integridade da membrana plasmatica da bactéria.

Molhoek et al. (2009) analisaram as propriedades relacionadas as ações imunomoduladoras do LL-37 e observaram que a hidrofobicidade em particular, desempenha um papel significativo no mecanismo de ação do peptideo LL-37 contra a membrana bacteriana.

Os efeitos inibitórios de LL-37 na formação de biofilme, bem como na fixação inicial de *Staphylococcus epidermidis* e *Pseudomonas aeruginosa* foram relatados (Hell et al., 2010; Overhage et al., 2008). Sabe-se que LL-37 tem forte atividade

bactericida contra *B. pseudomallei* (Kanthawong et al., 2009). Kanthawong et al. (2012) mostraram que o LL-37 foi o peptídeo mais efetivo tanto contra colônias isoladas quanto a formação de biofilme da *B. pseudomallei,* portanto, sugerem o uso deste como agente terapêutico no tratamento da melioidose, infecção causada por *B. pseudomallei*.

Outros estudos apontam que LL-37 causa hemólise e é tóxico para os leucócitos humanos e para o linfócito T *in vitro*, provavelmente, devido a interações hidrofóbicas com a membrana celular eucariótica (Oren et al., 1999). Entretanto, os efeitos citotóxicos do LL-37 quando liberados na circulação sanguinea são inibidos, pois o peptídeo se liga a apolipoproteína A-I do plasma (Wang et al., 1998; Ciornei et al., 2005).

Em peixe, são escassos os trabalhos que utilizaram o peptídeo LL-37 no controle de patógenos. Bridle et al. (2011) observaram atividade antimicrobiana do LL-37 contra *Yersinia ruckeri* e baixa atividade hemolítica em células sanguineas de salmão.

2.7 Parâmetros hematológicos e imunológicos em peixes teleósteos

Informações sobre variáveis hematológicas, imunológicas e bioquímicas podem proporcionar relevantes indicações de alterações no estado fisiológico dos peixes.

As análises citoquímicas apresentam fundamental importância nos estudos hematológicos em diferentes espécies animais. Infecções alteram os valores normais sanguíneos do hemograma nos peixes. Por tanto, o perfil hematológico dos peixes associado às condições de criação são variáveis importantes, utilizadas para indicar a presença de estresse (Tavares-Dias et al., 2009). De acordo com Oliveira-Ribeiro et al., (2000), a avaliação dos parâmetros sanguíneos nos peixes auxilia na detecção de alterações patológicas no organismo.

Alterações no número de eritrócitos e no hematócrito indicam anemia e agravamento do estado de saúde; diminuição na concentração de hemoglobina

pode ser indicativa de intoxicações que afetam o funcionamento das lamelas branquiais. Assim, o hematócrito, a concentração de hemoglobina e a contagem total do número hemácias podem ser indicadores da capacidade total de transporte de oxigênio nos peixes (Vosyliené,1999).

Os índices hematimétricos (Volume Corpuscular Médio, a Hemoglobina Corpuscular Média e a Concentração de Hemoglobina Média) podem ser utilizados no controle de patologias e estresse, evidenciando o estado fisiológico do animal (Tavares-Dias e Moraes, 2004).

Os processos de defesa não específico (inato) e específico (adaptativo) são constituídos pela imunidade mediada por células e a imunidade humoral. A imunidade celular inata envolve monócitos e leucócitos granulares. Estas células são responsáveis por processos de inflamação e fagocitose. A resposta humoral inata é composta por uma variedade de substâncias (lisozima, sistema complemento, entre outros) encontradas no muco e soro dos peixes, que impedem o crescimento de microorganismos infecciosos (Secombes, 1996; Yano, 1996).

A imunidade celular específica está relacionada à capacidade dos linfócitos T reconhecerem antígenos de células alvo. Quando um macrófago fagocita uma bactéria, estimula a proliferação de linfócitos T (Swain et al., 2006). O sistema humoral adaptativo é ativado pela entrada de um antígeno no organismo, os linfócitos B originam os plasmócitos (linfócitos B ativados) e células de memória que se encontram no sangue e migram para os locais da infecção (Kaattari e Piganelli,1996).

A ação modeladora do cortisol gera uma diminuição da atividade fagocitária e da migração de leucócitos para a região inflamada. A contagem de células auxilia no diagnóstico de distúrbios no sitema imune. Observa-se neutrofilia e linfopenia, quando o aumento de neutrófilos se deve a diminuição na migração de neutrófilos para o compartimento tecidual, e aumento do seu tempo na circulação. Já a diminuição de linfócitos se deve pela redistribuição dos linfócitos circulantes, assim como pela lise de alguns tipos de linfócitos nos linfonodos (Mazeaud et al. 1977; Rijnberk e Mol, 1997**).** A trombocitose (aumento do número de trombócitos) ocorre devido à contração esplênica, em que o baço concentra de ¼ a 2/3 do total de trombócitos para o compartimento circulante.

Além dos parâmetros hematológicos e imunológicos, a composição bioquímica do plasma sanguíneo, como concentração de albumina e proteínas totais mostra a situação metabólica dos tecidos animais, assim, por meio dela, é possível observar alterações no funcionamento dos órgãos, reação a uma infecção e a adaptação do animal diante dos desafios nutricionais ou fisiológicos (Higuchi et al., 2011).

Neste trabalho, busca-se ampliar o campo de conhecimento do uso desse peptídeo na aquicultura através da análise da ação bactericida do LL-37 contra *S. agalactiae*, parâmetros hematológicos e imunológicos e da atividade hemolítica em células sanguíneas de tilápia.

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CAPITULO 2: Haematological, biochemical and immunological biomarkers, antibacterial activity, and survival in Nile tilapia *Oreochromis niloticus* after treatment using antimicrobial peptide LL-37 against *Streptococcus agalactiae*[1]

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Abstract

Among the antimicrobial peptides (AMP), which act as natural antibiotics in preventing the colonization of pathogens, the LL-37 is noteworthy. It is a human cathelicidin characterized as a broad-spectrum antibiotic, with chemotactic and immunomodulatory properties. This study aimed to evaluate the effects of peptide LL-37 on the haematological, biochemical, and immunological profiles, antimicrobial activity and therapeutic effectiveness against streptococcosis caused by *Streptococcus agalactiae* in Nile tilapia. After *in vitro* studies of LL-37 cytotoxicity against Nile tilapia red blood cells (RBCs) and antimicrobial susceptibility testing (minimum inhibitory concentration and minimum bactericidal concentration, MIC and MBC, respectively) against *Aeromonas hydrophila* and *S. agalactiae,* fish were divided into 6 experimental groups. G1 and G2 were challenged by intraperitoneal injection with *S. agalactiae* and administered a single oral dose of LL-37 (15 mg/kg) and florfenicol (FFC) (15 mg/kg). G3 was challenged with *S. agalactiae* and administered a single intraperitoneal dose of LL-37 (15 mg/kg). The other groups were controls for infection (G4) and antimicrobial administration (G5 and G6). LL-37 showed weak hemolytic activity against Nile tilapia RBCs (24.4 % lysis at 625 µg/mL) and strong *in vitro* antimicrobial activity against *S. agalactiae* (MIC and MBC = 31.25 µg/mL). No significant effects were observed in the haematological, biochemical, and immunological profiles of fish medicated with LL-37 or FFC, compared to the control fish. Twenty days after the challenge, survival rates were 33.38% (G1), 38.51% (G2), 23.12% (G3), 25% (G4), and 100% (G5 and G6). Despite the strong *in vitro* antimicrobial activity, both antimicrobials were ineffective to control streptococcosis caused by *S. agalactiae* in Nile tilapia. These findings suggest that administration of LL-37 (15 mg/kg) is safe for Nile tilapia but not effective to control streptococcosis.

Keywords: Fish disease, *Oreochromis niloticus*, antimicrobial peptide, *Streptococcus agalactiae*

1. Introduction

Antimicrobial resistance, bacterial diseases and the lack of effective therapies are among the main constraints for aquaculture development. Although the number of studies on this topics has strongly grown over the last three decades, the disease control after an outbreak is often critical, mainly because of the widespread resistance to many antibiotics (Chideroli et al., 2017) and the ineffective antimicrobial treatments available for fish farmers. Currently, there are 25 vaccines (Assefa and Abunna, 2018) and 47 individual or combined antibiotics (Assane et al., 2019; Lulijwa et al., 2019) approved for global use in aquaculture (food species), most of them becoming irrelevant due to the emergence of antimicrobial resistance.

