LEONARDO MANTOVANI FAVERO

ANTIBIOTICOTERAPIA CONTRA BACTERIOSES EMERGENTES DE TILÁPIAS:

AVALIAÇÃO DA EFICÁCIA CLÍNICA DA OXITETRACICLINA CONTRA *FRANCISELLA ORIENTALIS* E PREDIÇÃO IN SILICO DE ALVOS DROGÁVEIS NO SOROTIPO III DE *STREPTOCOCCUS AGALACTIAE*

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Dissertação apresentada ao Programa de Pós-graduação em Ciência Animal da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Mestre.

Orientador: Prof. Dr. Ulisses de Pádua Pereira

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FAVERO, Leonardo Mantovani. **Antibioticoterapia contra bacterioses emergentes de tilápias**: avaliação da eficácia clínica da oxitetraciclina contra *Francisella orientalis* e predição *in silico* de alvos drogáveis no sorotipo III de *Streptococcus agalactiae*. 2021. 81 f. Dissertação (Mestrado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2021.

RESUMO

A produção de peixes segue em crescimento mundial e nacionalmente, principalmente devido a investimentos em tecnologia e intensificação da produção. A tilápia é umas das espécies mais produzidas devido a sua rusticidade e facilidade em se adaptar a sistemas intensivos. Apesar dos ganhos em produção, sistemas intensivos favorecem o surgimento de doenças. A franciselose e a estreptococose, ambas de etiologia bacteriana, destacam-se como as principais doenças causadoras de surtos com alta mortalidade em pisciculturas. A franciselose é causada por bactérias do gênero *Francisella* que possuem comportamento intracelular facultativo. Essa característica torna o desenvolvimento de vacinas dificultoso e não há ainda vacinas comerciais disponíveis para a profilaxia desta enfermidade. Há vacinas disponíveis para a profilaxia da estreptococose, porém devido à variedade de espécies e sorotipos de *Streptococcus* e a falta de imunidade cruzada entre eles, falhas vacinais são comuns. O sorotipo III de *S. agalactiae* tem emergido como patógeno nas pisciculturas brasileiras e destaca-se devido a descrição de linhagens multirresistentes e de genótipo (ST283) com potencial zoonótico. A principal estratégia de controle dessas bacterioses é utilização de antimicrobianos. Neste contexto, a presente dissertação avaliou a eficácia clínica da oxitetraciclina contra a infecção experimental por *F. orientalis* em tilápias e previu alvos de drogas no genoma do sorotipo III de *Streptococcus agalactiae* através de análises genômicas. No trabalho A, duas doses de oxitetraciclina foram fornecidas via oral, 100 mg·kg peso vivo^{-1} e 200 mg·kg peso vivo^{-1}, antes e após a infecção experimental, por 16 e 15 dias, respectivamente. Os dados obtidos neste experimento indicaram que a oxitetraciclina é segura, mesmo na maior dose, e eficaz para controlar a franciselose em tilápias. No trabalho B, 24 genomas completos de isolados humanos ou de peixes de *S. agalactiae* sorotipo III foram utilizados para obter o *core* genoma deste sorotipo. Através de critérios subtrativos e ferramentas de bioinformática, cinco proteínas foram identificadas como alvos potenciais para antimicrobianos. Uma biblioteca contendo 5008 compostos naturais foi confrontada com esses alvos através de *docking* virtual. As moléculas com as melhores características de ligação foram sugeridas para futuros testes *in vitro* e *in vivo*. Os resultados aqui apresentados acrescentarão segurança no emprego da oxitetraciclina em uma cadeia produtiva de grande importância e ainda trazem perspectivas para o desenvolvimento de novas moléculas antimicrobianas contra uma linhagem com descrição de multirresistência e com potencial zoonótico.

Palavras-chave: Bioinformática. Estreptococose. Franciselose. Tetraciclinas. Tratamento.

FAVERO, Leonardo Mantovani. **Antibiotic therapy against emerging bacterial diseases of tilapia**: clinical efficacy of oxytetracycline against *Francisella orientalis* and *in silico* prediction of druggable targets of *Streptococcus agalactiae* serotype III. 2021. 81pp. Dissertation (Master's degree in Animal Science) – Universidade Estadual de Londrina, Londrina, 2021.

ABSTRACT

Fish farming continues to grow worldwide and nationally, mainly due to investments in technology and intensification of production. Tilapia is one of the most cultivated species owing to its rusticity and ease in adapting to intensive systems. Despite the great outcomes in intensive production systems, emergence of diseases is favored by this kind of production. Francisellosis and streptococcosis, both bacterial diseases, stand out as the main outbreaks causes with high mortality in fish farms. Francisellosis is caused by bacteria of the genus *Francisella*. Bacteria from this genus are facultatively intracellular. This characteristic hampers the development of vaccines and there are no commercial vaccines available for the prophylaxis of this disease. Vaccines are available for the prophylaxis of streptococcosis, but due to the variety of species and serotypes of *Streptococcus* and a lack of cross-immunity between them, vaccine failures are common. *S. agalactiae* serotype III has emerged as a pathogen in Brazilian fish farms and stands out due to the description of multi-resistant strains and the existence of a potentially zoonotic genotype (ST283). The main strategy for controlling bacterial diseases is the use of antimicrobials. In this context, the present dissertation evaluated the clinical efficacy of oxytetracycline against an experimental infection with *F. orientalis* in tilapia and predicted drug targets in the genome of serotype III of *S. agalactiae* through genomic analyzes. In the study A, two doses of oxytetracycline were given orally, 100 mg·kg fish^{-1} and 200 mg·kg fish^{-1}, before or after the experimental infection, for 16 and 15 days, respectively. The data obtained from this experiment indicated that oxytetracycline is safe, even at the highest dose, and effective in controlling francisellosis in tilapia. In the study B, 24 complete genomes of human or fish *S. agalactiae* serotype III isolates were used to obtain the core genome of this serotype. Through subtractive criteria and bioinformatics tools, five proteins were identified as potential targets for antimicrobials. A library containing 5008 natural compounds was docked with these targets virtually. The molecules with the best binding characteristics were suggested for future *in vitro* and *in vivo* tests. The results herein will contribute in the safety of the use of oxytetracycline in a production chain of great importance and moreover bring perspectives for the development of new antimicrobial drugs against a strain with a description of multidrug resistance and with zoonotic potential.

Key-words: Bionformatics. Franciselosis. Streptococcosis. Tetracyclines. Treatment.

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LISTA DE QUADROS

LISTA DE ABREVIATURAS E SIGLAS

3D	*Three-dimensional*; em três dimensões
ALT	*Alanine aminotransferase*; alanine transaminase
ALP	*Alkaline phosphatase*; fosfatase alcalina
AST	*Aspartate aminotransferase*; aspartato transaminase
AUC	*Area under the curve*; área abaixo da curva
bp	*Base pairs*; pares de base
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CC	*Clonal complex*; complexo clonal
CcpA	*Catabolite control protein A*
CFU	*Colony forming units*; unidades formadoras de colônia
CHAH	*Cistine heart agar supplemented with 1 % bovine hemoglobin*; ágar cistina coração suplementado com 1 % de hemoglobina bovina
DEG	*Database of essential genes*; base de dados de genes essenciais
DNA	*Deoxyribonucleic acid*; ácido desoxirribonucleico
FAD	*Flavin adenine dinucleotide*
FMN	*Flavin mononucleotide*
GI	*Genomic island.*; ilha genômica
LC-MS	*Liquid chromatography-Mass spectrometry*; cromatografia líquida aclopada à espectrometria de massas
MBC	*Minimal bactericidal concentration*; concentração bactericida mínima
MCA	*Markov cluster algorithm*
MI	*Metabolic island*; ilha metabólica
MIC	*Minimal inhibitory concentration*; concentração inibitória mínima
MLST	*Multilocus sequence typing*
NCBI	*National Center for Biotechnology Information*; Centro Nacional de Informação Biotecnológica dos Estados Unidos da América

NGS	*Next generation sequencing*; sequenciamento de nova geração
OTC	*Oxytetracycline*; oxitetraciclina
PAI	*Pathogenicity island.*; ilha de patogenicidade
PCR	*Polymerase chain reaction*; reação em cadeia da polimerase
PD	*Pharmacodynamics*; farmacodinâmica
PK	*Pharmacokinetics*; farmacocinética
RI	*Resistance island.*; ilha de resistência
RNA	*Ribonucleic acid*; ácido ribonucleico
SEM	*Scanning electron microscopy*; microscopia eletrônica de varredura
SI	*Symbiotic island*; ilha de simbiose
SNP	*Single nucleotide polymorphism*
ST	*Sequence type*; tipo de sequência

SUMÁRIO

1 INTRODUÇÃO

Nos últimos 55 anos, o aumento na média anual de consumo de pescado superou o de consumo de carne de todos os animais terrestres juntos, 3,2 % contra 2,8 %. Em 2015, o pescado já representava 20% do consumo de proteína animal per capita no mundo (FAO, 2018). Mesmo enfrentando adversidades, com investimento em tecnologia e intensificação da produção, a piscicultura no Brasil conseguiu crescer 7,96 % de 2018 para 2019 (PEIXEBR, 2020). A tilápia do Nilo (*Oreochromis niloticus*) é a terceira espécie de peixe mais produzida no mundo (FAO, 2020). De toda a piscicultura brasileira, a tilápia contribui com o principal montante, representando 57 % e levando o país à 4ª posição no ranking mundial de maiores produtores da espécie (PEIXEBR, 2020).

Apesar das vantagens econômicas da intensificação da produção, o estresse decorrente desta é um fator que aumenta a susceptibilidade dos peixes a doenças causadas por bactérias, parasitas, fungos e deficiências nutricionais (ASENCIOS et al., 2016). As principais espécies ou gêneros de bactérias relacionados com enfermidades em tilápias no Brasil são *Streptococcus agalactiae*, *S. iniae*, *S. dysgalactiae*, *Flavobacterium columnare* e *Francisella orientalis* (MIAN et al., 2009; NETTO; LEAL; FIGUEIREDO, 2011; LEAL; TAVARES; FIGUEIREDO, 2014). Dentre esses, os gêneros *Streptococcus* e *Francisella* se destacam como os principais patógenos de peixes tropicais produzidos em todo o mundo (ELDAR et al., 1995; EVANS et al., 2002; AGNEW; BARNES, 2007; SHEWMAKER et al., 2007; LEAL; TAVARES; FIGUEIREDO, 2014).

Dados da literatura reportam a ocorrência de surtos de estreptococose em mais de 10 estados brasileiros, causados principalmente por *S. agalactiae* dos sorotipos Ib e III (MIAN et al., 2009; BARONY et al., 2017; CHIDEROLI et al., 2017). A mortalidade em surtos de estreptococose pode chegar a 90% do plantel (ELDAR et al., 1995; EVANS et al., 2002). Os surtos de franciselose em tilápias, causados por *F. orientalis*, relatados desde 2012 no sudeste e sul do Brasil, são caracterizados por elevada taxa de mortalidade em temperaturas menores ou iguais a 22º C (LEAL; TAVARES; FIGUEIREDO, 2014; FACIMOTO et al., 2019).

A vacinação é a principal estratégia de profilaxia para a estreptococose (LIU et al., 2016). Contudo, não há imunidade cruzada entre os diferentes sorotipos, genótipos e espécies de *Streptococcus* envolvidos na enfermidade (CHEN et al., 2012; LI et al., 2015). Não há vacinas comerciais para a profilaxia da franciselose e as possíveis candidatas

descritas na literatura esbarram em baixas taxas de sobrevivência a desafios heterólogos ou desafios de biosseguridade (SOTO et al., 2011; SHAHIN et al., 2019). Portanto, o uso de antimicrobianos figura como importante ferramenta no controle e tratamento de surtos em casos de doenças sem vacina disponível ou de falhas vacinais (CHATTOPADHYAY, 2014). O crescente surgimento de patógenos multirresistentes e a consequente racionalização do uso de antimicrobianos torna necessário o estudo e validação de estratégias vigentes de antibioticoterapia e a aplicação de novas metodologias na busca de novas moléculas contra organismos multirresistentes, como a bioinformática. A presente dissertação buscou avaliar a eficácia de duas doses de oxitetraciclina em dois regimes terapêuticos contra franciselose de tilápias e predizer, a partir de ferramentas de bioinformática, novos alvos para moléculas antibacterianas no genoma do sorotipo III de *S. agalactiae*, reportado como multirresistente.

2 REFERENCIAL TEÓRICO

2.1 TILAPICULTURA

A introdução da tilápia no Brasil ocorreu na década de 1970, quando o Departamento Nacional de Obras Contra a Seca povoou reservatórios públicos do Nordeste com alevinos de tilápia do Nilo (*Oreochromis niloticus*) e tilápia de Zanzibar (*Oreochromis hornorum*) (SCHULTER; VIEIRA-FILHO, 2017). Porém, foi apenas na década de 1990 que, com a introdução de tecnologias como a reversão sexual, que a exploração da espécie passou a ser economicamente atraente (KUBITZA, 2003). Hoje, a produção de tilápia está presente em todas as regiões do Brasil e seu aumento é constante. O crescimento entre 2018 e 2019 foi de 7,96 % (PEIXEBR, 2020).

Em 2019, a produção nacional de pescado aumentou 4,9 % em relação ao ano anterior e atingiu 758 mil toneladas, sendo a tilápia a espécie responsável por 57 % deste montante (PEIXEBR, 2020). Estes resultados garantiram ao Brasil a quarta posição entre os maiores produtores mundiais da espécie (PEIXEBR, 2020). Os resultados brasileiros seguem a tendência mundial de crescimento no setor. Entre 2000 e 2018, a contribuição da produção de espécies aquáticas na aquicultura mundial aumentou de 25,7 % para 46,0 % (FAO, 2020). A produção mundial de peixes chegou a 54,3 milhões de toneladas em 2018 (FAO, 2020).

O aumento constante da produção de peixes é, em grande parte, decorrência de tecnologia e intensificação da produção. A tilápia é uma espécie de peixe reconhecida por sua alta rusticidade e fácil adaptação a sistemas intensivos de produção. De fato, no Brasil a produção da espécie ocorre principalmente em sistemas semi-intensivos e intensivos, em tanques-rede ou viveiros escavados (SEBRAE, 2014). Apesar das vantagens econômicas da intensificação, o estresse a que os animais são submetidos nestes sistemas, aumenta a susceptibilidade a doenças causadas por bactérias, parasitas, fungos e deficiências nutricionais (ASENCIOS et al., 2016; LULIJWA; RUPIA; ALFARO, 2019). Dentre essas etiologias, os gêneros bacterianos *Francisella* e *Streptococcus* destacam-se como principais patógenos de peixes tropicais cultivados em todo o mundo (ELDAR et al., 1995; EVANS et al., 2002; AGNEW; BARNES, 2007; SHEWMAKER et al., 2007; LEAL; TAVARES; FIGUEIREDO, 2014).