Streptococcosis, caused by *Streptococcus agalactiae*, is considered worldwide to be the most important and devastating disease in Nile tilapia *Oreochromis niloticus* farming due to its potential to cause massive deaths at the end of the farming cycle (Chideroli et al., 2017). There are reports of vaccine failure and antibiotic ineffectiveness after disease outbreak caused by a highly virulent and multidrug-resistant serotype of *S. agalactiae* in Nile tilapia fish farm (Chideroli et al., 2017; de Oliveira et al., 2018). *Streptococcus* spp. has been reported to be resistant to multiple antibiotics, including gentamycin, kanamycin, trimethoprim, nitrofurantoin, ampicillin, spiramycin, oleandomycin, sulphamethoxazole, oxolinic acid, penicillin, erythromycin, oxytetracycline, and florfenicol (Assane et al., 2019; de Oliveira et al., 2018; Faria et al., 2014; Soto et al., 2015). However, therapy with antibiotics is still the main hope for farmers to stop massive economic loss during bacterial

disease outbreaks. Between 2008 and 2018, 67 individual or combined antibiotics were used in 11 of the 15 top aquaculture producing countries (Lulijwa et al., 2019).

The use of antimicrobial peptides (AMPs) is among the promising strategies to reduce antibiotics in aquaculture. In the last two decades, the number of studies on the potentialities of naturally occurring AMPs found in plants, insects, and vertebrates (Barksdale et al., 2016), including humans (Scheenstra et al., 2019; Yeaman and Yount, 2003; Zasloff, 2002) and fish (Chia et al., 2010; Masso-Silva and Diamond, 2014; Valero et al., 2018) has grown strongly.

The human cathelicidin LL-37 is a linear 37 amino acid sequence with Leu-Leu at the N-terminal (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) (Ji et al., 2007), a host defence peptide, cytotoxic to both bacterial and mammalian cells (Yeaman and Yount, 2003). LL-37 is known to be a broad-spectrum AMP (Fabisiak et al., 2016). Several reports describe its strong antimicrobial effect against Gram-positive (*Actinomyces naeslundii*, *A. israelii*, *Lactobacillus acidophilus*, *L. casei*, *L. rhamnosus*, *L. brevis*, *L. fermentum*, *Listeria monocytogenes*, *Staphylococcus aureus*, *S. epidermidis*, *S. sanguines*, *Streptococcus mutans*, *S. sobria*, *S. salivarius*, *S. sanguis*, *S.mitis*), Gram-negative (*Escherichia coli*, *Fusobacterium nucleatum*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*,), and alcohol-acid resistant (*Mycobacterium tuberculosis*) bacteria isolated from humans (Ciornei et al., 2005; Ji et al., 2007; Kreling et al., 2016; Ouhara et al., 2005; Rivas-Santiago et al., 2013; Turner et al., 1998). Bacteria are less likely to develop resistance against LL-37 because its target and kills the bacteria *via* different mechanisms (Zsila and Beke-Somfai, 2020), as well as modulate the immune system during bacterial infection (Nagaoka et al., 2006; Scheenstra et al., 2019), and binds and neutralizes bacterial lipopolysaccharides (LPS) (Sigurdardottir et al., 2006; Turner et al., 1998).

Despite the high potential to be a substitute to the conventional antimicrobials, the application of LL-37 in medicine is hampered by the fact that it is cytotoxic to mammalian cells (Sigurdardottir et al., 2006). Several attempts have been made to find a less toxic synthetic analogue with similar or stronger antimicrobial activity to be used in humans (Ciornei et al., 2005; Consuegra et al., 2013; Sigurdardottir et al., 2006). Taking these aspects into account, LL-37 can be a suitable candidate to use in aquaculture as a substitute to the conventional treatments. Thus, this study aimed to assess the potential effects of synthetic AMP LL-37 on haematological, immunological and biochemical profiles, antimicrobial effect and therapeutic effectiveness against streptococcosis caused by *S. agalactiae* in Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), since this information is not available in the literature.

2. Material and methods

2.1. Antimicrobials

Antimicrobial peptide (AMP) LL-37 (LLGDFFRKSKEKIGKEFKRIVQ-RIKDFLRNLVPRTES-NH2) (Ji et al., 2007) (≥95% purity) was synthesized at the Institute of Chemistry, UNESP, Araraquara, São Paulo, Brazil. Solid-phase peptide synthesis (SPPS) (Merrifield, 1963) was performed using Fmoc (9-fluorenylmethyloxycarbonyl) protocols on the Rink amide MBHA resin. Coupling and deprotection were assessed by the ninhydrin test (Kaiser et al., 1970). Cleavage from the resin and removal of the protecting groups from the amino acid residue side chains were performed simultaneously with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIS) for 2 h (Santos-Filho et al., 2015). After synthesis peptides were analyzed using electrospray mass spectrometry to confirm the correct synthesis. Purification of crude peptides was performed using semipreparative HPLC

on a Shimadzu system (Japan) with a reverse-phase C18 column. Purity degree of peptides reached at least 95% was determined by analytical HPLC.

The analytical standard of florfenicol–FFC (Sigma–Aldrich®, Lot: MKBV1157V, ≥99.0% purity) was purchased from Sigma–Aldrich® Brazil and stored following the manufacturer's recommendations.

2.2. Cytotoxicity studies by haemolytic activity

In vitro cytotoxic activity of LL-37 against red blood cells (RBCs) was analysed by examining LL-37 haemolytic activity against Nile tilapia *Oreochromis niloticus* RBCs using 1% (v/v) Triton X–100 solution as the positive control (PC) (100% lysis) and phosphate-buffered saline (PBS, pH 7.4) as a negative control (NC) (0% lysis). Briefly, fresh blood was collected from healthy juvenile fish to perform the haemolysis assay. RBCs were washed three times by centrifugation (500*x g* for 5 min, 4 ºC) with PBS. A suspension of 1% (v/v) erythrocyte (1% ER) was prepared with packed RBCs suspended in PBS. Microtubes tubes (2 mL) containing 1% ER and different concentrations of LL-37 (4–0.01% (v/v)) or control solution (1% (v/v) Triton X-100 or PBS) were incubated for 1 h at 28 ºC, and then the samples were centrifuged at 500*x g* for 5 min (4ºC). 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 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 were calculated from triplicate assays. Sample normality and homogeneity of variance were studied, and rare non-normal data were normalized using square root

transformation to evaluate significant differences between concentrations through one–way ANOVA followed by Holm-Sidak post hoc test ($\alpha = 0.05$).

2.3. Antimicrobial susceptibility testing (AST)

2.3.1. Bacterial strains

One clinical isolate of pathogenic *Streptococcus agalactiae* (GenBank accession no. KU605571) and one reference strain, *Aeromonas hydrophila* ATCC® 7966™ (GenBank accession no. NC_008570), both from the Strain Bank of the Laboratory of Microbiology and Parasitology of Aquatic Organisms at the Aquaculture Center of Unesp were used to determine the antimicrobial effect of LL-37. The clinical isolate was previously recovered from the brain of Nile tilapia positively diagnosed with a bacterial infection, collected during disease outbreaks in a fish farm located in Nova Avanhandava reservoir (Brazil) in 2015. The reference strain *Escherichia coli* ATCC® 25922™ and florfenicol (FFC) were used for antimicrobial susceptibility testing (AST) quality control (QC).

2.3.2. Determinations of the minimum inhibitory concentration and minimum bactericidal concentration (MIC and MBC)

The approved microdilution broth method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014) was used to determine MICs and MBCs. Briefly, LL-37 (≥95% purity) and FFC (≥99.0% purity) were weighed, dissolved and diluted in sterile distilled water to prepare 5.000 µg/mL and 1.280 µg/mL antimicrobial stock solutions, respectively**.** The ranges of antimicrobials concentrations in the tests were 250 – 0.4883 µg/mL **(**LL-37) and 128 – 0.25 µg/mL (FFC).

Streptococcus agalactiae (KU605571) and *A. hydrophila* (NC_008570) were recovered and cultured in brain heart infusion (KASVI®, Lot: 901091) (BHI) Agar (Biolog®, Lot: BA1890827) (BHIA) with 5% sheep blood and Mueller-Hinton Agar (Sigma – Aldrich®, Lot: BCBS4007V) (MHA), respectively. Cation-adjusted Mueller-Hinton Broth – CAMHB (BD™ Difco™, Lot: 8096574) and CAMHB supplemented with 5% (v/v) lysed horse blood (LHB) were used for the susceptibility testing of *A. hydrophila*, and *S. agalactiae*, respectively. *S. agalactiae* or *A. hydrophila* bacterial suspension (1.5 x 10^8 colony-forming units (CFU)/mL) was diluted in CAMHB or 0,9% NaCl solution to prepare 1 x 10^6 CFU/mL inoculum. 100 µL aliquots were inoculated in each well of 96-well microplates containing 3 identical series of ten 2-fold dilutions of LL-37 or FFC, and controls for growth and sterility. Plates were incubated at 28 ºC for 24-28 h, and MIC endpoints were determined by visual inspection.

The lowest concentration of each antimicrobial at which no bacterial growth was observed in the culture suspension was defined as the MIC. Such culture suspensions without visible bacterial growth were then spread on Mueller Hinton Agar plates and incubated for 24-28 h in triplicate. The lowest concentration at which no growth was observed visually on the plate was regarded as the MBC.

Quality control ranges were determined using CAMHB and plates were prepared and incubated as described above.

2.4. Therapeutic effectiveness against streptococcosis

2.4.1. Experimental groups

Six hundred healthy Nile tilapia (49.7± 9.9 g) were obtained from a commercial fish farm (Itajobi, São Paulo, Brazil) and transported to Aquaculture Center of Unesp (Jaboticabal,

São Paulo, Brazil). After an acclimation period of two weeks, 170 fish (70.9 ± 29.9 g) were distributed randomly in 500 L 17 tanks (10 fish/tank) with constant aeration and open water flow.

Fish were divided randomly into six experimental groups with three (G1, G2, G3, G4, G5) or two (G6) replicates as follows: G1: challenged by intraperitoneal injection with *S. agalactiae* and administered single oral dose (by gavage) of LL-37 (15 mg/kg); G2: challenged by intraperitoneal injection with *S. agalactiae* and administered single oral dose (by gavage) of FFC (15 mg/kg); G3: challenged by intraperitoneal injection with *S. agalactiae* and administered a single dose of LL-37 (15 mg/kg) by intraperitoneal injection; G4: challenged by intraperitoneal injection with *S. agalactiae* and not medicated; G5: administered with PBS (pH 7.4) both by gavage and intraperitoneal injection; and G6: administered PBS (pH 7.4) by intraperitoneal injection.

Fish were fed twice a day with commercial feed (Omnivorous feed - ADM®) appropriate to their stage, at a rate of 3% of body weight. Water quality parameters were measured every three days using a U – 52G probe (Horiba®, Japan).

All the measured parameters were within the recommended range for the Nile tilapia culture (dissolved oxygen: 7.71± 0.45 mg/L, temperature: 28.4± 0.41ºC, pH 8.2± 0.05, electrical conductivity: 167.5 mS/cm, and salinity: 0.08 %).

The experiment followed the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation and recommended by the Animal Welfare Committee (CEUA) of the School of Agricultural Sciences and Veterinary Medicine of Unesp (protocol number 011794/19).

2.4.2. Experimental infection with *S. agalactiae*

S. agalactiae KU605571.1 was cultured in BHIA with 5% sheep blood (28ºC for 24-28h), inoculated in BHI broth, and incubated overnight at 28ºC. The bacteria were then washed twice with PBS (pH 7.4) by centrifugation (3000 × g, 4 °C for 10 min) and adjusted to 1×10^6 UFC/mL for inoculation.

2.4.2.1. Inoculation of *S. agalactiae* KU605571.1

The lethal dose-50 (LD_{50}) of *S. agalactiae* KU605571.1 in Nile tilapia was determined experimentally by intraperitoneal (i.p.) injection to verify the optimum bacterial concentration to perform the bacterial challenge, before the challenge. For inoculation, fish were anaesthetized in 100 mg/mL benzocaine (Sigma–Aldrich®, Lot: SLCB4354, ≥99% purity) solution, and challenged by i.p. injection (1 mL syringe with needle 27G – 13 x 0.45 mm) of 100 µL bacterial suspension (G1, G2, G3, and G4) or PBS (G5 and G6).

2.4.2.2. Antimicrobial administration

LL-37 and FFC were dissolved in sterile distilled water and diluted in PBS. The antimicrobials were then administered through gavage (OD) (G1, and G2) or i.p. injection (IA) (G3), using a 1 mL syringe connected to a catheter (OD) or a 13×0.45mm needle (IA) (Assane et al., 2019). Two antimicrobial solutions were prepared: 15 mg/mL LL-37 (G1, and G3), and 15 mg/mL FFC (G2). The control groups, G5 and G6, were administered PBS by gavage and i.p. injection, respectively.

2.4.3. Effectiveness

The effectiveness of the antibiotic dose or therapy method was assessed based on cumulative survival rates in each group, recorded four times a day for 20 consecutive days. Along with mortality the clinical signs and feeding and swimming behaviour were recorded. Recently dead animals were collected for microbiological examination (streptococcosis confirmation) following standard microbiological procedures.

On the 20th day after challenge, blood was collected from the caudal vessel of survived fish anaesthetized in 100 mg/mL benzocaine solution. Following, fish were euthanized by an overdose of benzocaine and necropsied. Brain, kidney, liver, spleen, and intestines were collected for microbiological and histological analyses.

2.4.3.1. Histological analyses

The brain, kidney, liver, spleen, and intestines were fixed in buffered formalin (10%) for 24 h. Subsequently, they were dehydrated in increasing concentrations of alcohol, diaphanized in xylol, impregnated and included in paraffin. Histological sections (5µm) were stained with a periodic acid-Schiff (PAS) and hematoxylin and eosin (H&E) stain for the histological description under an optical microscope (Nikon Eclipse E200®) connected to a camera (Moticam® 2300). Individual slides of each tissue were analysed, and photomicrographs were captured and used for histological description.

Histological changes were described following the method described by Schwaiger et al., (1997) and Brum et al., (2018). The degree of intensity was classified as 0 (absence of alteration), 1 (mild alteration, corresponding to < 25% of the tissue area), 2 (moderate alteration, 25 to 50% of the tissue area) and 3 (severe alteration, > 50% of the tissue area) (Brum et al., 2018). Photomicrographs of the intestines taken at magnification 100x and 400x

were used to describe the intestinal morphology by measuring the length and width of the villi (100x) and quantifying the goblet cells and intraepithelial lymphocytes (IELs) (400x). Intestinal villi were measured using the software Image-Pro Plus®, and the quantification of intestinal epithelial cells was performed in Microsoft Windows PowerPoint software® using a grid with a systematic set of random windows of known area (Gundersen, 1977). IELs data were reported as the number of IELs per 100 enterocytes.

2.5. Blood parameters

The blood from survived fish was used for haematological, biochemical and immunological analysis.

2.5.1. Haematological parameters

The haematological analyses were performed as described by Valladão et al. (2019). Briefly, an aliquot of blood was heparinized to perform the total RBC count following the method recommended by Hesser (1960) and to quantify the respiratory burst activity. The remaining blood (without heparin) was used to prepare blood smears and left to clot for 2 h at room temperature. Blood smears were stained with a May Grünwald-Giemsa-Wright (MGGW) stain and used for total counts, differential leukocyte counts (Ranzani-Paiva et al., 2013), and examination of erythrocyte abnormalities (Fenech et al., 2003). Clotted blood was centrifuged at 3000×g for 10 min (4 °C) to obtain serum for biochemical and immunological analyses. Total thrombocytes and leukocytes were quantified by counting 2000 RBCs, and a differential count of 200 white blood cells was performed (Ranzani-Paiva et al., 2013). The haemoglobin concentration (Hb) was determined by the cyanmethemoglobin method described by Collier (1944). The mean corpuscular volume (MCV), mean corpuscular

haemoglobin (MCH**)** and mean corpuscular haemoglobin concentration (MCHC) were determined using the method described by Wintrobe (1934).