2.2 FRANCISELOSE

A franciselose é uma doença infectocontagiosa de caráter crônico em peixes (PRADEEP et al., 2017; NGUYEN et al., 2019) que pode causar mortalidades de até 95% de peixes jovens (COLQUHOUN; DUODU, 2011), principalmente em temperaturas abaixo de 22ºC (ORTEGA et al., 2016). É uma doença de etiologia bacteriana, causada por bactérias Gram-negativas, intracelulares facultativas, altamente infecciosas do gênero *Francisella* (ORTEGA et al., 2016). Devido às exigências destes microrganismos e, consequentemente, dificuldades em seu cultivo, muitas espécies de *Francisella* são atribuídas a partir de evidências moleculares, mesmo sem isolamento (BERRADA; TELFORD, 2010). O gênero abriga tanto bactérias ambientais, quanto patogênicas. As espécies reconhecidamente como patógenos para peixes são *Francisella noatunensis* e *Francisella orientalis* (RAMIREZ-PAREDES et al., 2020).

A franciselose é uma doença cosmopolita e, no Brasil, *F. orientalis* é relatada em tilápias desde 2012 (LEAL; TAVARES; FIGUEIREDO, 2014). Pode afetar tanto espécies de água salgada, como de água doce e já foi descrita, além de em tilápias, em peixes roncadores (KAMAISHI et al., 2005), peixes ornamentais (CAMUS et al., 2013; LEWISCH et al., 2014) e bacalhau do Atlântico (OLSEN et al., 2006; BAKKEMO et al., 2016; VARGAS-LAGOS et al., 2019). A apresentação clínica é inespecífica e os sinais que podem ser observados são anorexia, letargia, melanose, natação errática e mortalidade elevada em alevinos e juvenis (JEFFERY et al., 2010; ASSIS et al., 2017; NEETHIRAJAN et al., 2017; SEBASTIÃO et al., 2017). Os achados macroscópicos incluem esplenomegalia e granulomas multifocais em baço, rins, fígado e brânquias (SOTO et al., 2009; SEBASTIÃO et al., 2017; FERNANDEZ-ALARCON et al., 2019). Na histopatologia, a principal lesão é inflamação granulomatosa com predomínio de macrófagos (SOTO et al., 2009). Além da alta mortalidade em estágios iniciais do desenvolvimento, a doença pode causar prejuízos no final da cadeia produtiva. A carcaça de peixes acometidos por franciselose que atingem peso de abate pode ser condenada devido a acúmulos de melanomacrófagos na musculatura, observados como pontos pretos (MAUEL et al., 2007; JUNIOR et al., 2020).

Um dos principais mecanismos de patogenicidade da *F. noatunensis* é a evasão do sistema imune pela capacidade de sobrevivência dentro de macrófagos, através de estratégias similares aos da *F. tularensis* (SOTO et al., 2009), causador da tularemia em humanos e potencial arma biológica (OYSTON; SJOSTEDT; TITBALL, 2004; PECHOUS;

MCCARTHY; ZAHRT, 2009). Há homologia entre genes do *locus* de crescimento intracelular (*igl*) das duas espécies (SOTO et al., 2013). O comportamento intracelular facultativo destas bactérias dificulta o desenvolvimento de profilaxia através de vacinas. Atualmente, não há vacinas comerciais para o controle da franciselose em peixes e as possíveis candidatas na literatura esbarram em problemas como baixa biosseguridade, por uma possível reversão de virulência de uma cepa atenuada (SOTO et al., 2011), ou baixa proteção frente a desafio por cepas heterólogas à utilizada na produção da bacterina (SHAHIN et al., 2019). Assim, a profilaxia da doença é baseada em medidas de biosseguridade e rastreamento de lotes e o controle de surtos é através da utilização de antimicrobianos.

2.3 ESTREPTOCOCOSE

A estreptococose é uma doença septicêmica que atinge uma variedade de espécies de peixes e está amplamente disseminada geograficamente (OLIVARES-FUSTER et al., 2008). É de etiologia bacteriana, causada por cocos Gram-positivos de diferentes espécies do gênero *Streptococcus*. As espécies que podem estar envolvidas são: *S. agalactiae* (EVANS et al., 2002), *S. iniae* (AGNEW; BARNES, 2007), *S. dysgalactiae* (NOMOTO et al., 2004), *S. phocae* (GIBELLO et al., 2005) e *S. ictaluri* (SHEWMAKER et al., 2007). As duas espécies com destaque como patógeno em tilápias são *S. agalactiae* e *S. iniae*, sendo a primeira mais prevalente (LIU et al., 2016).

Apesar de orginalmente isolado de golfinhos em 1976, *S. iniae* é uma das principais espécies causadoras de estreptococose em peixes (AGNEW; BARNES, 2007). No Brasil, o primeiro relato de *S. iniae* causando surtos de doença em tilápias foi em 2012, no estado do Paraná (FIGUEIREDO et al., 2012). Contudo, a espécie que se destaca como principal agente etiológico de estreptococose no Brasil é *S. agalactiae* (FIGUEIREDO et al., 2006). Além de importante patógeno para peixes, *S. agalactiae* é comensal dos tratos urinário e gastrointestinal de humanos, e atua como patógeno oportunista em recém nascidos, idosos e pessoas com alguma doença primária predisponente (ARMISTEAD et al., 2019; RAABE; SHANE, 2019). Essa espécie pode ainda infectar bovinos, sendo a mastite de vacas leiteiras sua principal apresentação (PEREIRA et al., 2010).

De acordo com características de cápsula, os isolados de *S. agalactiae* podem ser classificados em 10 sorotipos antigenicamente distintos: Ia, Ib, II a IX (SLOTVED et al., 2007). Além da classificação sorológica, os isolados podem ser classificados em

genótipos (*sequence types*, ST), baseados em *multilocus sequence typing* (MLST), que são agrupados em complexos clonais (CC) de acordo com a similaridade entre si (BARONY et al., 2017). A maioria dos isolados brasileiros são do sorotipo Ib e, ocasionalmente são isolados sorotipos Ia e outros não sorotipáveis (GODOY et al., 2013). Em 2016, foi identificado pela primeira vez, no estado do Piauí, um isolado de *S. agalactiae* sorotipo III com perfil de resistência a várias classes de antimicrobianos (CHIDEROLI et al., 2017). A emergência do sorotipo III no Brasil é preocupante. Surtos de doença invasiva em humanos imunocompetentes por *S. agalactiae* sorotipo III pertencente ao genótipo ST283 foram relacionados ao consumo de peixe cru em Singapura (TAN et al., 2016; KALIMUDDIN et al., 2017). Ainda, outros isolados brasileiros do sorotipo III foram identificados como pertencentes ao ST283 e agruparam juntos com os isolados asiáticos de peixes e humanos (LEAL et al., 2019). Especula-se que o sorotipo III tenha emergido em pisciculturas brasileiras através de quebra de barreira interespécies (CHIDEROLI et al., 2017) ou através da importação de peixes vivos provenientes da Ásia (LEAL et al., 2019).

Os sinais clínicos da estreptococose em peixes incluem anorexia, letargia, natação errática, em espiral e na superfície, escoliose, opacidade ocular, hemorragia periorbital e intraocular e exoftalmia (EVANS et al., 2002). Morte súbita, sem outros sinais clínicos, também pode ser observada (ZAMRI-SAAD; AMAL; SITI-ZAHRAH, 2010). Os achados microscópicos mais comuns são epicardite, esplenite, coroidite e meningite granulomatosas ou linfohistiocíticas (SOTO et al., 2016). No Brasil, surtos com alta mortalidade já foram descritos nas regiões nordeste, sudeste e sul (MIAN et al., 2009; CHIDEROLI et al., 2017). Os principais fatores de risco para surgimento de surtos são temperaturas da água acima de 27ºC e manejos muito intensos (MIAN et al., 2009).

A principal estratégia de profilaxia para a estreptococose é a vacinação (LIU et al., 2016). Contudo, não há imunidade cruzada entre os diferentes sorotipos e genótipos de *S. agalactiae* (CHEN et al., 2012; LI et al., 2015) e entre as diferentes espécies de *Streptococcus* envolvidas nesta enfermidade. Existem duas vacinas disponíveis no mercado brasileiro, uma protege contra o sorotipo Ib de *S. agalactiae* (Aquavac Strep® SA, MSD) e a outra protege contra os sorotipos Ia e III (Aquavac Strep® SA1, MSD). A falta de um produto único com os dois principais sorotipos de *S. agalactiae* circulantes no país (Ib e III) favorece uma má cobertura vacinal e consequentes surtos por infecção heteróloga. Na tentativa de mitigar essa situação, as duas vacinas comerciais podem ser administradas de forma conjunta, com uma vacinadora de fonte dupla que mistura e inocula os dois produtos de uma

única vez no peixe. Entretanto, o custo com a aquisição de duas vacinas e do equipamento se torna alto e pouco atrativo para produtores.

2.4 Uso de Antimicrobianos na Piscicultura

O uso de antimicrobianos na produção animal é constantemente revisado e, principalmente quando empregado como promotor de crescimento, cada vez mais restrito. Especialmente na aquicultura, devido ao grande acúmulo de resíduos favorecido por seus sistemas em águas adjacentes, nas espécies animais e vegetais (LULIJWA; RUPIA; ALFARO, 2019). Porém, os antimicrobianos são importante ferramenta para controle e tratamento de surtos em casos de doenças sem vacina disponível ou de falhas vacinais (CHATTOPADHYAY, 2014), como ocorre com a franciselose e a estreptococose em peixes.

Um levantamento considerando 11 dos 15 países com as maiores produções de pescado no mundo entre 2008 e 2018, encontrou 67 diferentes moléculas antimicrobianas em uso, uma média de 15 por país, sendo oxitetraciclina, sulfadiazina e florfenicol as mais comumente utilizadas (LULIJWA; RUPIA; ALFARO, 2019). No Brasil, apenas produtos à base de oxitetraciclina e florfenicol são licenciados pelo Ministério da Agricultura, Pecuária e Abastecimento para uso na piscicultura (SINDAN, 2020). Contudo, há evidências de que produtores utilizam irregularmente produtos à base de outras moléculas licenciadas para uso na produção de outras espécies animais. Isto fica claro no Plano Nacional de Controle de Resíduos e Contaminantes, em que é previsto o monitoramento de resíduos de 23 outras moléculas no pescado (BRASIL, 2015).

A oxitetraciclina é utilizada mundialmente na aquicultura desde a década de 1950 (RIGOS; SMITH, 2013). É uma molécula bacteriostática por ligação a subunidade 30S ribossomal que possui baixos custo e toxicidade, além de alta penetração tecidual (SIDHU et al., 2018). A farmacocinética de drogas varia drasticamente em diferentes espécies de peixes e em situações ambientais variadas, como salinidade e temperatura, mesmo na mesma espécie (RIGOS; SMITH, 2013; SIDHU et al., 2018; RAIRAT et al., 2019). Assim, há poucas informações disponíveis de farmacocinética mesmo para espécies de peixes muito produzidas e para moléculas liberadas e utilizadas amplamente, como a tilápia e a oxitetraciclina (RIGOS; SMITH, 2013; SIDHU et al., 2018). Estudos constantes que visem determinar a segurança do emprego de antimicrobianos tanto na fisiologia dos peixes, quanto para a segurança alimentar do produto final são mandatórios. Além disso, deve-se

determinar a real eficiência dessas moléculas em combater linhagens bacterianas circulantes nos plantéis nacionais.

2.5 Estudos Genômicos e Ferramentas *in silico* na Busca por Novos Antimicrobianos

O início dos estudos genômicos ocorreu na segunda metade do século XX. Dentre as tecnologias de sequenciamento de nucleotídeos que surgiram, destacou-se a metodologia de “Sanger”, que utilizava dideoxi-nucleotídeos para amplificar fragmentos de diferentes tamanhos de uma mesma região genômica e, pela migração desses fragmentos em eletroforese, determinar sua sequência (SANGER; NICKLEN; COULSON, 1977). A associação de programas computacionais ao sequenciamento de Sanger possibilitou a montagem de fragmentos maiores de sequências de nucleotídeos através da sobreposição de fragmentos pequenos sequenciados, como a metodologia *shotgun* (STADEN, 1979). Devido ao alto custo e tempo demandado para montar as sequências e obter genomas completos, normalmente apenas um genoma de uma determinada espécie bacteriana era montado, chamado de “referência”, e utilizado para desenhar estudos de evolução molecular, função e manipulação genética (GIANI et al., 2020).

A corrida para a obtenção do genoma humano nos anos 1990 destacou o potencial econômico dos estudos genômicos e empresas privadas passaram a desenvolver as tecnologias de sequenciamento hoje chamadas de “nova geração” (*next generation sequencing – NGS*) (GIANI et al., 2020). Resumidamente, as diferentes plataformas de NGS sequenciam genomas através da fragmentação do material genético de uma amostra para formar uma biblioteca de fragmentos, pareados ou não (HEAD et al., 2014), que são amplificados simultaneamente e a sequência é determinada pela detecção de sinais emitidos, que podem ser físicos ou químicos, a cada ciclo de amplificação (MARGULIES et al., 2005; METZKER, 2010; LOMAN et al., 2012). As grandes vantagens das tecnologias de NGS foram baixo custo e menor tempo para obtenção de sequências (GIANI et al., 2020).

O número de genomas completos de microrganismos em bancos de dados públicos aumentou exponencialmente desde a introdução de tecnologias de NGS, permitindo análises comparativas a nível de genoma (MUKHERJEE et al., 2020). O aumento do número de genomas disponíveis para uma mesma espécie ou gênero, permitiu a determinação do pangenoma, conceito introduzido primeiramente em estudos genômicos de *S. agalactiae* (TETTELIN et al., 2005). O pangenoma é a coleção completa de todos os genes encontrados

em um gênero ou espécie. De acordo com sua distribuição nos representantes estudados, os genes dentro do pangenoma são classificados em genes *core*, aqueles presentes em todos os genomas, genes compartilhados, genes presentes em dois ou mais genomas, e os genes *singleton*, que são encontrados em apenas um dos genomas analisados (JAISWAL et al., 2020).

A aplicação de estudos genômicos comparativos e seus desdobramentos (proteômica e metabolômica) integrados a ferramentas de bioinformática, também chamadas de ferramentas *in silico*, são de grande valor na identificação de alvos para drogas em patógenos (MONDAL et al., 2015). A aplicação dessas estratégias torna o processo de desenvolvimento de novas drogas mais econômico e menos trabalhoso, eliminando anos de pesquisas de identificação e isolamento de alvos *in vitro* ou *in vivo* (TIMO et al., 2019). De fato, estudos genômicos associados a ferramentas de bioinformática já se mostraram eficientes no combate a microrganismos multirresistentes. Estudos de seleção *in silico* de moléculas com efeitos antimicrobianos e com validação da inibição em ensaios *in vitro* são descritos para isolados multirresistentes de *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Escherichia coli*, and *Vibrio cholerae* (ZANG et al., 2016; SANDHAUS; CHAPAGAIN; TSE-DINH, 2018; TIWARI et al., 2019).

Os principais critérios na busca por potenciais alvos de ação farmacológica no genoma de um patógeno são genes que codifiquem proteínas que não sejam homólogas ao do hospedeiro, para evitar qualquer efeito adverso, que estejam presentes no *core* genoma da espécie, que sejam citoplasmáticas, devido a seu papel na manutenção celular (VILELA RODRIGUES et al., 2019), e que sejam essenciais à sobrevivência do microrganismo (MONDAL et al., 2015).

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4 HIPÓTESES

i. O fornecimento de oxitetraciclina via oral para tilápias nos primeiros momentos de infecção não é hepatotóxico e é capaz de controlar o surgimento de sinais clínicos de franciselose, reduzir mortalidade pela doença e impede o estado de carreador assintomático.

ii. Através do emprego de análises genômicas comparativas e ferramentas de bioinformática é possível predizer alvos drogáveis no *core* genoma do sorotipo III de *Streptococcus agalactiae* e selecionar compostos naturais que se liguem fortemente a esses alvos e inativem o patógeno bacteriano.

5 OBJETIVOS

5.1 Objetivo Geral

Avaliar a eficácia clínica da oxitetraciclina contra a infecção experimental por *Francisella orientalis* em tilápias e prever alvos de drogas no genoma do sorotipo III de *Streptococcus agalactiae*.

5.2 Objetivos Específicos

i. Determinar a concentração bactericida mínima da oxitetraciclina e seus efeitos na morfologia celular contra a cepa F1 de *Francisella orientalis*
ii. Avaliar os efeitos fisiológicos do fornecimento via oral de oxitetraciclina, nas doses 100 mg·kg de peso vivo^{-1} e 200 mg·kg de peso vivo^{-1} através de biomarcadores séricos de lesão hepática e histopatologia de tecidos hepáticos
iii. Avaliar a eficácia da oxitetraciclina nas doses 100 mg·kg de peso vivo^{-1} e 200 mg·kg de peso vivo^{-1} via oral, fornecidas um dia antes ou após a infecção, em controlar evolução de sinais cínicos, mortalidade e carreadores assintomáticos em tilápias desafiadas experimentalmente por *Francisella orientalis*
iv. Obter o *core* genoma do sorotipo III de *Streptococcus agalactiae* através de genomas completos de cepas isoladas de peixes ou humanos disponíveis no banco de dados do *National Center for Biotechnology Information*
v. Predizer alvos proteicos para antimicrobianos no *core* genoma do sorotipo III de *Streptococcus agalactiae* através de ferramentas de bioinformática e critérios subtrativos
vi. Sugerir moléculas que se liguem fortemente aos alvos identificados no *core* genoma através de *docking* virtual entre uma biblioteca de compostos naturais e que possam ser utilizados como antimicrobianos eficazes contra o sorotipo III de *Streptococcus agalactiae*

6 ARTIGO A – ADMINISTRATION OF DEHYDRATED OXYTETRACYCLINE EFFECTIVELY REDUCES FRANCISELLOSIS MORTALITY IN NILE TILAPIA

Artigo submetido para a revista “Aquaculture Research” em 23 de outubro de 2020

Administration of dehydrated oxytetracycline effectively reduces francisellosis mortality in Nile tilapia

Running title “Oxytetracicline for tilapia francisellosis”

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ABSTRACT

Francisellosis is a disease responsible for severe economic losses in aquaculture. Currently, this disease is controlled via biosecurity measures and treatment of affected batches. In this study, tilapia juveniles were challenged with *Francisella orientalis* via immersion, and fed with oxytetracycline-medicated feed, at 100 mg kg fish^{-1} and 200 mg kg fish^{-1}, under prophylactic and therapeutic strategies for 16 and 15 days, respectively. All doses and treatment strategies reduced the fish mortality. The detection of the pathogen from the spleen were lower in the treated groups. Histopathology analysis showed that challenged fish that received the highest dosage presented fewer granuloma in their spleens and less inflammatory infiltrate in their livers than untreated fish. Fish that received the prophylactic treatment, regardless of the dose, presented fewer granuloma in their livers than untreated fish. Fish treated with 100 mg kg fish^{-1} had better scores for hepatocyte accumulations than fish treated with the higher dosage. Furthermore, 20 % of the unchallenged fish that received the higher dosage presented discreet inflammatory infiltrate in their livers. No differences were observed in the serum hepatocyte lesion enzyme quantifications. The results indicate that oxytetracycline-medicated feed is safe, even at the higher dose, and effective at preventing and controlling francisellosis in tilapia.

KEYWORDS: carrier identification; dosage; francisellosis treatment; hepatological lesion scores; tetracyclines.

1 INTRODUCTION

Fish production often faces health challenges, especially outbreaks of bacterial diseases. Francisellosis is a major disease affecting tilapiculture and has been identified as the cause of outbreaks in at least 11 countries: Taiwan (Chen et al., 1994), the United States of America (Mauel et al., 2005), Costa Rica (Soto, Hawke, Fernandez, & A Morales, 2009), Indonesia (Ottem, Nylund, Karlsbakk, Friis-Møller, & Kamaishi, 2009), the United Kingdom (Jeffery, Stone, Feist, & Verner-Jeffreys, 2010), Colombia (Iregui, Vasquez, Rey, & Verjan, 2011), Brazil (Leal, Tavares & Figueiredo, 2014), Thailand (Nguyen, Dong, Senapin, Pirarat, & Rodkhum, 2016), China (Lin et al., 2016), Mexico (Ortega et al., 2016). and Honduras (Soto et al., 2019). The disease is characterized by chronic granulomatous inflammation of the spleen, liver, and kidney. Francisellosis is responsible for large economic losses, as severe outbreaks can result in mortalities of up to 95 % (Chern & Chao, 1994; Colquhoun & Duodu, 2011). Additionally, granulomas characterized by central necrosis and the presence of melanomacropahges in adults' muscles, macroscopically described as black spots, caused by francisellosis are reported to result in the condemnation of fillets at slaughterhouses (Junior et al., 2020; Mauel, Soto, Moralis, & Hawke, 2007).

Piscine francisellosis is caused by Gram-negative coccobacilli of the species *Francisella noatunensis* and *Francisella orientalis* (Ramirez-Paredes et al., 2020). Controlling this disease can be challenging as these bacteria are facultatively intracellular (Colquhoun & Duodu, 2011), which hampers the development of effective vaccines. Furthermore, currently, there is no commercially available vaccine against piscine francisellosis. In the literature, only two experimental vaccine formulations are reported to have good results. However, one is a live-attenuated vaccine (Soto, Wiles, Elzer, Macaluso, & Hawke, 2011), which is not considered safe, owing to the risk of virulence recovery; and the other is an adjuvanted bacterin that was first reported with positive results (Ramírez-Paredes et al., 2019). However, a lower relative percent survival (RPS) to heterologous challenges was reported in a subsequent study from the same group (Shahin et al., 2019). Thus, the current control of piscine francisellosis is based on biosecurity policies and the treatment of affected fish.

Although seriously revised and restricted globally as growth promoters, the use of antibiotics in veterinary medicine can be an important tool in the control, prevention, and treatment of diseases in naive populations (Chattopadhyay, 2014). Their use is of great importance for diseases that cause massive mortality outbreaks where no vaccine is available, such as piscine francisellosis. Fish mortality caused by intracellular pathogens, such as *Francisella* and *Piscirickettsia*, has been successfully controlled by oxytetracycline (OTC)-medicated feed (Chern & Chao, 1994; Ostland et al., 2006). Mauel et al. (2005) described a decrease in tilapia mortality to near zero, after the fifth day of treatment with OTC-medicated feed. Additionally, 0.5 μg mL^{-1} of OTC has been reported as the minimum inhibitory concentration (MIC) of an *F. philomiragia* (syn. *F. noatunensis* subsp. *noatunensis*) salmon isolate (Bohle et al., 2009), and 0.25 μg mL^{-1} for *F. orientalis* (Soto, Griffin, Wiles, & Hawke, 2012). Recently, a study on the suitability of *Galleria mellonella* larva as an infection model for piscine francisellosis showed that early tetracycline treatment increases larval survival when infected with fish-isolated *F. noatunensis* subsp. *orientalis* (syn. *F. orientalis*) (Djainal, Shahin, Metselaar, Adams, & Desbois, 2020). However, the effectiveness of different OTC dosages at different stages of *Francisella* spp. infections, and their effects on tilapia tissues, are poorly addressed in the literature.

Oxytetracycline is an antibiotic that binds to the 30S ribosomal subunit, and acts on bacterial protein synthesis (Sidhu et al., 2018). It has been used in aquaculture since the 1950s (Rigos & Smith, 2013) and its major advantages are good penetration in the fluids and tissue, low cost, and low toxicity (Sidhu et al., 2018). OTC is one of the three most used antibiotics, of 67 compounds identified as in

use by 11 of the 15 countries that are major aquaculture producers (Lulijwa, Rupia, & Alfaro, 2019). The literature on OTC pharmacokinetics (PK) and pharmacodynamics (PD) is vast, but restricted, and the divergence in experimental methodologies makes comparisons and assumptions difficult (Rigos & Smith, 2013). Furthermore, the literature lacks diversity regarding the target fish species, and specifically, information on OTC PK and PD in tilapia species is poor (Rigos & Smith, 2013; Sidhu et al., 2018). What is known of OTC PK in tilapia is: i) medicated feed results in great variations in the plasma and tissue concentrations within the same population (Chen, Getchell, Wooster, Craigmill, & Bowser, 2004); ii) the depletion of OTC is not compromised in infected tilapia (*Streptococcus iniae* and *Vibrio vulnificus*) when compared to healthy tilapia (Chen & Bowser, 2005); and iii) OTC absorption and elimination are slower in freshwater and brackish water maintained tilapia when compared to those in saltwater (Sidhu et al., 2018);

Although OTC is licensed for use in aquaculture and its use against francisellosis is reported to be successful, these reports either describe OTC field use and its outcome; or a specific strain susceptibility to the drug. There is no information available on whether OTC eliminates *Francisella* cells from the hosts, or if the treated fish remain as carriers. Additionally, OTC is still not licensed specifically for tilapia francisellosis. For safer and more evidence-based use of OTC-medicated feed against this disease, this study evaluated the effects of a dehydrated OTC-based feed additive (TM700®, Phibro Animal Health) on the growth and cell morphology of *F. orientalis in vitro*. To evaluate the ability of the OTC to control *F. orientalis* infections, tilapia juveniles were fed two different doses of OTC-medicated feed (i.e., 100 mg kg fish^{-1} and 200 mg kg fish^{-1}), and prophylactic and early therapeutic treatment strategies were evaluated. Possible carrier fish were identified using microbiological, PCR, and spleen macroscopic lesion analyses. Additionally, the possible hepatotoxic effects of the treatments were assessed using histopathology and the quantification of serum biomarkers for the hepatocyte lesions.

2 MATERIALS AND METHODS

2.1 Bacterial strain and growth conditions

Francisella orientalis strain F1 was used in all *in vitro* and *in vivo* trials. It was isolated from an outbreak in a tilapia farm in southeastern Brazil (Facimoto et al., 2019). Strain F1 was grown in cystine-heart agar supplemented with 1 % bovine hemoglobin (CHAH) (Himedia®, India), at 28 °C for 96 h.

2.2 Antimicrobial susceptibility

A 100 mg mL^{-1} initial solution of the feed additive was prepared in sterile saline (0.85 % NaCl) and then serially 2-fold diluted 16 times. Owing to the 77.8 % OTC levels in the product, the OTC concentrations ranged from 77.8 mg mL^{-1} to 1.19 µg mL^{-1}. Agar diffusion and minimum bactericidal concentration (MBC) analyses were performed. The agar diffusion test was conducted based on the disk-diffusion test, standardized by the Clinical and Laboratory Standard Institute (CLSI, 2006). The adaptations were mandatory, owing to the fastidious nature of the *F. orientalis*, which would not grow on Mueller-Hinton agar; and because it was not a purified antimicrobial that was being tested. Briefly, an F1 strain inoculum with 0.5 McFarland's scale turbidity was streaked onto CHAH plates using a sterile swab. After the plates were dried, 10 µL of each dilution was inoculated into the center of the dishes. After complete absorption of the dilutions by the medium, the dishes were incubated at 28 °C for 48 h. Inhibition zones were measured in millimeters. Each dilution was tested in duplicate. The MBC was assessed in 96-well plates by mixing each product dilution and an F1 strain inoculum, with 0.5 McFarland's scale turbidity in a 1:1 volume proportion, followed by incubation at 28 °C for 48 h. Then, 1 µL from each well was streaked on CHAH and incubated at 28 °C for 96 h. The MBC

was determined as the lowest dilution of the product able to prevent bacterial growth after subculture on antibiotic-free media (CHAH). MBC was performed in triplicate for each dilution. In both analyses, sterile saline was used as a control.

2.4 Scanning electron microscopy

An inoculum of F1 strain with a McFarland's scale turbidity of 2 was exposed to two dilutions (1:1 v/v) of the product tested: 781 µg mL^{-1} and 48.8 µg mL^{-1} (607.8 µg mL^{-1} and 37.9 µg mL^{-1} of OTC, respectively), at 28 °C for 6 h. An additional sample was exposed to sterile saline as a control. The samples were centrifuged for 5 min at 500 × *g*, resuspended in 100 µL of fixative (2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.0) and transferred to 24-well polystyrene microtiter plates (Fisher Scientific, Kamstrupvej, Roskilde, Denmark) with glass coverslips, pre-coated with a thin layer of poly-L-lysine (Sigma, Saint Louis, MO, USA). After 1 h, the volume was adjusted to 500 µL using the fixing solution to avoid cell adherence to the coverslips and they were incubated at 25 °C for 12 h. The samples were postfixed in 1 % OsO4 (Electron Microscopy Sciences, Washington, PA, USA) and dehydrated in an ethanol series (30, 50, 70, 90, and 100 °GL). Samples were subjected to critical-point drying with a CO_2 BALTEC CPD 030 Critical Point Dryer (Shapiro Center for Engineering and Physical Science Research, New York, USA), coated with gold in a BALTEC SDC 050 Sputter Coater (Capovani Brothers Inc., New York, USA), and observed using a scanning electron microscope (FEI Quanta 200, Netherlands).