2.5.2. Biochemical parameters

The total serum protein test (Labtest, 2014a) was used to measure the total amount of protein in the blood and albumin (ALB) (Labtest, 2013), by spectrophotometry (Unico 2100 Spectrophotometer). The total globulin (g/dL) was determined by subtracting the albumin from the total protein. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by spectrophotometry (UV/VIS spectrophotometer Genesys 10S) (Labtest, 2014b, 2012). ALT and AST values were reported in UI/L.

2.5.3. Immunological parameters

Leukocytes respiratory burst activity was measured by nitro blue tetrazolium (Sigma–Aldrich®) reduction test using a spectrophotometer (Biller-Takahashi et al., 2013). The activity was expressed as the optical density (OD).

The serum lysozyme concentration (SLC) was determined using an ELISA reader (Thermoplate Reader Mn), following the lysozyme assay protocol proposed by Demers and Bayne (1997). The assay was based upon the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus.*

The complement system activity (ACH50) in serum was determined following the method described by Zanuzzo et al. (2017) using rabbit erythrocytes. Each sample was measured as the time (minutes) required for the initial optical density to be reduced by one-half (50% of RaRBC hemolysis by the alternative pathway).

2.5.4. Evaluation of micronuclei and nuclear abnormalities in erythrocytes

Erythrocyte abnormalities were evaluated by the presence of micronucleus (MN) and other erythrocyte nuclear abnormalities (ENAs). The blood smears stained with MGGW stain were used to examine 2000 erythrocytes under the light microscope (1000× magnification) to determine the frequencies of MN and ENAs in each fish. The MN and ENAs were identified as proposed by Fenech et al. (2003), and Carrasco et al. (1990), respectively. Non-parametric Kruskal–Wallis test followed by Dunn test was applied for MN and ENAs analysis.

2.6. Statistical analysis

Data were examined for normality and homogeneity of variance, and through one-way ANOVA followed by Holm-Sidak or Bonferroni's posthoc comparisons tests at a significance level of 0.05.

3. Results

3.1. Hemolytic activity of LL-37 against Nile tilapia red blood cells

The antimicrobial peptide (AMP) LL-37 showed weak hemolytic activity against Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) red blood cells (RBCs) (less than 25% at the highest concentration tested 63.0 μg/mL) (Figure 1). At lower concentrations, the hemolysis rate was 18.5% (0,31 mg/mL), 0.16% (0,16 mg/mL), 0.08% (0,08 mg/mL), 0, 04% (0,04 mg/ mL) and 0.02% (0,02 mg/mL) (Figure 1).

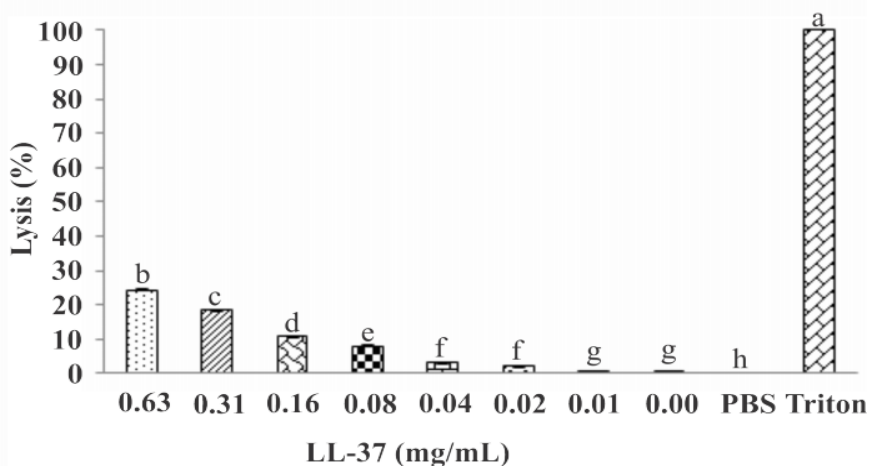

Figure 1. Hemolytic activity of the LL-37 to Nile tilapia red erythrocytes.

3.2. In vitro antimicrobial activity

The minimum inhibitory concentration (MIC) of florfenicol (FFC) for the quality control strain (QC), Escherichia coli ATCC® 25922™ (MIC = 8 µg/mL) was within the range established by the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014).

The MIC of AMP LL-37 and FFC against *Aeromonas hydrophila* ATCC® 7966™ was > 250 µg /mL and 8 µg /mL, respectively. FFC showed bactericidal (minimum bactericidal concentration (MBC)/MIC = 2) activity against *A. hydrophila* ATCC® 7966™. The MIC of AMP LL-37 against *Streptococcus agalactiae* KU605571.1 was 31.25 µg/mL. LL-37 showed bactericidal activity (MBC/MIC = 1) against *S. agalactiae* KU605571.1.

3.3. Therapeutic effectiveness against streptococcosis

The first death was recorded 48 h post-challenge. Only fish from bacterial challenges died during this period (Figure 2). The first death on the challenged and medicated groups (G1, G2, and G3) was recorded 48 h after the first death in G4.

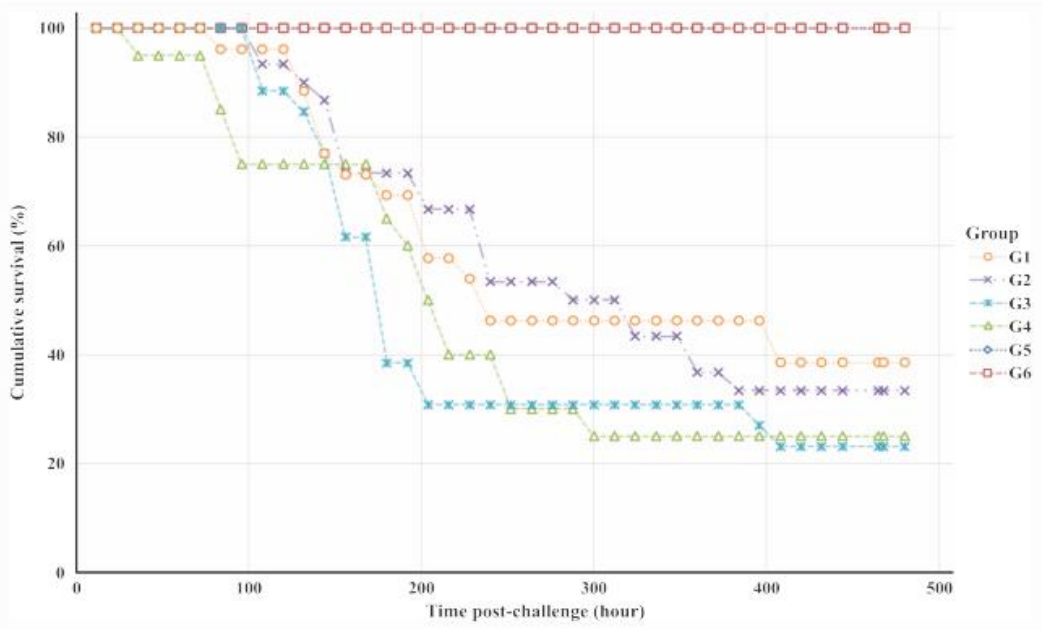

Figure 2. Cumulative survival (%) of Nile tilapia challenged with *Streptococcus agalactiae* and medicated with LL-37 or florfenicol (FFC). G1: challenged and medicated with 15 mg/kg bw LL37 by gavage; G2: challenged and medicated with 15 mg/kg bw FFC by gavage; G3: challenged and medicated with 15 mg/kg bw LL-37 by intraperitoneal injection; G4: challenged and not medicated; G5: administered PBS by gavage and intraperitoneal injection; and G6: administered PBS by intraperitoneal injection.

The mortality stopped sixteen days post-challenge. There was no mortality, clinical signs of disease or pathological changes in the control groups administered phosphate-buffered saline (PBS) (G5, and G6) throughout the experimental period (20 days). On the other hand, high mortality rates, ranging between 61.49% (G1) and 76.88% (G3), clinical signs of disease, and pathological changes were observed in all challenged groups (G1, G2, G3, and G4).