2.5 Fish

A total of 900 Nile tilapia juveniles (mean weight 15 g) were obtained from a commercial hatchery in Paraná State, Brazil, without a history of francisellosis. The fish were distributed in 18 150 L tanks (50 fish per tank) and underwent a 15-day acclimatization period before the experimental trial. Fish were kept in a 12 h light-dark photoperiod in dechlorinated water with continuous renewal. The temperature was maintained at approximately 25 °C, and oxygen was provided by two aerators, resulting in an average dissolved oxygen of 5.4 mg mL^{-1}. Fish were fed four times a day until apparent satiety (4 to 6 % of body weight per day), with commercial extruded feed, which had 32% crude protein. Water pH (6.8 to 7.2), total ammonia (< 0.4 mg/L), and chlorine presence were assessed daily and maintained throughout the experimental period. To confirm the health status of the fish, a sample of 10 fish were euthanized with benzocaine (200 mg mL^{-1}) for microbiological assessments. Brain, kidney, and spleen samples were streaked in CHAH and Mueller-Hinton agar (Kasvi, Brazil) enriched with 5 % defibrinated bovine blood. Plates were incubated at 28 °C for 5 days. No bacterial growth was observed in any of the samples. All animal procedures were approved by the Ethics Committee on Animal Use of State University of Londrina (Approval number CEUA/UEL-18705.2018.39). All experiments were performed following relevant guidelines and regulations.

2.6 Experimental design

Two OTC doses and two treatment strategies were analyzed. The first dose was 100 mg kg $fish^{-1}$. This dose was used as it is commonly adopted by fish farmers in Brazil for the treatment of francisellosis. The second dose was 200 mg kg^{-1}, and this was selected to assess the possible effects of an overdose on the product. The treatment strategies analyzed were prophylactic (treatment initiated 1 day before bacterial challenge) and therapeutic (treatment initiated 1 day after bacterial challenge). The prophylactic and therapeutic groups were fed medicated feed for 16 and 15 days, respectively. Three control groups were included as follows: a group neither treated nor challenged (negative control), a group not treated but challenged (positive control), and a group treated with 200

mg kg^{-1} dosage but not challenged, as a control of the possible deleterious effects. Therefore, the experimental groups were as follows:

$OTC100_{BC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day before bacterial challenge;
$OTC100_{AC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day after bacterial challenge;
$OTC200_{BC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day before bacterial challenge;
$OTC200_{AC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day after bacterial challenge
NC: negative control, fish neither treated nor challenged;
PC: positive control, fish not treated but challenged;
TMC: control treated with 200 mg kg^{-1} dosage but not challenged.

Groups $OTC100_{BC}$, $OTC100_{AC}$, $OTC200_{BC}$, and $OTC200_{AC}$ were conducted in triplicate and control groups were conducted in duplicate. Fish were monitored twice a day for 45 days after the end of the treatment.

2. 7 Medicated feed

The following data were considered to calculate the amount of product added to the feed: 77.8 % antimicrobial availability in the product, that the fish consumed 4 % of their body weight per day, and that there was a loss of 15 % of the product due to leaching. Consequently, 6.7 g and 13.4 g of product were incorporated into 1.5 kg of feed, to obtain 100 mg kg $fish^{-1}$ and 200 mg kg $fish^{-1}$ in the OTC-medicated feed, respectively. The product was homogeneously distributed and mixed into the feed as one-third of the total amount at a time, followed by the incorporation of 5 % (w/v) of the universal binding agent (carboxymethylcellulose-based; Vansil, Descalvado, SP, Brazil) and homogenization. The prepared feed was dried at 18 °C, overnight. The concentration of the OTC in each medicated feed was assessed before the experimental trials with liquid chromatography-mass spectrometry (LC-MS). The analyses were performed at Phibro Animal Health™ (Guarulhos, Brazil). Both medicated feeds contained the expected concentration of OTC.

2.8 Bacterial challenge

One day prior to the bacterial challenge, the water temperature was gradually decreased to approximately 21 °C, to better mimic the natural infection conditions (Suphoronski et al., 2019). Immediately before the bacterial challenge, the volume of the water in the tanks was reduced to approximately 30 L, and oxygenation was maintained. Then, via immersion, an F1 strain suspension containing 1.2 10^8 CFU mL^{-1} was added to groups 1–4 and the positive control. This dose induced a 50 % mortality rate in a previous lethal dose trial with experimental conditions similar to those used in this study (data not shown). After 2 h of the bath treatment, the volume of the tanks was restored. The negative and the treated controls were handled similarly, but without exposure to the bacteria.

2.9 Carrier identification and Histopathology analysis

At the end of the 45-day monitoring period, the remaining fish were euthanized with a benzocaine overdose (200 mg L^{-1}). Spleen and liver samples were then collected from the fish. Spleen fragments were collected and either: i) immediately seeded in CHAB and incubated at 28 °C for 96 h; ii) fixed in 10 % buffered formalin for histopathological analysis; and iii) frozen at −20 °C until DNA extraction. Macroscopic lesions in the spleen were observed at the moment that the samples were taken. The fish that were considered healthy (without macroscopic spleen lesions), but in which it was possible to reisolate *F. orientalis* from the spleen samples, were considered asymptomatic carriers. Liver fragments were fixed in 10 % buffered formalin for histopathological analysis. DNA was extracted from the tissue samples with the commercial DNeasy Blood & Tissue kit (QIAGEN,

Hilden, Germany), following the manufacturer's instructions. *Francisella* genus PCR identification was conducted with the DNA samples, using the primers described by Forsman et al. (1994). Fixed tissue samples were embedded in paraffin at 60 °C to obtain cross-sections, with a thickness of 5 µm and stained with hematoxylin-eosin. The slides (Entellan, Darmstadt, Germany) were mounted and subjected to microscopic evaluation. The spleen slides were classified by the stage of their granuloma lesions, based on the francisellosis histopathological lesion developments previously described by Soto et al. (2013). Further, a value/score was attributed to each class as: 0 - no structural changes; 1 - enlargement of perivascular macrophage sheath; 2 - macrophage vacuolization extending beyond the perivascular sheath; 3 - incipient granulomas around the perivascular sheaths; and 4 - multifocal to coalescent granulomas replacing the normal splenic structure. Hepatocytes were classified according to their accumulations as accentuated and diffuse glycogen accumulation (+); moderate and diffuse/multifocal glycogen accumulation (++); or discrete glycogen accumulation concomitant to multifocal or diffuse fatty accumulation (+++). The accumulation scores were based on the normal glycogenic accumulations in healthy captive-reared fish (Wolf et al., 2015) and on the presence of fatty accumulations after a starvation period in the tilapia (Dias-Junior et al., 2016). The liver slides were also evaluated for the presence and intensity of granuloma and inflammatory infiltrate.

2.10 Serum hepatic enzymes quantification

On days 4 and 7 after the treatment began, five fish samples were collected from groups $OTC100_{BC}$ and $OTC200_{BC}$. An additional sample of five fish was collected from NC as a control. The fishes were anesthetized with benzocaine (50 mg L^{-1}), and their blood was collected by puncturing the caudal vessels with 3 mL syringes (21 G) without an anticoagulant. Blood samples were transferred to microtubes, allowed to coagulate, and centrifuged at 1400 × *g* for 10 min, to obtain the serum. Serum samples were stored at −20 °C until analysis. The serum activities of the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were measured using an automated spectrophotometer (Dimension Xpand Plus® Siemens, Brazil), with specific commercial reagents (Flex® reagent cartridge, Siemens, Brazil).

2.11 Statistical analysis

The cumulative mortality was analyzed using Fisher's exact test with a significance level of 5 % with OpenEpi, version 3.01 (https://www.openepi.com/Menu/OE_Menu.htm). Serum hepatic enzyme data that tested positive for normality and homogeneity were submitted for the analysis of variance (ANOVA) and Tukey's test to compare their arithmetic means, adopting a significance level of 5 % (i.e., ALP). The data that did not present normality or homogeneity were submitted to Kruskal-Wallis' nonparametric test, followed by Dunn's test, with a significance level of 5 % (i.e., ALT and AST). The aforementioned analyses were performed using R version 3.6 (R Core Team, 2019).

3 RESULTS

Different dilutions of the tested product resulting in OTC concentrations ranging from 77.8 mg mL^{-1} to 1.19 µg mL^{-1} were tested *in vitro* against *F. orientalis* strain F1, using agar diffusion and MBC tests. The lowest OTC concentration capable of satisfactorily inhibiting the growth of the F1 strain was 303.9 µg mL^{-1}, resulting in a 30 mm halo. Since a 10 µL drop of the dilution was used, it could be considered that 3.04 µg generated the 30 mm halo. The MBC technique was applied over minimal inhibitory concentrations (MICs), owing to the turbidity of the product dilutions that could interfere in the analysis interpretation. The MBC of OTC was 37.9 µg mL^{-1}.

Scanning electron microscopy (SEM) was performed to visualize the effects of the product directly on the morphology of the *F. orientalis*. A suspension of the F1 strain was exposed for 6 h to a 4-fold

higher than the MBC concentration (607.8 µg OTC mL^{-1}), to the MBC (37.9 µg OTC mL^{-1}), and saline, as a control. Figure 1A shows *F. orientalis* untreated cells with their characteristic coccoid to coccobacillus morphology. The morphologies of the cells treated with 607.8 µg mL^{-1} of OTC (Figure 1B) were altered, as they displayed a shrunken appearance and an uneven surface. Some cells maintained their coccoid shape, although they seemed larger than the untreated cells, and this was probably due to an effect of the antimicrobial. The MBC-treated cells were mostly shrunken, as observed at the highest concentration (Figure 1C). Neither of the two concentrations resulted in a decrease in cell numbers.

All evaluated doses and treatments successfully reduced the fish mortality rate (Figure 2). The mean mortality rate for the positive control was 49.0 %, and the highest mortality observed for the treated groups was 6.7 % ($OTC200_{BC}$). There was a significant difference in the mortalities between the prophylactic groups ($p < 0.05$). No mortality was observed in $OTC100_{BC}$ and a 6.7 % mortality was observed in $OTC200_{BC}$. On the other hand, there was no significant difference between the therapeutic groups, although a 1.3 % mortality was observed in the $OTC100_{AC}$. No mortality was observed in the NC and TMC groups.

In addition to the reduced mortality rates, the frequencies of the macroscopic lesions and the detection of *Francisella* spp., in both the CHAH bacterial culture and the PCR, were lower in all the treated groups than in the positive control (Table 1). The histopathological evaluation of spleen samples corresponded with the macroscopic findings. The mean score for the positive control was 3.8, while it was lower for the treated groups. The mean scores of the groups treated with 100 mg kg^{-1} were lower ($OTC100_{BC}$, 1.3 and $OTC100_{AC}$, 1.05) than those of the groups treated with 200 mg kg^{-1} ($OTC200_{BC}$, 1.55 and $OTC200_{AC}$, 1.25). Models for scoring the splenic granuloma lesions are shown in Figure 3.

The liver slides were evaluated for hepatocyte accumulation, presence of granuloma, and intensity of the inflammatory infiltrate. Examples for scoring the hepatocyte accumulation are shown in Figure 4. The negative control and the treated control slides were scored as normal (+). Sixty percent of the PC slides were classified as score +++, and the remainder were classified as ++. In Table 2, it is notable that the distribution of the scores' frequency was similar when comparing the two treatment strategies in the groups that received the same dosage. Groups that received the highest dosage scored worse more frequently. No granuloma were observed in the groups that were not exposed to the bacteria (NC and TMC), however, a high frequency of granuloma was observed in the positive control. Sixty percent of the PC samples had multifocal granuloma, and 15 % had bifocal granuloma. In groups that received the prophylactic treatments ($OTC100_{BC}$ and $OTC200_{BC}$), the frequency of the granuloma was low (≤ 15 %). In the group that received the 100 mg kg^{-1} therapeutic treatment, focal granuloma were observed in 40 % of the samples, while no granuloma were observed in the group that received the 200 mg kg^{-1} therapeutic treatment. For the inflammatory infiltrate evaluation, the frequency of the infiltrate in both of the 200 mg kg^{-1} administration strategies was lower than in the other groups. In 25 % of the samples from $OTC200_{BC}$, focal mononuclear infiltrate was observed. Eosinophils were present in 5 % of the infiltrates observed in the $OTC200_{BC}$ samples. Focal mononuclear infiltrate was observed in 20 % of the $OTC200_{AC}$ samples. Eosinophils were not observed in the $OTC200_{AC}$ samples. Inflammatory infiltrate was more frequent and more extensive in the groups treated with 100 mg kg^{-1}. Multifocal mononuclear infiltrate with the presence of eosinophils (25 %), and focal mononuclear infiltrate without eosinophils (20 %), were observed in the liver samples from $OTC100_{BC}$. In 60 % of the $OTC100_{AC}$ samples, a multifocal mononuclear infiltrate was observed. Eosinophils were present in 15 % of the infiltrates observed in $OTC100_{AC}$. In all the PC liver samples, inflammatory infiltrate was observed, and most (70 %) were mononuclear

and multifocal. No inflammatory infiltrate was observed in the samples from NC. However, in 20 % of the TMC samples, a mild multifocal mononuclear infiltrate was observed.

Table 3 shows the serum activity of ALT, AST, and ALP after four and seven days of treatment with 100 mg kg^{-1} or 200 mg kg^{-1} of OTC-medicated feed. There was no significant difference between the groups, suggesting that neither dose induced hepatocyte damage within seven days.