Despite the delayed death of fish from challenged and medicated groups, LL-37 and FFC were inefficient in controlling fish mortality caused by *S. agalactiae* KU605571.1 infection throughout the experimental period. All challenged groups had a significantly ($p <$

0.01) higher mortality percentage (G1–66.62%, G2–61.49%, G3–76.88%, and G4–75%) compared to control groups administered PBS (G5, and G6 – 0%). There was no significant difference between the survival rates of fish challenged and medicated with LL-37 (G1, and G3: 15 mg/kg bw) or FFC (G2: 15 mg/kg bw), and challenged and not medicated (G4).

Died fish exhibited typical signs of streptococcosis, including lethargy, erratic swimming, dorsal rigidity, exophthalmia, corneal opacity, hepatomegaly, haemorrhages around the brain, and hemorrhagic kidney (Figure. 3). *S. agalactiae* was recovered from dead fish, confirming their cause of death.

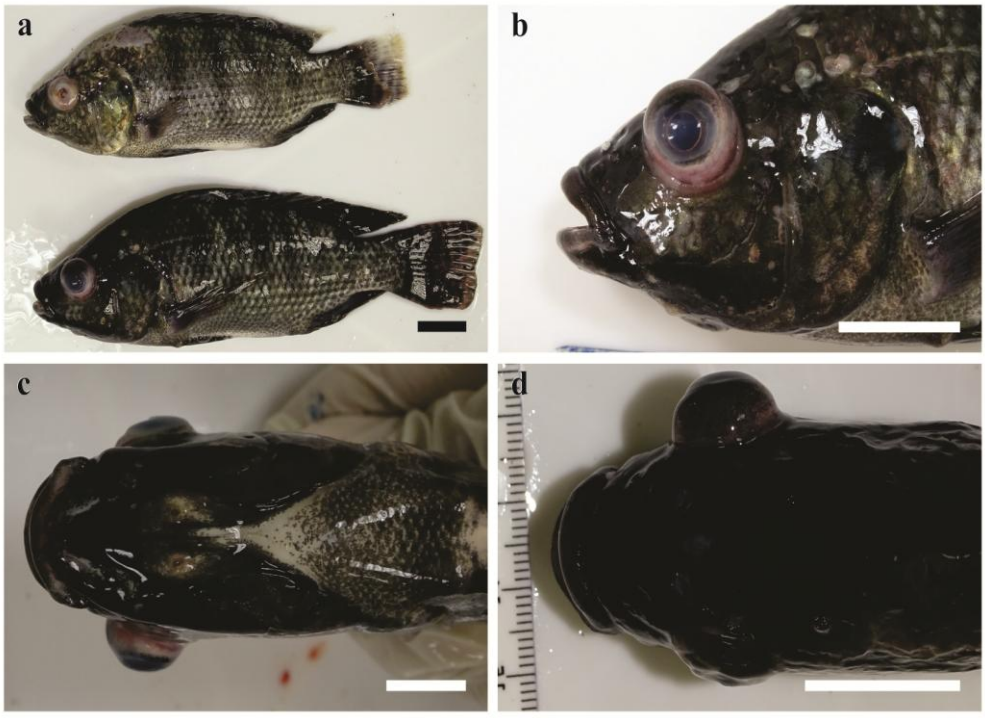

Figure 3. Typical signs of streptoccosis (exophthalmia, corneal opacity) in fish dying during the experimental period, scale bar – 2cm.

At 20 days post-challenge, clinical signs of disease, such as exophthalmia and corneal opacity were still present in 40% of the surviving fish from the challenged and not medicated group (G4). *S. agalactiae* was recovered from all the surviving fish from G2 and G4, and none from G1, and G3.

3.3.1. Histological analyses

Severe histological changes were observed in the liver, spleen, kidney and brain of all dead fish, during the first 15 days post-challenge. No change was observed in intestine. Mild to severe organ changes were observed 20 days post-challenge in all surviving fish. Mild histopathological alterations were found in the tissues from both the untreated control group. On the other hand, mild to moderate alterations were observed in the liver, spleen, kidney and brain tissue of all fish medicated with LL-37 (G1, and G3) (Figure 4).

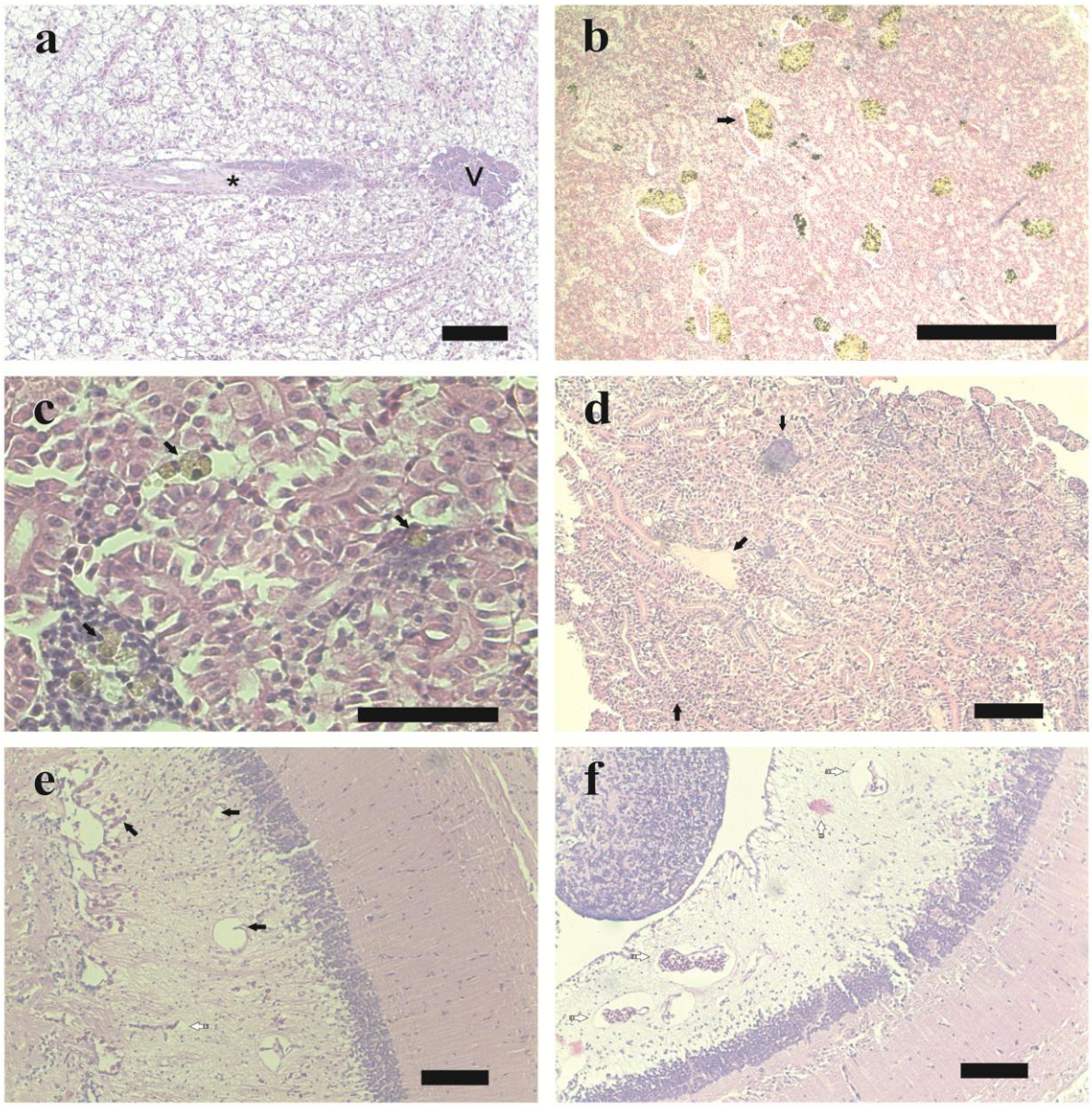

Figure 4. Tissue section of nile tilapia. (a) Histological changes in liver: thrombus in the in of portal blood vessel (asterisk) and congestion of blood vessel (V), scale bar -1000 µm; (b) Increased melanomacrophage center in the spleen (arrows), scale bar-500 µm; (c) Kidney tissue changes: hemosiderin deposits (arrows), scale bar-50 µm; (d) Tubular degeneration (upward black arrow) and thrombosis in hematopoietic tissue (south west arrow), scale bar -1000 µm; (e, f) Brain of nile tilapia showing capillaries swelling and spaces indicating oedema (arrows), scale bar -1000 µm.