4 DISCUSSION

Although fish production is increasing, health issues and disease outbreaks among aquaculture species are constant causes for concern (Leung & Bates, 2013). In this study, we assessed the efficacy of OTC against francisellosis, a challenging disease that affects tilapia. To represent the tools that are available for fish farmers in the field, all analyses were performed using a commercial feed additive rather than purified OTC. Consequently, the *in vitro* results should be interpreted under the bias that a purified molecule was not used. The main limitations of testing the *in vitro* antimicrobial activity of a feed additive were its unavailability in disks, for the disk-diffusion tests; and the dark-yellow turbidity when in suspension, which did not allow for MIC interpretation. Owing to that, the methodologies were adapted to an agar diffusion, applying the different product dilutions directly to the agar; and to MBC, subcultures of the F1 strain after exposure to the different product dilutions. Furthermore, part of its product design for incorporation into feed, is that it has low solubility, so losses owing to lixiviation are small. Thus, susceptibility results, e.g., the MBC, may be overvalued.

Although the MIC is the primary PD index for antibiotics, it should be integrated with PK information for improved treatment outcomes and dosing choices (Onufrak, Forrest, & Gonzalez, 2016). Sidhu et al. (2018) investigated the PK of OTC administered to tilapia in different water salinities, and after a single dose of 50 mg kg^{-1} OTC via oral gavage, they found a fast absorption time in all salinities (30 min), and the highest plasma concentration reported for tilapia in freshwater was 1.221 µg mL^{-1} after 8 h. Moreover, the area under the concentration-time curve ($AUC_{0–last}$; time calculated between the first and last detection of OTC in plasma) for the tilapia in the freshwater was 165 h µg mL^{-1}. The MBC found in this study (i.e., 37.9 OTC µg mL^{-1}) was high considering the plasma concentration described by Sidhu et al. (2018), although only 3.04 µg of OTC was capable of producing a 30 mm halo in the agar diffusion. A MIC of 64 µg mL^{-1}, even higher than the MBC found in the present study, was previously reported for *F. noatunensis* (Isachsen, Vågnes, Jakobsen, & Samuelsen, 2012). In contrast, low MICs of 0.25 and 0.5 µg mL^{-1} have also been reported for *F. orientalis* (Soto et al., 2012) and *F. phylomiragia* (syn. *F. noatunensis*) (Bohle et al., 2009), respectively. The high MBC found in this study could be influenced by two factors: the possible overestimation owing to the low solubility of the product; and a high MBC should be expected since OTC is a bacteriostatic drug. Nevertheless, comparing the PK data from Sidhu et al. (2018) and the MICs found by Soto et al. (2012) and Bohle et al. (2009), good outcomes should be expected from the use of OTC against francisellosis.

SEM was performed after a 6 h exposure of an F1 strain inoculum to the MBC (Figure 1C) and a 4-fold higher dilution than the MBC (Figure 1B). When compared to the control, only discreet alterations to the cells were observed (i.e., shrunken and uneven surface) for both dilutions. None of the dilutions tested were able to decrease the number of cells, and these results are consistent. Owing to the long generation time of *Francisella* spp. (Cowley, Gray, & Nano, 2000; Furevik, Pettersen, Colquhoun, & Wergeland, 2011) and the bacteriostatic nature of OTC, only a few structural changes could be observed after 6 h of exposure.

Both treatment strategies efficiently reduced francisellosis mortality after the challenge, when compared to the PC, which had a 49.0 % mortality. In the therapeutic groups ($OTC100_{AC}$ and $OTC200_{AC}$), although no significant differences were observed, the group $OTC100_{AC}$ had a 1.3 % mortality, in contrast to no mortality in $OTC200_{AC}$. However, in the prophylactic groups, the mortality in $OTC200_{BC}$ was statistically higher (6.7 %). These results indicate that the 200 mg kg fish^{-1} OTC-medicated feed successfully controlled mortality when used therapeutically ($OTC200_{AC}$). However, the higher mortality in $OTC200_{BC}$ suggests that a longer use of the 200 mg kg fish^{-1} dose could induce physiological stress, which may result in minor deleterious effects. Furthermore, the mortality results reinforce that the practice of using antibiotics prophylactically is unreasonable. Monitoring batches for early infection detection and initiation of antibiotic therapy can successfully control francisellosis progression.

Macroscopic splenic lesions, reisolation, and PCR detection results were smaller in all challenged groups than in the PC, enhancing the ability of the OTC to reduce live bacteria in the tissues after treatment. *F. orientalis* was reisolated from only 1 and 2 fishes in $OTC100_{BC}$ and $OTC200_{BC}$, respectively, that did not have macroscopic lesions. Curiously, both groups had received prophylactic treatment. According to the histopathological analysis of the spleen samples, granulomatous lesions were less frequent in the fish from groups that received 100 mg kg fish^{-1} OTC-medicated feed.

Dose-dependent results were observed in the liver histopathological analysis. Granulomatous lesions and inflammatory infiltrate were observed less frequently in the groups that received the highest dose. However, the hepatocyte accumulation of the glycogen or lipids score was better in the groups that received 100 mg kg fish^{-1} OTC-medicated feed. The hepatocyte accumulation score applied here, considers that normal and healthy captive-reared tilapia have glycogenic accumulation, owing to dietary influences and that anorexia and fasting followed by stressful events induces the consumption of hepatic glycogen and a decrease in vacuolization of these cells (Wolf et al., 2015). Moreover, fasting induces lipid mobilization through the lipolysis of adipose tissues and increases lipid storage in the liver (Dias-Junior et al., 2016). Accordingly, the slides scored better (+) when the hepatocytes accumulated glycogen, and worse (+++) when the glycogen storage was decreased and some lipid vacuolization occurred in the hepatocytes. Julinta et al. (2019) found that the OTC-medicated feed itself reduced the feed intake of the tilapia. This information could indicate that the hepatocyte accumulations in the fish from the groups treated with the highest dosage, scored worse more frequently, owing to the anorexia induced by the OTC. However, in the TMC, which also received 200 mg kg fish^{-1} OTC-medicated feed, all samples were classified as normal (+), indicating that the worse scores in groups $OTC200_{BC}$ and $OTC200_{AC}$ could be synergic anorexia induced by both the OTC and the disease. Besides, in 20 % of the TMC samples, a mild mononuclear inflammatory infiltrate was observed, further indicating a possible discreet deleterious effect from the higher dosage.

In this study, no significant differences were observed in the activities of the ALS, AST, and ALP between the NC and OTC-treated fish after 4 or 7 days, suggesting that the OTC-medicated feed did not induce hepatotoxic lesions, even at the higher dose. On the other hand, all biomarkers evaluated by Julinta et al. (2019) after a 10-day OTC-medicated feed treatment for tilapia (i.e., glucose, ALT, AST, creatinine, and C-reactive protein) were significantly higher in the treated groups than in the non-treated control. Forty-two days posttreatment, despite an observed reduction in the serum levels of the biomarkers, glucose, ALT, AST, and creatinine levels remained higher than those of the control. This was considered to be a response of the fish to a stressful event, and it was hypothesized that discontinuation of OTC-medicated feed would reverse the levels of the biomarkers back to normal.

CONCLUSIONS

The administration of OTC-medicated feed at doses of 100 mg kg fish^{-1} and 200 mg kg fish^{-1} for 15 days to tilapia experimentally infected with *F. orientalis* efficiently reduced the onset and progression of francisellosis using both prophylactic and early therapeutic strategies. These results reinforces that prophylactic antibiotic treatment is a practice that is no longer tolerable. Monitoring batches regularly for appearance of any clinical signs, specially associated to risk factors, e.g. low temperatures, is a practice of easy implementation and training of the farms staff, and allows an early initiation of OTC-medicated feed, which was successful in controlling fracisellosis progression. Unpubished results of late antibiotic treatments, after the onset of mortality, were unable to control the progression of diseases owing to the severe hyporexia and low medicated-feed intake. Dose-adjusment studies are being conducted for better treatment outcomes in late francisellosis. The results herein indicate that 100 mg kg fish^{-1} is a safe dosage for OTC-medicated feed. The 200 mg kg fish^{-1} dose could also be considered safe, but its use should be restricted in the early onset of francisellosis, especially in endemic regions and in farms where monitoring of the batches is routinely undertaken. Moreover, these results suggest that a 200 mg kg fish^{-1} loading dose, followed by a 100 mg kg fish^{-1} dose for a regular period, could successfully control the progression of francisellosis outbreaks, without possible deleterious effects.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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TABLE 1 Results of the presence of macroscopic lesions, isolation in CHAH, and detection of *Francisella* spp. by specific PCR in spleen samples from tilapia from different groups that survived the experimental infection with *F. orientalis*. NC: negative control, fish neither treated nor challenged; PC: positive control, fish not treated but challenged; $OTC100_{BC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day before bacterial challenge; $OTC100_{AC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day after bacterial challenge; $OTC200_{BC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day before bacterial challenge; $OTC200_{AC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day after bacterial challenge.

Group	Amount of survivors	Fish with macroscopic lesions in spleen (%)	Fish with positive isolation (%)	Fish with positive confirmation in PCR (%)
NC	100/100	0	0	0
PC	51/100	43.1	49.0	70.6
$OTC100_{BC}$	150/150	18.0	16.0	18.7
$OTC100_{AC}$	148/150	21.6	23.6	26.4
$OTC200_{BC}$	140/150	17.9	19.3	20.7
$OTC200_{AC}$	150/150	7.3	9.3	10.0

TABLE 2 Frequency (%) of hepatocyte accumulation, presence of granuloma, and presence of inflammatory infiltrate in histopathological sheets of liver samples from different groups of tilapia treated with dehydrated oxytetracycline and experimentally challenged with *F. orientalis*. NC: negative control, fish neither treated nor challenged; TMC: treated control, treated with 200 mg kg^{-1} dosage but not challenged; PC: positive control, fish not treated but challenged; $OTC100_{BC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day before bacterial challenge; $OTC100_{AC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day after bacterial challenge; $OTC200_{BC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day before bacterial challenge; $OTC200_{AC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day after bacterial challenge.

		NC	TMC	PC	$OTC100_{BC}$	$OTC100_{AC}$	$OTC200_{BC}$	$OTC200_{AC}$
Accumulation score	+	100	100	0	10	40	0	0
	++	0	0	40	85	50	70	70
	+++	0	0	60	5	10	30	30
Presence of granuloma		0	0	75	10	40	25	0
Presence of inflammatory infiltrate		0	20	100	45	60	25	20

TABLE 3 Serum activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) of tilapia treated or not with oxytetracycline for 4 and 7 days. NC: negative control, fish neither treated nor challenged; $OTC100_{BC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day before bacterial challenge; $OTC200_{BC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day before bacterial challenge.

Group (treatment length)	ALP (IU L^{-1})				AST (IU L^{-1})				ALT (IU L^{-1})			
	$\bar{x} \pm SD$	1stQ	M	3rdQ	$\bar{x} \pm SD$	1stQ	M	3rdQ	$\bar{x} \pm SD$	1stQ	M	3rdQ
NC	17.0 ± 4.24	15.0	17.0	19.0	2.89 ± 3.02	0.0	2.0	6.0	211.33 ± 179.71	86.0	106.0	415.0
$OTC100_{BC}$ (4 d)	17.4 ± 2.61	15.0	17.0	19.0	2.00 ± 2.55	0.0	1.0	3.0	232.80 ± 196.04	88.0	184.0	242.0
$OTC100_{BC}$ (7 d)	16.4 ± 3.84	13.0	17.0	19.0	0.60 ± 0.89	0.0	0.0	1.0	45.20 ± 30.64	31.0	34.0	43.0
$OTC200_{BC}$ (4 d)	19.8 ± 7.66	14.0	19.0	26.0	3.80 ± 3.27	1.0	4.0	4.0	168.40 ± 147.31	81.0	149.0	186.0
$OTC200_{BC}$ (7 d)	16.8 ± 1.92	16.0	17.0	18.0	2.60 ± 1.82	2.0	3.0	3.0	144.20 ± 141.86	61.0	104.0	109.0

$\bar{x} \pm SD$: mean ± standard deviation; 1stQ: first quartile; M: medium; 3rdQ: third quartile.

FIGURE 1 Scanning electron microscopy visualization (total magnification 12000 ×) of the effects of a oxytetracycline (OTC)-based feed additive on *F.* orientalis cells after 6 hours of exposure. **A**: untreated cells. White arrows indicate normal morphology (coccoid cells). **B**: cells treated with 607.8 μg OTC·mL^{-1}, 4× higher than the minimal bactericidal concentration (MBC). White arrows indicate coccoid cells, but with a larger aspect. Black arrows indicate *F. orientalis* cells with a shrunken appearance. **C**: cells treated with the MBC (37.9 μg OTC·mL^{-1}). White arrows indicate coccoid cells. Black arrows indicate cells with shrunken morphology.

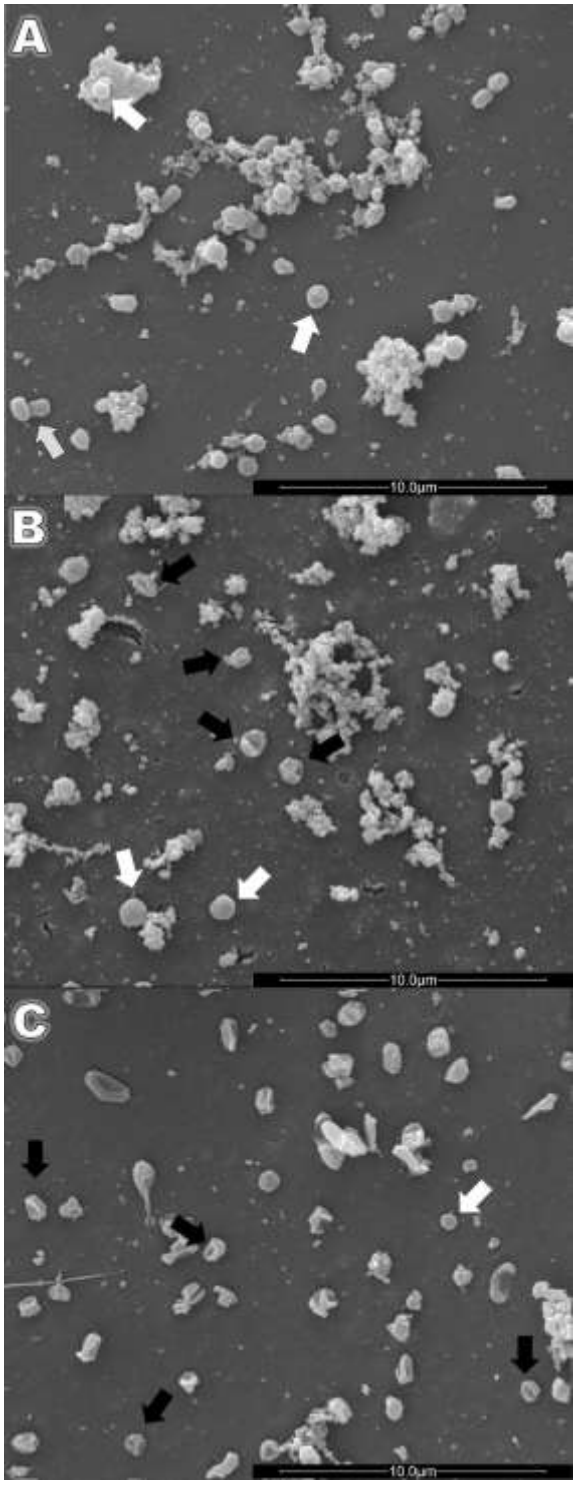


FIGURE 2 Cumulative mortality observed in different groups after experimental challenge with *F. orientalis*. NC: negative control, fish neither treated nor challenged; PC: positive control, fish not treated but challenged; $OTC100_{BC}$: fish treated with 100 mg kg^{-1} of oxytetracycline (OTC) initiated 1 day before bacterial challenge; $OTC100_{AC}$: fish treated with 100 mg kg^{-1} of OTC initiated 1 day after bacterial challenge; $OTC200_{BC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day before bacterial challenge; $OTC200_{AC}$: fish treated with 200 mg kg^{-1} of OTC initiated 1 day after bacterial challenge.