The liver parenchyma showed necrosis, presence of hemosiderin, and vacuolation of hepatocytes and melanomacrophage centres (MMCs). In addition to that, infiltrations of mononuclear cells, thrombus and occurrence of congestion were also observed (Figure 4a). The kidney of G4 and G2 showed a great variety of lesions as congestion, hemorrhagic areas and tubular degeneration with high intensity (Figure 4c, and Figure 4d).

The spleen showed degeneration, necrosis and focal haemorrhages in G2, G3 and G4 groups. Areas of hemosiderin deposits and an increase of MMCs were also observed in spleen tissue of fish from G1 to G4 groups (Figure 4b).

Severe alterations with signs of meningoencephalitis were observed in the brain of fish from G4 (Figure 4e,f). It was visible by the presence of haemorrhages, mononuclear cell infiltration and increase intercellular space indicating oedema. Severe alterations (scored 3) was found in fish from groups G4 and G2 for all organs (Table 1).

Table 1. Histological changes according to the degree of intensity: absence of alteration (-), mild alteration (+), moderate alteration (++) and severe alteration (*) in Nile tilapia *Oreochromis niloticus* 20 days post-challenge with *Streptococcus agalactiae* and administration of LL-37 or florfenicol (FFC).

Alterations	Liver						Spleen						Kidney						Brain					
	G1	G2	G3	G4	G5	G6	G1	G2	G3	G4	G5	G6	G1	G2	G3	G4	G5	G6	G1	G2	G3	G4	G5	G6
Congestion	-	*	+	++	-	-	-	-	-	-	-	-	-	++	+	*	-	-	-	-	-	-	-	-
Hemosiderin deposition	-	-	-	*	-	+	+	+	+	++	-	+	-	+	-	++	-	-	-	-	-	-	-	-
Vacuolization	+	++	+	*	+	++	-	-	+	-	-	-	-	+	-	++	-	-	-	+	-	+	-	-
Necrosis	+	++	+	*	-	-	-	+	+	++	-	-	-	+	-	++	-	+	-	-	-	-	-	-
Haemorrhage	-	+	-	++	-	-	-	+	+	++	-	-	+	+	-	++	-	-	-	++	+	*	+	-
Melanomacrophage centers	-	+	-	+	-	-	-	++	-	*	-	-	-	+	-	*	-	-	-	-	-	-	-	-
Infiltration of inflammatory cell	-	++	-	++	-	-	-	+	-	*	-	-	-	++	-	*	-	-	-	+	+	*	-	-
Tubular degeneration	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	++	-	+	-	-	-	-	-	-
Tubular necrosis	+	+	+	++	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
Inflamed neurons	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	-	-
Meningoencephalitis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	-
Oedema	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	++	-	-

There was no significant difference in the morphometric parameters of the intestine (Table 2). Twenty days post-challenge, the width of the villi was not significantly different in fish treated with antimicrobials compared with the control. Regarding the mean of length of the villi of fish from G2 was higher than G5. The number of goblet cells and intraepithelial lymphocytes in the intestinal villi was not significantly different ($p > 0.05$) in fish of all groups.

Table 2. Intestinal morphometry of Nile tilapia treated with LL-37 or florfenicol (FFC), at 20th day after challenge with *Streptococcus agalactiae*.

Parameters	Group*					
	G1	G2	G3	G4	G5	G6
Height of villi (μm)	271.9 ± 45.8	329.1 ± 69.4	267.3 ± 43.6	272.0 ± 46.4	251.4 ± 61.8	302.5 ± 70.8
Width of villi (μm)	140.6 ± 28.3	145.6 ± 30.3	152.5 ± 37.4	137.8 ± 31.6	139.0 ± 6.9	153.3 ± 43.9
Number of goblet cells	7.6 ± 4.7	7.5± 4.3	6.4± 4.0	7.5± 3.3	7.0± 2.6	7.0± 3.1
Number of Intraepithelial lymphocytes	60.6 ± 8.6	58.9 ± 14.4	63.3 ± 20.4	60.6 ± 11.9	57.9 ± 7.8	63.5 ± 9.6

3.4. Haematological analyses

The blood cells of Nile tilapia challenged with *S. agalactiae* and medicated with LL-37 or FFC did not show significant changes 20 days post-challenge (Table 3). The total leukocyte and thrombocyte counts did not differ among the treatments ($p > 0.05$). There was a predominance of lymphocytes, followed by neutrophils, monocytes and basophils in the differential leukocyte count. Basophils were rarely observed and eosinophils were absent.

Table 3. Haematological parameters of Nile tilapia *Oreochromis niloticus* 20 days post-challenge with *Streptococcus agalactiae.*

Cells	Group*					
	G1	G2	G3	G4	G5	G6
RBC (10^6/µL)	2.05± 0.40	1.76± 0.24	1.97± 0.44	1.7± 0.23	1.97± 0.36	2.14± 0.37
Htc (%)	35.16 ± 7.16	30.87 ± 5.19	32.5± 3.0	35.62± 7.69	34.81± 5.4	33.31± 3.0
Hb (g/dL)	8.09 ± 1.47	7.24 ± 1.56	8.11 ± 1.31	8.46± 3.57	7.67± 1.10	8.73± 4.29
MCV (fL/µL)	163.2± 19.11	176.6 ± 30.41	169.0± 30.86	211.0± 52.36	181.3± 42.0	158.8± 25.34
MCHC (g/dL)	40.26± 6.30	40.75± 5.78	41.49± 3.26	50.21± 21.16	22.35± 3.92	26.77± 15.31
MCH (pg/µL)	24.98 ± 5.25	23.89 ± 5.84	24.91 ± 2.69	24.02 ± 9.69	39.82± 7.96	42.0± 21.64
Neutrophils (10^3/µL)	5.94± 349.1	5.93± 929.7	5.83± 729.2	4.83± 399.6	6.09± 427.2	5.10± 358.2
Lymphocytes (10^3/µL)	6.50± 4.11	6.42± 3.15	6.21± 4.36	5.73± 1.92	7.00± 2.11	5.71± 1.50
Basophils (10^3/µL)	1.68± 848	1.37± 5.70	1.93± 771.6	1.23± 936.6	3.24± 1.75	1.93± 943.9
Monocytes (10^3/µL)	551.5± 156.6	485.3± 346.5	580± 668.5	496.3± 258.1	552.7± 543.9	588.2± 91.7
Thrombocytes (10^3/µL)	12.72± 4.88	12.05 ± 4.10	12.71± 2.67	11.86± 2.20	12.47± 4.53	13.90± 3.53

The total red blood cells (RBC), mean corpuscular haemoglobin concentration and mean corpuscular haemoglobin did not differ among the groups studied. However, the mean

corpuscular volume of G4 showed a higher value compared to the control (G6). No significant difference in hematocrit (Htc), and haemoglobin (Hb) was observed among groups.

3.5. Biochemical analyses

Administration of 15 mg/kg bw LL-37 or FFC did not affect the biochemical parameters of fish challenged with *S. agalactiae*. The concentration of total protein, albumin, globulin, AST and ALT did not differ among groups ($p > 0.05$) (Table 4).

Table 4. Serum biochemical parameters of Nile tilapia *Oreochromis niloticus* 20 days post-challenge with *Streptococcus agalactiae*, and administration of LL-37 or florfenicol (FFC).

Parameter	Group*					
	G1	G2	G3	G4	G5	G6
TP (g/dL)	3.96±0.31	4.20±0.18	3.84±0.30	3.92±0.55	3.68±0.58	3.83±0.53
ALB(g/dL)	1.75±0.40	1.54±0.32	1.68±0.47	1.58±0.33	1.64±0.29	1.38±0.20
ALT (UI/L)	10.7±6.2	10.9±9.6	19±22.9	14.5±15.6	11.2±8.55	17.1±19.7
AST (UI/L)	37.6±24.5	49.4±34.8	35.9±14.3	31.1±19.1	42.1±66.0	40.1±37.1

*Group- G1: challenged and medicated with 15 mg/kg bw LL37 by gavage; G2: challenged and medicated with 15 mg/kg bw FFC by gavage; G3: challenged and medicated with 15 mg/kg bw LL-37 by intraperitoneal injection; G4: challenged and not medicated; G5: administered PBS by gavage and intraperitoneal injection; and G6: administered PBS by intraperitoneal injection. TP: total protein, ALB: albumin. AL: alanine aminotransferase and AST: aspartate aminotransferase.