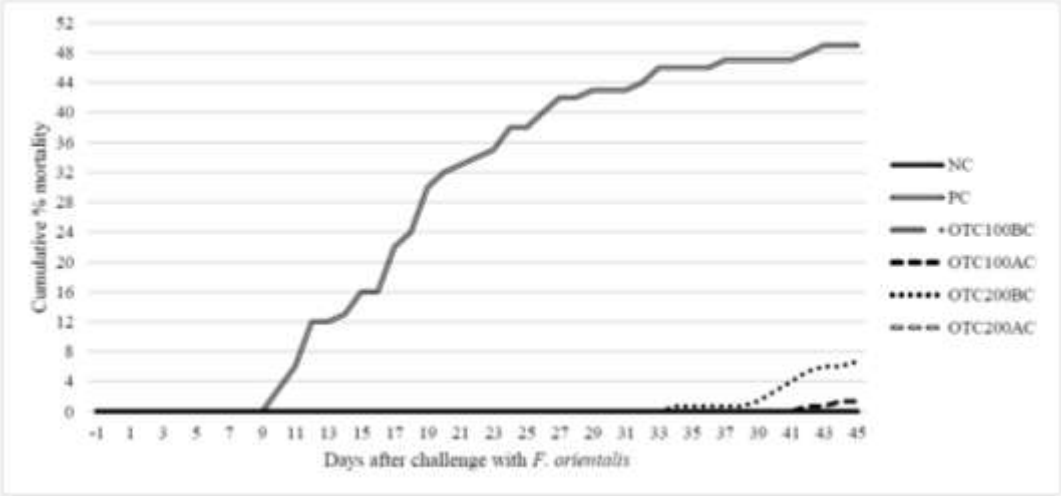


FIGURE 3 Digital microscopy of spleen from tilapia infected with *F. orientalis* and treated with oxytetracycline medicated-feed. Total magnification 40x. Models for scoring splenic damage. **A**: Score 0. No damage. **B**: Score 1. Enlargement of the perivascular macrophage sheath. **C**: Score 2. Macrophage vacuolization extending beyond the perivascular sheath. **D**: Score 3. Incipient granulomas around perivascular sheaths. **E**: Score 4: Multifocal to coalescent granulomas replacing the normal splenic structure.

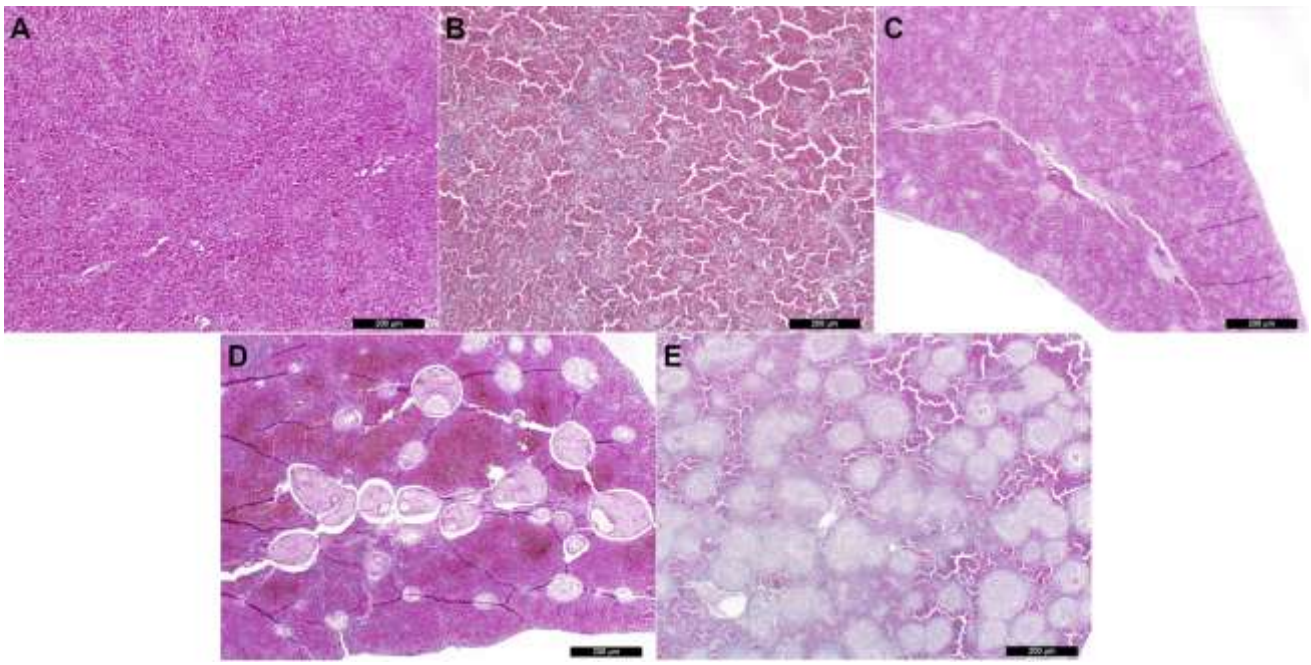
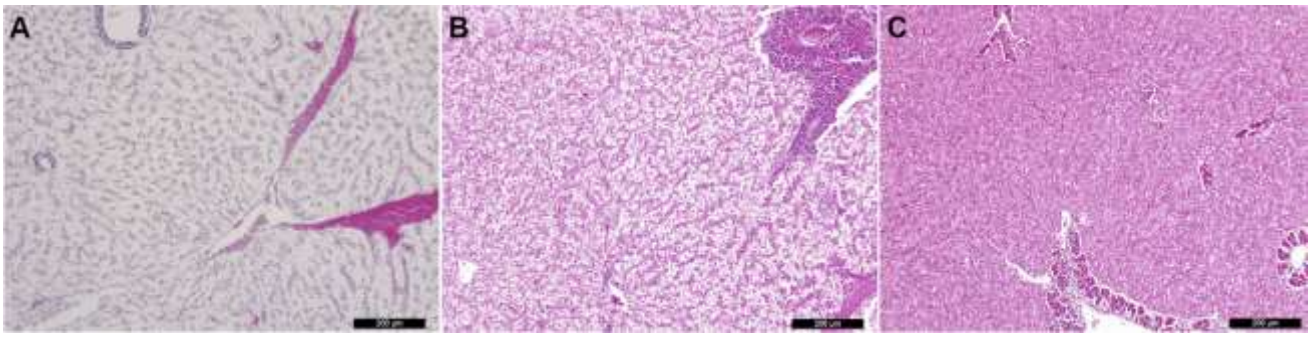


FIGURE 4 Digital microscopy of liver from tilapia infected with *F. orientalis* and treated with oxytetracycline medicated-feed. Total magnification 40 ×. Models for scoring glycogenic accumulation in hepatocytes **A**: Score (+). Accentuated and diffuse glycogen accumulation. **B**: Score (++). Moderate and diffuse/multifocal glycogen accumulation. **C**: Score (+++). Mild diffuse glycogen accumulation concomitant with mild multifocal fatty accumulation.



7 ARTIGO B – *IN SILICO* PREDICTION OF NEW DRUG CANDIDATES AGAINST THE MULTIDRUG-RESISTANT AND POTENTIALLY ZOONOTIC FISH PATHOGEN SEROTYPE III *Streptococcus agalactiae*

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In silico prediction of new drug candidates against the multidrug-resistant and potentially zoonotic fish pathogen serotype III *Streptococcus agalactiae*

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Abstract

Streptococcus agalactiae is an invasive multi-host pathogen that causes invasive diseases mainly in newborns, elderly, and individuals with underlying health complications. In fish, *S. agalactiae* causes streptococcosis, which is characterized by septicemia and neurological signs, and leads to great economic losses to the fish farming industry worldwide. These bacteria can be classified into different serotypes based on capsular antigens, and into different sequence types (ST) based on multilocus sequence typing (MLST). In 2015, serotype III ST283 was identified to be associated with a foodborne invasive disease in non-pregnant immunocompetent humans in Singapore, and the infection was related to raw fish consumption. In addition, a serotype III strain isolated from tilapia in Brazil has been reported to be resistant to five antibiotic classes. This specific serotype can serve as a reservoir of resistance genes and pose a serious threat to public health. Thus, new approaches for the control and treatment of *S. agalactiae* infections are needed. In the present study, 24 *S. agalactiae* serotype III complete genomes, isolated from human and fish hosts, were compared. The core genome was identified, and, using bioinformatics tools and subtractive criteria, five proteins were identified as potential drug targets. Furthermore, 5008 drug-like natural compounds were virtually

screened against the identified targets. The ligands with the best binding properties are suggested for further *in vitro* and *in vivo* analysis.

1 Introduction

Streptococcus agalactiae is a multi-host, invasive, Gram-positive pathogen, identified in several species of terrestrial and aquatic mammals, reptiles, amphibians, and fish (Delannoy et al., 2013). It is a commensal organism of the human gastrointestinal and lower genital tracts, with the potential to cause diseases mainly in newborns, elderly, and individuals with underlying medical conditions (Armistead et al., 2019; Raabe e Shane, 2019). Outbreaks of *S. agalactiae* infection, characterized by septicemia, exophthalmia, and meningoencephalitis, has been reported among both farmed and wild fish species, which leads to high mortality of fishes and serious economic losses (Olivares-Fuster et al., 2008; Mian et al., 2009; Soto et al., 2015).

S. agalactiae strains can be classified into ten serotypes based on their capsular antigens (Slotved et al., 2007). Based on multilocus sequence typing, the strains are classified into sequence types (ST), which are further grouped into clonal complexes (CC) (Barony et al., 2017). In 2015, an *S. agalactiae* human outbreak in Singapore raised concerns about a specific *S. agalactiae* genotype, serotype III ST283. Although *S. agalactiae* isolated from human and cow hosts had been proven to infect fish (Pereira et al., 2010; Chen et al., 2015), and this ST had already been identified in human (Ip et al., 2006; Salloum et al., 2010) and tilapia (Delannoy et al., 2013) infections, this was the first ever case of human infection linked to consumption of raw farmed fish (Tan et al., 2016). Unlike the general nosocomial *S. agalactiae* infection in humans, this outbreak affected younger, non-pregnant, and immunocompetent individuals also (Kalimuddin et al., 2017). Even though this particular ST emerged as a regional threat, it has already spread and has caused outbreaks among farmed fish in Brazil owing to aquaculture and global food trades (Barkham et al., 2019; Leal et al., 2019), supporting the initial concerns to worldwide public health. In addition, the first Brazilian serotype III isolated from diseased tilapia was reported to be multi-drug resistant (i.e., resistant to ampicillin, norfloxacin, aminoglycosides, fluoroquinolone, sulfamethoxazole, and tetracycline) (Chideroli et al., 2017).

Antimicrobial resistance of pathogens is a critical concern to human and animal health. Furthermore, aquaculture systems and their products have been reported to be hotspots for horizontal transfer of gene, particularly antimicrobial resistance genes, from a donor bacteria (which harbor resistance gene) to the environment's and/or to consumers and handler's microbiota (Watts et al., 2017). Therefore, it is important to develop novel strategies for the prevention and treatment of infections by multidrug-resistant pathogens. Integration of bioinformatics tools with metabolomics, proteomics, and comparative genomics is of great value for the identification of drug targets in a pathogen (Mondal et al., 2015); moreover, this strategy can reduce the number of *in vitro* trials, rendering the drug discovery process more economical and less laborious (Timo et al., 2019). Promising results have been obtained by integrating *in silico* and *in vivo* techniques for inhibiting multidrug-resistant pathogens, including *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Escherichia coli*, and *Vibrio cholerae* (Zang et al., 2016; Sandhaus et al., 2018; Tiwari et al., 2019).

S. agalactiae serotype III serves as a reservoir of antibiotic resistance genes and poses a great threat to public health; hence, newer strategies are required for the control and treatment of *S. agalactiae* infections. Thus, through comparative genomics and bioinformatics approaches, this study screened all the available genomes of *S. agalactiae* isolated from human and fish hosts in GenBank of National Center for Biotechnology Information (NCBI) database for conserved proteins that are not homologous to the host's proteome and can serve as potential drug targets, and virtually screened for drug-like natural compounds that could bind to the identified drug targets.

2 Materials and Methods

2.1 Genomes

From the 114 *S. agalactiae* complete genomes available at the NCBI database, 24 were selected based on the following two inclusion criteria: serotype III strains; and isolates from human (n = 19) or fish hosts (n = 5). Genomes with lack of information about host source at NCBI Biosample page were not included. Both FASTA and GenBank files were downloaded. For comparative genomic analysis, the complete genome of a non-pathogenic *Streptococcus thermophilus* strain isolated from dairy products was downloaded. The genome information available at NCBI database is summarized in Supplementary Table 1.

2.2 Genomic analysis

A phylogenetic analysis was performed to select representative genomes for better visualization of the concentric alignment, further described. The genomes of the 24 *S. agalactiae* strains and the *S. thermophilus* strain were submitted to Gegenees v2.2 using the following settings: fragment-size, 500 bp; and step-size, 500 bp. This software performs genomic analysis through whole-genome fragmentation followed by an all-against-all BLAST comparison (Ågren et al., 2012). The output is a heatmap showing the similarity between the genomes. The heatmap was exported as a distance matrix in a nexus file, which served as the input for construction of a phylogenetic tree using SplitsTree4 software (Huson e Bryant, 2006). The neighbor-joining method was employed for phylogenetic tree construction according to Ågren et al. (2012), and *S. thermophilus* served as the out-group.

The Brazilian multidrug-resistant fish isolate strain S73, described by Chideroli et al. (2017), was selected to predict genomic islands (GIs) using the software GIPSy, which predicts pathogenicity, metabolic, resistance, and symbiotic islands (Soares et al., 2016). Next, to visualize the presence of selected drug targets in the predicted genomic islands, a concentric ring alignment was generated using BRIG software (Alikhan et al., 2011). The following data were used for this alignment: genome of S73 strain as the reference genome (center ring), 32 predicted GIs, 48 essential non-host homologous proteins as drug targets, and a representative genome from each cluster of the phylogenetic tree. When more than one ST or host were present within a cluster, a representative from this ST or host was also included. The clusters in the phylogenetic tree and the rings in the alignment were identified with same colors.

For identification of conserved proteins among the genomes, the 24 *S. agalactiae* serotype III FASTA files were submitted to the Orthofinder software under its default parameters to identify the core genome. Through BLAST searches and Markov Cluster Algorithm (MCA), this software infers homologous regions and calculates the orthogroups (Emms e Kelly, 2015). Next, core, shared, and singleton genes were identified using in-house scripts. The first genes were present in all genomes, the following genes were present in few genomes, and the later genes were present in only one genome. The core genes were then subjected to subtractive genomics analysis to identify the most suitable proteins for drug binding analysis.

2.3 Subtractive analysis of core proteins

To eliminate targets with any similarity to the hosts' proteome, the core proteins were subjected to BLASTp searches against human (*Homo sapiens*, taxid: 9606) and tilapia (*Oreochromis* sp., taxid: 8139) proteomes using the NCBI database. Sequences with any similarity to host proteins were excluded. Using Orthofinder software, proteins that were non-homologous to the human and *Oreochromis* sp. proteome were selected for the subsequent steps.

The software SurfG+ was employed to predict subcellular localization of the proteins based on the identification of peptide signals, retention signals, transmembrane helices, and protein secretion pathways (Barinov et al., 2009). SurfG+ is not downloadable and can be found as a part of the Mature Epitope Density Server at https://med.compbio.sdu.dk/. Only cytoplasmatic proteins were selected for drug discovery owing to their importance in the maintenance of cell viability (Vilela Rodrigues et al., 2019).

Three-dimensional (3D) model structures of cytoplasmatic proteins were predicted by Protein Data Bank (PDB) homology modeling utilizing the MHOLline software (http://www.mholline2.lncc.br/). This software combines HMMTOP, BLAST, BATS, MODELLER, and PROCHECK programs to generate 3D protein models with structural and functional information (Capriles et al., 2010). Only models with identity ≥ 50 % and e-value ≤ 0.3 (good to very high-quality sequences, according to MHOLline) were selected for further analysis.

The amino acid sequences of the proteins were subjected to BLASTp search against the Database of Essential Genes (DEG, http://www.essentialgene.org/). This online platform comprises information on essential genes from bacteria, archaea, and eukaryotes, essential noncoding RNAs, promoters, regulatory sequences, and replication origins (Luo et al., 2014). Proteins with bit-score ≥ 100 and e-value $\leq 10^{-4}$ were selected for further analysis (Mondal et al., 2015).

Following the subtractive strategy, only proteins in or at the edge of predicted genomic islands were selected for subsequent analysis. As a final selection criterion, the druggability of the proteins was assessed using the DoGSiteScorer algorithm, available at the ProteinPlus web server (https://proteinsplus.zbh.uni-hamburg.de). This algorithm identifies potential binding sites (referred to as pockets) in the protein 3D models, provides the amino acid composition of these pockets, and infers a druggability score for each, ranging from zero to one (Volkamer et al., 2012; Fährrolfes et al., 2017). Only proteins with pockets having druggability score ≥ 0.8 were retained, and from these, only the pocket with the highest score was selected for drug binding analysis.

Two databases were employed to obtain more information about the proteins selected as drug targets. The protein annotation database UniProt (The Uniprot Consortion, 2019) was used to access information regarding the functions of the selected proteins and the pathways in which they were involved; and the DrugBank database (Wishart et al., 2018) was used to determine whether the selected proteins have already been tested as drug targets.

2.4 Drug binding analysis

A library of 5008 natural drug-like molecules was downloaded from the ZINC database in SDF format (Sterling e Irwin, 2015). These ligands were then converted into the PDBQT format required for docking using OpenBabel (O'Boyle et al., 2011) and the python script "prepare_ligand4.py", described in AutoDockTools MGL user guide web pages (http://autodock.scripps.edu/). The proteins selected as drug targets were submitted to AutoDockTools MGL (Morris et al., 2009) for visualization of the previously identified druggable pocket; a grid box containing all the amino acid residues of the pocket was created, and the protein model was converted into the PDBQT format.

The ligand library was screened against each target using AutoDock Vina, and the best 10 ligands were identified based to their binding affinity using the python script "vina_screen_get_top.py" (Trott e Olson, 2009). Next, the Chimera program was applied to visualize the different interactions between the identified ligand and the active site of its target protein (Pettersen et al., 2004). The ligand forming the most number of hydrogen bonds with its target and having the lowest energy binding affinity ($kcal \cdot mol^{-1}$) was defined as the best drug candidate (Thomsen e Christensen, 2006).

3 Results and Discussion

3.1 Genomic analysis

A phylogenetic tree (Figure 1) was constructed based on the heatmap of whole-genome comparison (Supplementary Figure 1) between the 24 *S. agalactiae* serotype III genomes and the *S. thermophilus* genome. It is notable that the genomes of serotype III isolates were found to be highly conserved, and the lowest similarity observed was 82 %. In the phylogenetic tree, isolates from the same ST were grouped together. The only two exceptions were strains HU-GS5823 (ST335) and H002 (ST736); though these STs were grouped in the ST19 cluster, they belong to the clonal cluster 19 (Usein et al., 2014; Chen et al., 2015; Emaneini et al., 2016).

The ST283 cluster was divided into two subclusters, one included mainly fish isolates (green subcluster) and the other included human isolates (yellow subcluster); however, in both the subclusters, one isolate from the other species was present. This reinforces the ability and adaptation of this ST to infect both species and cause zoonotic infections, like the Singapore outbreak in 2015 (Tan et al., 2016; Kalimuddin et al., 2017). Furthermore, a phylogenetic tree constructed based on ST283 isolated from human and fish hosts in Southeast Asian countries showed that human and fish isolates were grouped together, with zero single nucleotide polymorphism (SNP) difference in locations where samples were collected together, which was not observed for samples collected from different countries (Barkham et al., 2019). In the phylogenetic tree presented here, the S73 strain was present in a branch separated from the Asian ST283 isolates, which corroborates the findings of Leal et. al (2019). In the study of Leal et. al (2019), serotype III Brazilian isolates were identified as ST-283, using whole-genome MLST approach, and the Brazilian isolates grouped together with fish and human isolates from Asia. Although, the strains from Brazil and Asia (fish isolates) exhibited high genetic diversity, with loci variations ranging from 3.84 % to 14.26 % (Leal et al., 2019).

S73 is the first serotype III isolated from diseased fish in Brazil, in 2016. It is a multidrug-resistant strain isolated from a streptococcosis outbreak in a northeastern Brazilian fish farm by our group (Chideroli et al., 2017). A total of 32 GIs were predicted in the S73 genome, of which seven were pathogenicity islands (PAI), ten were metabolic islands (MI), six were resistance islands (RI), five were symbiosis islands (SI), and the remaining four GIs shared the characteristics of two or more island types (Supplementary Table 2).

The concentric alignment (Figure 2) confirms that the genomes of serotype III isolates, particularly isolates from the same ST or CC, are conserved. It can be noticed that all S73 genomic islands were present in the genomes of all serotype III isolates, though some of them were not well conserved. Even in strain SGEHI2015-25 (dark green ring), which was isolated from a Singaporean fish in 2015 (Kalimuddin et al., 2017), certain gaps in islands PAI1 and SI5 have been observed. This, along with the discussed possible introduction of serotype III ST283 in South America through trading of live fish from Asia (Barkham et al., 2019; Leal et al., 2019), indicates that the Brazilian strain could have acquired certain genes after the introduction of the strain in the country.

The Orthofinder software identified 1473 core, 131 shared, and 127 singleton genes. In a study comparing the serotype III isolates only from human hosts, 1610 core genes were predicted in the pan-genome (Lannes-Costa et al., 2020). The closeness in the sizes of the core genomes observed in the aforementioned study and the present study reinforces the possibility of a strong genomic similarity among serotype III isolates.

3.2 Selection of drug targets based on subtractive criteria

To ensure that the probability of drug side-effects is small, it is important that the proteins selected as drug targets do not bear homology to the host's proteome (Sakharkar et al., 2008). After running a BLASTp search of the core genes against human and *Oreochromis* spp. proteomes, 857 and 773 non-

host homologous genes were obtained, respectively. An additional Orthofinder round identified 666 core genes non-homologous to both hosts.

SurfG+ analysis indicated that, among the core non-host homologous proteins, 408 were cytoplasmatic, 154 were membrane proteins, 80 were potentially surface-exposed proteins, and 24 were secreted proteins. Among these, cytoplasmatic proteins were selected as drug targets owing to their important role in cell maintenance (Vilela Rodrigues et al., 2019).

After MHOLline 3D-structure prediction, only 76 proteins were listed as good-quality proteins or above (identity $\geq$ 50 %; e-value $\leq$ 0.3). As essential genes are considered to be great drug targets (Duffield et al., 2010; Mondal et al., 2015), the essentiality of the proteins was assessed by BLASTp against the DEG database; and 48 proteins were found to be essential to the survival of *S. agalactiae* under the established criteria.

GIs are one of the sources of DNA acquired through horizontal gene transfer, and they carry gene clusters that enable the cell to perform certain special activities (Soares et al., 2012). They can contain virulence (pathogenicity), symbiotic, resistance, and metabolic genes (Soares et al., 2016). Owing to their importance in increasing the fitness and pathogenicity of not only the pathogen in which they occur but also a population of organisms, only the proteins encoded by the genes present in the GIs of S73 strain were selected. Although GIs are generally considered a part of the accessory genome, the proteins encoded by the genes present in the GIs have already been filtered as core and essential, indicating that they are probably well-fixated in the genome of this species. Considering that GIs are comprised of mobile genetic elements (in the distant evolutionary past) and that bioinformatic tools can incur minor errors, proteins at the edge of GIs were also selected. Of the 48 essential proteins, three were present in the GIs and the other three were present at the edge of GIs.

Druggable proteins are defined as proteins that bind and are responsive to drug-like molecules (Keller et al., 2006). From a known dataset of druggable features, including size, compactness, and physicochemical properties, of the pockets (Volkamer et al., 2012), the DoGSiteScorer algorithm returned the druggable pockets within the selected proteins with their area, volume, active amino acid residues, and a druggability score. Five of the six selected proteins had pockets with druggability score > 0.8 and were selected for molecular docking and drug discovery (Supplementary Table 3). Therefore, the final drug target candidates were proteins WP_000077187, WP_001068667, WP_001090621, WP_001067088, and WP_000282567. The subtractive steps, the number of proteins identified at each step, and the inclusion criteria are summarized in Table 1.

The protein WP_000077187 showed 100 % identity to phosphopentomutase (UniProt accession number Q8CMH7), encoded by *deoB1* gene, of *S. agalactiae* serotype III strain NEM316. This protein was predicted to be encoded by the Metabolic-Island-10 of the S73 strain. Phosphomutases are enzymes responsible for rearranging of phosphate within a substrate molecule. In bacteria, phosphopentomutases catalyze the transfer of phosphate group between C1 and C5 of a pentose, and can act on both ribose- and deoxyribose-phosphates (Tozzi et al., 2006; Panosian et al., 2011); thus, they are directly involved in nucleic acid biosynthesis and energy production in the absence of glucose. Phosphopentomutases can be considered good drug targets because they are distantly related to the human isoform of the enzyme (Panosian et al., 2011). Moreover, a *deoB*-disrupted *Francisella tularensis* mutant has been reported to be less lethal to chicken embryos and defective in entering human phagocytic cells and cultured embryonic kidney cells (Horzempa et al., 2008). There is no record of phosphopentomutase as a drug target in the Drugbank database. These data indicate that phosphopentomutase is a promising target for new drugs as its inhibition can affect both metabolism and virulence of *S. agalactiae*.

The protein WP_001068667 showed 100 % identity to the 50S ribosomal protein L19 (Uniprot accession number Q8E6H6), encoded by *rplS* gene, of *S. agalactiae* strain NEM316. According to GIPSy prediction, the gene encoding protein L19 is present at the edge of a GI predicted to be a resistance-, symbiosis-, and pathogenicity-island (-2, -3, and -5, respectively). In *E. coli*, L19 is one of the proteins responsible for joining of the small and large ribosomal subunits and is essential for protein translation and subsequently for cell viability (Persson et al., 1995; Soung et al., 2009). L19 has phosphorylation sites at Ser, Thr, and Tyr amino acids. The phosphorylation of ribosomal proteins can reduce up to 50 % of their activity (Mikulík et al., 2001), although it seems that phosphorylated L19 is the active form of the protein that contribute to the assembly and decoding processes (Soung et al., 2009). Moreover, a mutagenic study of the *rplS* gene in *E. coli* revealed that, despite the restraint in translation, the defective mutants did not exhibit problems in 30S–50S association or cell growth (VanNice et al., 2016). There is no record of L19 protein as a drug target in the DrugBank database. Owing to its importance in ribosomal structure, the precise effects of targeting L19 on *S. agalactiae* ribosome structure and function should be addressed in further studies.