3.6. Immunological analyses

The respiratory burst activity of blood leukocytes (BURST), serum lysozyme concentration (SLC), and the hemolytic activity of the alternative pathway of the complement

system (ACH50) of Nile tilapia challenged with *S. agalactiae* were not affected by LL-37 or FFC ($p > 0.05$) (Figure 5).

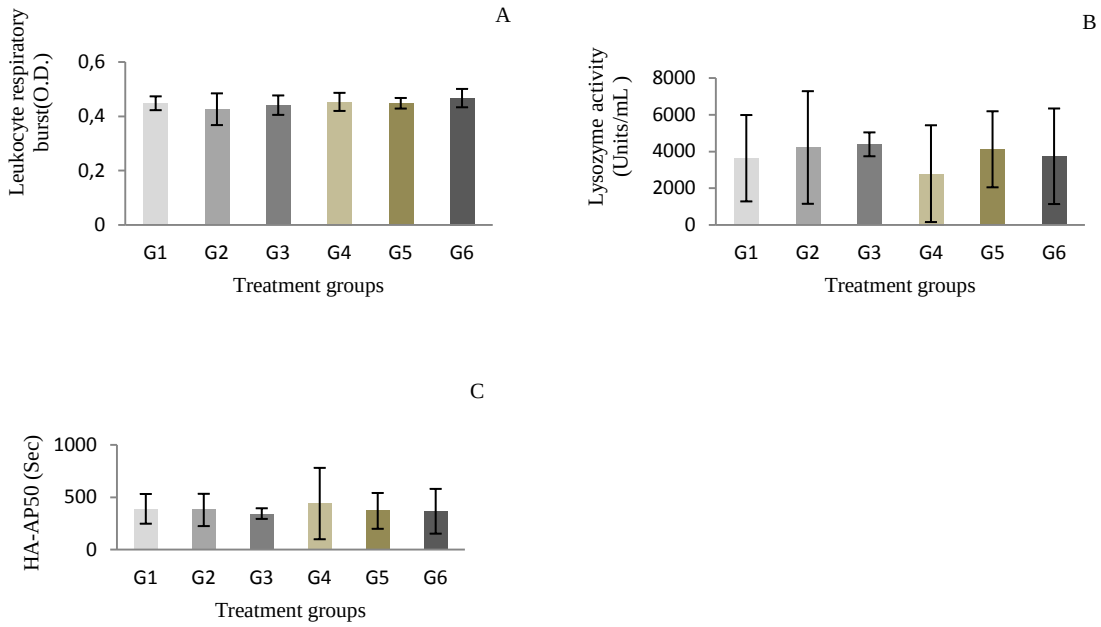

Figure 5. Mean (S.E.) of respiratory burst (A), Serum lysozyme concentration (B) and Hemolytic activity of the alternative pathway of the complement system (C) of surviving *O. niloticus* in 20th day post treatment with LL-37 peptide.

3.7. Erythrocytes abnormalities

Presence of micronuclei and nuclear abnormality of erythrocytes (Figure 6) were observed in Nile tilapia challenged with *S. agalactiae* 20 days pos-challenge.

Despite the higher value of abnormalities including notched, lobed, and vacuolized nuclei observed in erythrocytes from fish of medicated groups (G1, G2, and G3), there were no significant differences ($p < 0.05$) among groups (Table 5).

Table 5. Comparison of micronuclei and other nuclear abnormalities in Nile tilapia *Oreochromis niloticus* 20 days post-challenge with *Streptococcus agalactiae* and administration of LL-37 or florfenicol (FFC).

Erythrocytes abnormalities	Group*					
	G1	G2	G3	G4	G5	G6
Micronuclei	0.80 ± 0.81	0.75± 0.5	0.85± 1.0	0.66± 0.51	0.63± 0.75	0,0± 0,0
Notched	1.16 ± 0.75	1.75± 1.25	1.20± 0.81	1.66± 0.81	1.03± 0.75	1.25± 0.5
Lobed	0,5 ± 0,57	0,0± 0,0	0,75± 0,05	0,0± 0,0	0,0± 0,0	0,0± 0,0
Blebbed	3.16 ± 0.75	1.25 ± 0.5	2.75± 2.06	1.83± 1.16	1.0± 1.2	2.0± 0.81
Vacuolated nuclei	0.5± 0.54	0,0± 0,0	0.25 ± 0.5	0,0± 0,0	0,0± 0,0	0,0± 0,0

Bud nuclei were observed in a single fish, so it was not quantified (Figure 6a). Also, a high number of notched nuclei was observed in erythrocytes from fish of medicated groups.

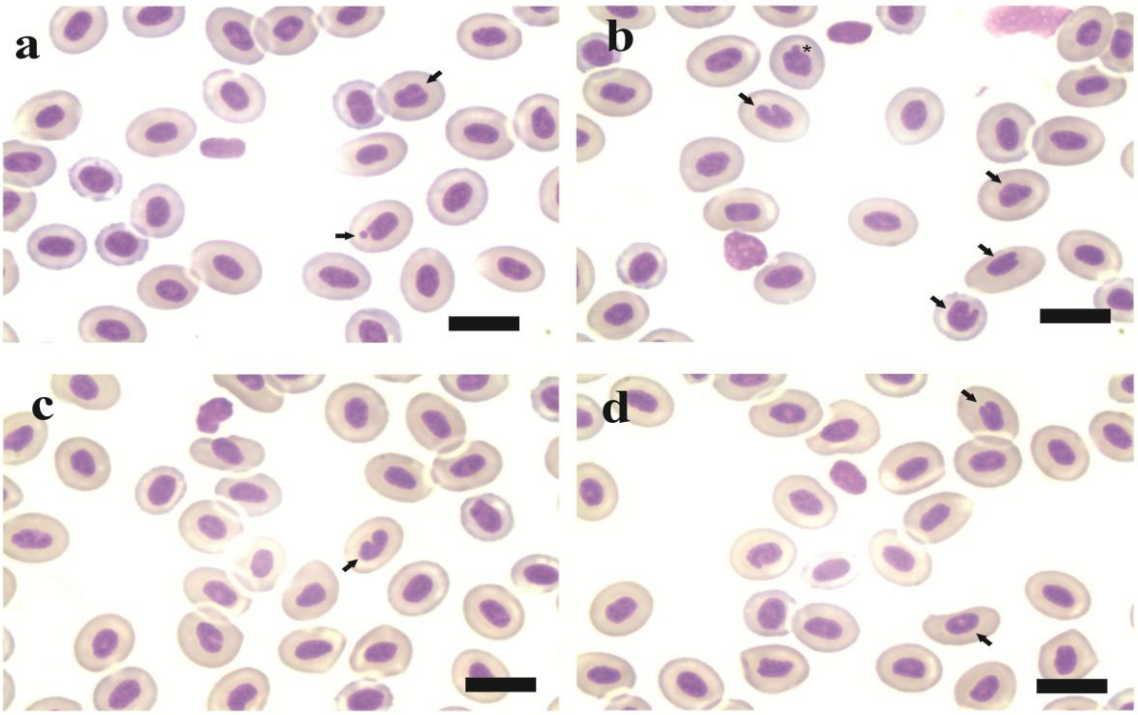

Figure 6. Photomicrographs of erythrocytes with abnormalities of nucleus. (a) Micronuclei (black rightwards arrow) and lobed (southwest arrow); (b) nuclear abnormalities like blebbed nuclei (arrows), bud nuclei (asterisk); (c) notched nuclei (arrow); (d) vacuolated nuclei (northwest arrow), blebbed nuclei (southwest arrow) (scale bar -10 µm).

4. **Discussion**

The widespread resistance to many of the currently available antibiotics (Cabello et al., 2016; Chideroli et al., 2017) and the consequent lack of effective therapies against bacterial diseases in aquaculture are among the main constraints for the growth and development of the sector (Subasinghe et al., 2000). In this study, we provide a promising antimicrobial candidate in the fight against bacterial diseases in aquaculture. The human cathelicidin LL-37 antimicrobial peptide (AMP) showed strong *in vitro* antimicrobial activity against *Streptococcus agalactiae* and no significant effects on the haematological, biochemical and immunological parameters of Nile tilapia *Oreochromis niloticus* (Linnaeus,

1758). Moreover, the AMP had a low toxic effect against Nile tilapia red blood cells (RBCs), suggesting its potential as an alternative to the currently available antibiotics.

The cytotoxicity to host cells (including erythrocytes), even at therapeutic concentrations, is among the potential limitations in the clinical use of many AMPs (Sigurdardottir et al., 2006). Indeed, despite the potential to be used as a substitute for the conventional antibiotics, LL-37 is not applicated in medicine mainly due to its cytotoxicity against mammalian cells, and less toxic synthetic analogues are being investigated (Ciornei et al., 2005; Consuegra et al., 2013; Sigurdardottir et al., 2006). Nevertheless, the haemolytic activity of LL-37 against Nile tilapia RBCs was very low in this study. The highest concentration tested (625 μg/mL) resulted in 24.2 ± 0.2% haemolysis. The concentration (39.06 μg/mL) similar to the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *S. agalactiae* KU605571.1 (31.25 μg/mL) resulted in 3.28 ± 0.24% haemolysis. The MIC was below the concentrations previously reported to not have cytotoxic effects against human odontoblast-like cells (62.5 μg/mL) (Caiaffa et al., 2019) and Atlantic salmon *Salmo salar* erythrocytes (250 μM (~1,123.25 μg/mL) LL-37 combined with Atlantic salmon serum) (Bridle et al., 2011).

The lysis capacity of LL-37 is mainly based on a toroidal pore carpet-like mechanism, where the peptide bind parallels to the surface of the outer membrane of cells, with the positively charged amino acids with the head groups of phospholipids, originating small pores in the cell membrane that lead to severe leaks of intracellular material and lysis (Turner et al., 1998; Vandamme et al., 2012). Due to the hydrophobic interactions that occur between bacterial cell membrane with mammalian cells (Yeaman and Yount, 2003), LL-37 not only interact with bacteria but also with eukaryotic cells, causing toxicity. However, no differences in erythrocyte abnormalities (EAs) were observed in the *in vivo* experiment, suggesting low *in*

vivo cytotoxic effect of LL-37 in Nile tilapia. LL-37 cytotoxic effects appear to be attenuated by one or several factors present in the serum. Bridle et al. (2011) and Johansson et al. (1998) reported significantly high reduction and even loss of biological effects of LL-37 in *in vitro* experiments following the addition of serum from different animals or dilution of AMP in serum.

The presence of micronuclei (MN) and other EAs in all groups, including in the control groups, is probably a result of spontaneous DNA breaks resulting from errors during DNA metabolism, without the influence of antimicrobials (Ribeiro et al., 2003). Besides, serum activities of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also similar in all groups, indicating that there was no damage to the hepatocytes or liver tissue of fish 20 days post-medication.

The MIC and MBC (31.25 μg/mL) of LL-37 against *S. agalactiae* KU605571.1 were within the range of MICs (25 – 50 μg/mL) previously reported as the MIC of LL-37 against different clinical isolates of *Streptococcus* from the human oral cavity, including *S. mutans, S. sobria, S. salivarius, S. sanguis, S.mitis* (Ouhara et al., 2005). To the best of our knowledge, this is the first report of antimicrobial activity of LL-37 against clinical isolate of *Streptococcus* from fish. The most similar available study is from Bridle et al., (2011), who reported the antimicrobial activity of LL-37 against *Yersinia ruckeri* and *Vibrio anguillarum* isolated from Atlantic salmon.

In this study, both florfenicol (FFC) (15 mg/kg) and LL-37 (15 mg/kg) were ineffective in controlling streptococcosis caused by *S. agalactiae* KU605571.1 in Nile tilapia, although they had low MIC and MBC against the tested strain (Assane et al., 2019). This could be attributed to many factors that affect the clinical outcome of therapy and were not evaluated in this study, including virulence of the pathogen, antimicrobial pharmacokinetics,

interactions, and dose regimen (Giguère et al., 2013). LL-37 *in vivo* effects are reduced depending on the blood components. Johansson et al. (1998) and Caiaffa et al. (2019) reported a reduction or loss of antibacterial and cytotoxic effects of LL-37 in the presence of a human, porcine, or fish serum.

The challenge with *S. agalactiae* KU605571.1 resulted in an acute onset of streptococcosis, with external clinical signs of disease and inflammatory alterations in the examined organs (Asencios et al., 2016; Filho et al., 2009; Soto et al., 2016). However, the low intensity and less severity of histological changes in fish treated with LL-37 suggest the absence of an inflammatory focus in fish that survived up to 20 days post-challenge since tissue alterations with a degree of severity 3 can be irreversible and cause loss of functionality of the organ (Poleksic and Mitrovic-Tutundzic, 1994). Furthermore, it was not observed an increase in leukocytes respiratory activity, which could indicate the activation of the innate immunity system (Jeney and Anderson, 1993; Jørgensen and Robertsen, 1995).

It is well known that intestinal morphology plays an important role in fish health and well-being through the modulation of the ability of intestinal immune cells to fight infection and improvement of nutrient absorption capacities by the intestinal absorptive epithelium (Ringø et al., 2016; Stroband and Debets, 1978). The intestinal morphology, evaluated by the number of goblet cells, height, and width of the intestinal villi of fish challenged with *S. agalactiae* and medicated with LL-37 (15 mg/kg) or FLO (15 mg/kg) had no significant differences from the control fish 20 days pos-challenge.

No haematological, biochemical, or immunological changes were observed in medicated fish 20 days post-challenge. Most of these parameters were within the ranges reported as normal for healthy Nile tilapia under experimental conditions (Azevedo et al., 2006; Hisano et al., 2007; Hrubec et al., 2000; Nagata et al., 2009; Tavares-Dias et al., 2000;

Tavares-Dias and Faustino, 1998; Valladão et al., 2019). It seems that one single dose of LL-37 administered by gavage or injection is not enough to promote changes in such parameters. In general, most of these parameters are affected by multiple-dose regimens and long periods (more than 2 weeks) of administration (Valladão et al., 2019). Therefore, it cannot be excluded that the multiple-dose regimen of LL-37, as well as more effective administration methods, could lead to changes in haematological and biochemical parameters and improved immune system. There are reports of improvements in fish resistance to bacterial infection after prolonged administration of multiple-doses of AMPs (Chettri et al., 2017; Pan et al., 2012).

Although there have been poor clinical outcomes using both FFC or LL-37, the absence of *S. agalactiae* 20 days post-infection in fish medicated with LL-37, even using a relatively lower dose (15 mg/kg), indicates that LL-37 could be of great interest in preventing recurrent *S. agalactiae* infection and chronic meningoencephalitis in fish, by preventing the emergence of a persistent subpopulation of bacteria. On the other hand, the isolation of *S. agalactiae* in fish treated with FFC (15 mg/kg) suggests the occurrence of a persistent subpopulation of *S. agalactiae* after a single dose of antibiotic. The occurrence of a persistent subpopulation of *S. agalactiae* and recurrent infection in Nile tilapia has been reported by Faria et al. (2014) and de Oliveira et al. (2018) after therapy with oxytetracycline and FFC, respectively.

Studies show that persister cells cause a reduction in the effective intracellular concentration of the antimicrobial (Brauner et al., 2016) and AMPs can be more effective to kill bacterial persister cells by permeabilization of the bacterial membrane (Andersson, 2019; Beaudoin et al., 2018; Chen et al., 2011; Mwangi et al., 2019).

In conclusion, this study indicates that LL-37 is a promising antimicrobial candidate in the fight against bacterial diseases in aquaculture, since it showed strong *in vitro* antimicrobial activity against the most important and devastating pathogens of Nile tilapia and no significant effects on the haematological, biochemical and immunological parameters of the host, following a single oral dose of 15 mg/kg.

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