The protein WP_001090621 showed 100 % identity to RegM protein (Uniprot accession number Q8E0M3), encoded by *sag0707* gene, of *S. agalactiae* serotype V, and 93.1 % identity to catabolite control protein A (CcpA; Uniprot accession number Q9A118), encoded by *ccpA* gene, of *Streptococcus pyogenes* serotype M1. In the S73 genome, this protein is localized in Resistance-Island-3. CcpA is a regulatory protein conserved in Gram-positive organisms, and it plays an important role in carbon catabolite repression (a system of rapid adaptation to a preferred carbon source) and expression of virulence factors (Lang et al., 2014; Liao et al., 2017). Giammarinaro and Paton (2002) first described RegM protein while searching for CcpA homologs in *Streptococcus pneumoniae*. In their study, they found that, in addition to the involvement of RegM in carbohydrate catabolism, RegM could regulate the expression of the capsular gene *cps*, and *regM* knockout-mutants were less virulent in mice (Giammarinaro e Paton, 2002). In the presence of high glucose concentration, CcpA, encoded by *sag0707*, is downregulated in *S. agalactiae* serotype V ((Di Palo et al., 2013). Moreover, targeting CcpA for antimicrobial purposes has been proven to be efficient. Liao et al. (2017) demonstrated that silver ions could bind CcpA and subsequently inhibit the growth of *S. aureus*, toxin expression, and biofilm formation; Huang et al. (2020) reported that a small compound, bis(4-hydroxy-3-methylphenyl) sulfide, inhibited the expression of *ccpA* and α-hemolysin in *S. aureus*. In the DrugBank database, four drugs have been registered to bind to regulators similar to RegM/CcpA in other bacterial species (DrugBank Accession Numbers DB02283, DB01862, DB08297, and DB02430). Virulence regulators remain unexplored in the field of drug discovery, and the available data show that they can serve as great targets (Huang et al., 2020), reinforcing the potential of RegM/CcpA as a drug target in *S. agalactiae*.

The protein WP_001067088 showed 98.7 % identity to the flavin mononucleotide (FMN)-binding oxidoreductase (UniProt accession number Q8DZN9), encoded by gene *sag1061*; and protein WP_000282567 showed 97.4 % identity to a flavoprotein-related protein (Uniprot accession code Q8DZN7), encoded by *sag1063*, of *S. agalactiae* serotype V strain 2603V/R. Both the genes are predicted to be present in the Metabolic-Island-4 of the S73 strain. FMN and flavin adenine dinucleotide (FAD) function as cofactors of flavoproteins, and flavoproteins are related to several essential and vital functions in living beings (Sebastián et al., 2018). Most prokaryotic FAD synthetases, which synthesize FMN, FAD, and flavoproteins, are different from mammalian FAD synthetases; this allows specific targeting of the prokaryotic proteins and cofactors for antimicrobial purposes (Cremades et al., 2005; Rodríguez-Cárdenas et al., 2016; Sebastián et al., 2018). In *S. agalactiae* serotype III, the flavoprotein type 2 NADH dehydrogenase (NDH-2), described as the only entry point for electrons in the respiratory chain, has already been suggested to be a great drug target (Lencina et al., 2018). As WP_001067088 and WP_000282567 are related to flavoproteins and

their cofactors, they can serve as drug targets in *S. agalactiae*. WP_001067088 matched with four drug targets registered in the DrugBank, and 11 different drugs are listed to bind to these drug targets (DrugBank Accession Numbers DB03147, DB03247, DB03461, DB03698, DB01676, DB02060, DB03651, DB04528, DB07373, DB02508, and DB11090). On the other hand, WP_000282567 matched with one drug target in the DrugBank database that can bind to two drugs (DrugBank Accession Numbers DB02431 and DB03403). Information regarding the identified drug targets is summarized in Table 2.

Although this study focused on targeting serotype III isolates, drug targets homologous to all or the majority of *S. agalactiae* serotypes would be more desirable and economically attractive. A BLASTp search against representative strains from other *S. agalactiae* serotypes showed that these five proteins identified herein are highly conserved in serotypes Ia, Ib, II, IV, V, and VI (Supplementary Table 4). This information indicates that these proteins may serve as drug targets in multiple serotypes.

3.3 Molecular docking and compound identification

Natural compounds have been and still are the main sources of most classes of antibiotics (Genilloud, 2019). Three out of five new compound classes released as antibiotics between 2000 and 2015 for humans were based on natural products (Harvey et al., 2015). Natural compounds have greater chemical diversity as compared to synthetic compounds, and are easily absorbed and metabolized in the body despite their complex structures (Strohl, 2000). Therefore, a library of 5008 drug-like natural compounds was downloaded from the ZINC database to screen for new candidates against the five proteins identified in *S. agalactiae* serotype III isolates obtained from humans and fish hosts.

Guided by a grid containing the amino acids of the most druggable pocket of each protein (identified by the DoGSiteScorer algorithm), the AutoDock Vina software was employed to screen the ligand library against the five selected drug target proteins. Next, 10 ligands having the lowest binding affinity for each protein were identified using a python script (Supplementary Box 1). These 50 compounds were docked against their respective targets using Chimera software. In this step, the best drug-like molecule to each protein was elected based on the lowest binding affinity and the greatest number of hydrogen bonds between the ligand and the protein (Table 3). The 3D images showing the docked ligands and their respective targets are presented in Figure 3.

Molecular docking analysis revealed that the five selected targets were effective in binding to natural compounds. The best docked protein and ligand, identified based on binding energy and hydrogen bonds, were RegM/CcpA (WP_001090621) and ZINC04236030, respectively. The ligand bound RegM/CcpA with three hydrogen bonds to the amino acids ARG 136 and ARG143, with −8.4 $kcal \cdot mol^{-1}$ binding affinity. The involvement of RegM/CcpA in carbohydrate metabolism and regulation of virulence factor expression (Giammarinaro e Paton, 2002; Lang et al., 2014) grounds the potential of targeting this particular protein in further *in vitro* and *in vivo* trials using the ligand herein identified. Promising results have been observed in targeting *S. aureus* Ccpa for antibiotic purposes (Liao et al., 2017; Huang et al., 2020). In addition, RegM/CcpA, being a conserved protein in Gram-positive bacteria (Liao et al., 2017), it can be targeted as broad-range for infections caused by this group of bacteria.

The integration of genome-driven platforms and culture-based approaches may be the key to discover new innovative antibiotics (Genilloud, 2019). With the support of genomic analysis and bioinformatics tools, this study predicted the core conserved proteins in the potentially zoonotic *S. agalactiae* serotype III isolates obtained from human and fish hosts. Following the subtractive criteria, five potential drug targets and drug-like molecules that can bind to them were proposed. Amongst the identified targets, the most promising target, according to the criteria established herein,

is the protein WP_001090621, an analog of RegM/CcpA that is involved in both metabolism and virulence regulation; and the best ligand for this target was the compound ZINC04236030. The targets and drugs predicted here can be readily tested *in vitro* and *in vivo*, and they support the development of new strategies for control and treatment of *S. agalactiae* infection that causes serious losses in fish farming and poses a serious threat to public health.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statemente

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/SupplementaryMaterial.

Author Contributions

LMF and UPP conceived the study. LMF, ST, UPP, NMLB, and VACA performed the research. LMF, RTC, NAF, and ST performed bioinformatics analysis under the supervision of UPP and VACA. LMF wrote the manuscript and produced the figures under the supervision of UPP. All authors contributed to discussing the manuscript. All authors have read and approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.01024/full#supplementary-material

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Figures and Tables

Figure 1 Phylogenetic tree based on whole genomes of *Streptococcus agalactiae* serotype III isolates from human and fish hosts constructed using neighbor-joining method. The scale bar represents a difference of 1 % in average BLASTN score similarity. Different colors indicate different clusters. Purple: ST23 cluster; blue: ST12 cluster; green: ST283 subcluster; yellow: ST283 subcluster; orange: ST17 cluster; red: CC19 cluster.

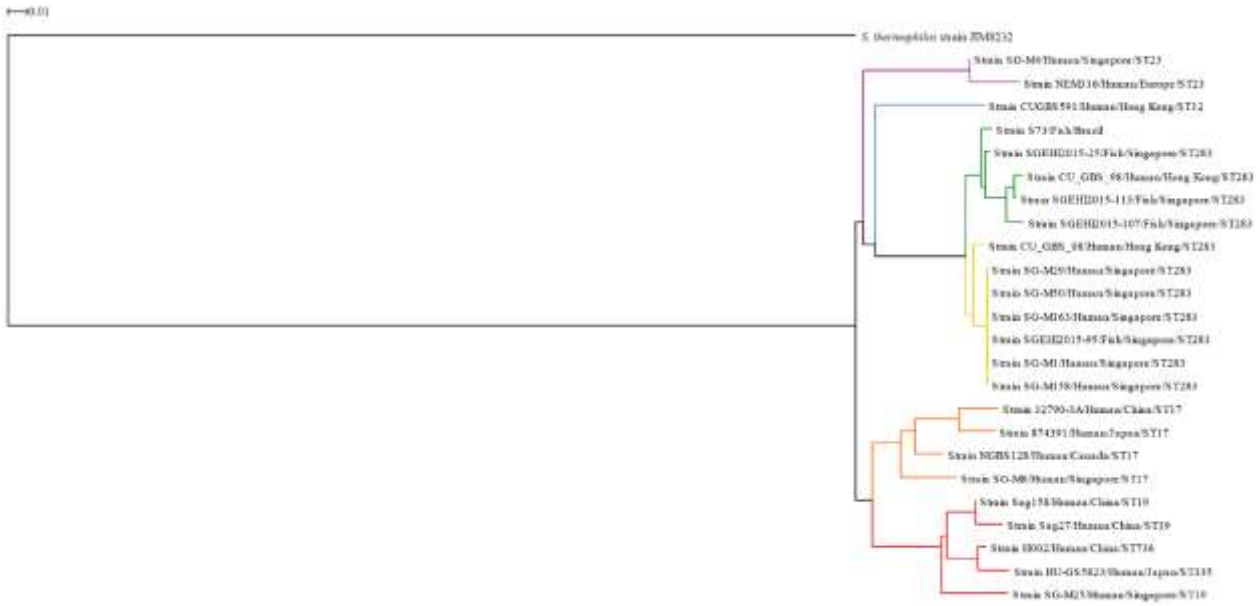

Figure 2 Circular alignment of genomes of representative *Streptococcus agalactiae* serotype III strains. The intensity of the ring color indicates the identity between that genome and the S73 strain, which was used as reference for the alignment. Rings of the same color indicate genomes of strains from the same cluster in the phylogenetic tree. Arranged from the center to the edge: GC content and GC skew of strain S73; strain SG-M4; strain CUGBS591; strain SGEHI2015-25; strain CU_GBS_98; strain SG-M29; strain SGEHI2015-95; strain 32790-3A; strain Sag158; strain H002; strain HU-GS582; predicted genomic islands in strain S73; and essential non-host homologous proteins of strain S73.

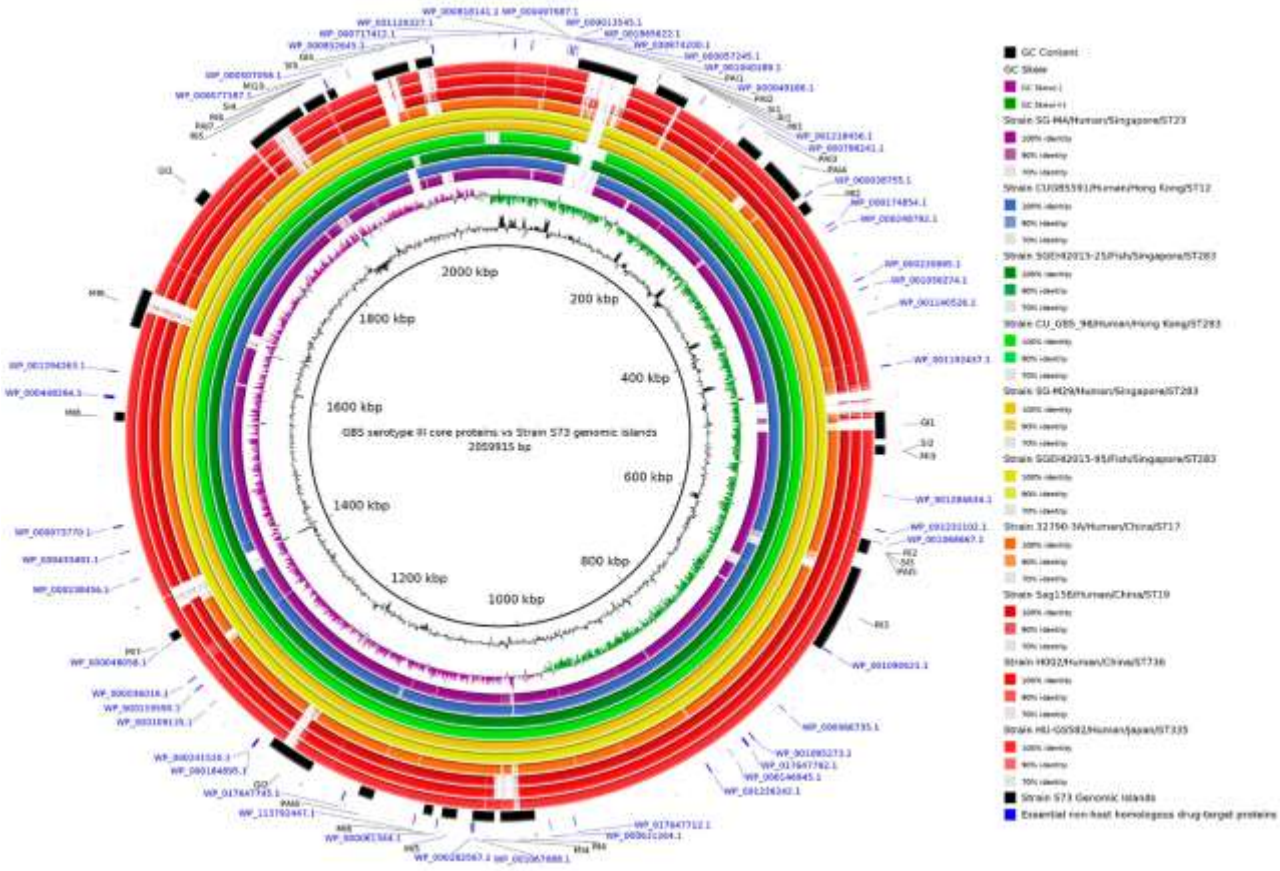

Figure 3 Three-dimensional representation of the interaction between drug-like natural compounds and *Streptococcus agalactiae* serotype III drug target proteins. The blue areas indicate the druggable pocket of the protein. Hydrogen bonds are represented in dark yellow and the amino acid residues involved are identified. **(A)** and **(B)** represent interaction between WP_000077187 (phosphopentomutase) and ZINC05410520; **(C)** and **(D)** represent interaction between WP_001068667 (ribosomal protein L19) and ZINC03838587; **(E)** and **(F)** represent interaction between WP_001090621 (RegM/CcpA) and ZINC04236030; **(G)** and **(H)** represent interaction between WP_001067088 (FMN-binding oxidoreductase) and ZINC03839958; and **(I)** and **(J)** represent interaction between WP_000282567 (flavoprotein-related protein) and ZINC04222225.

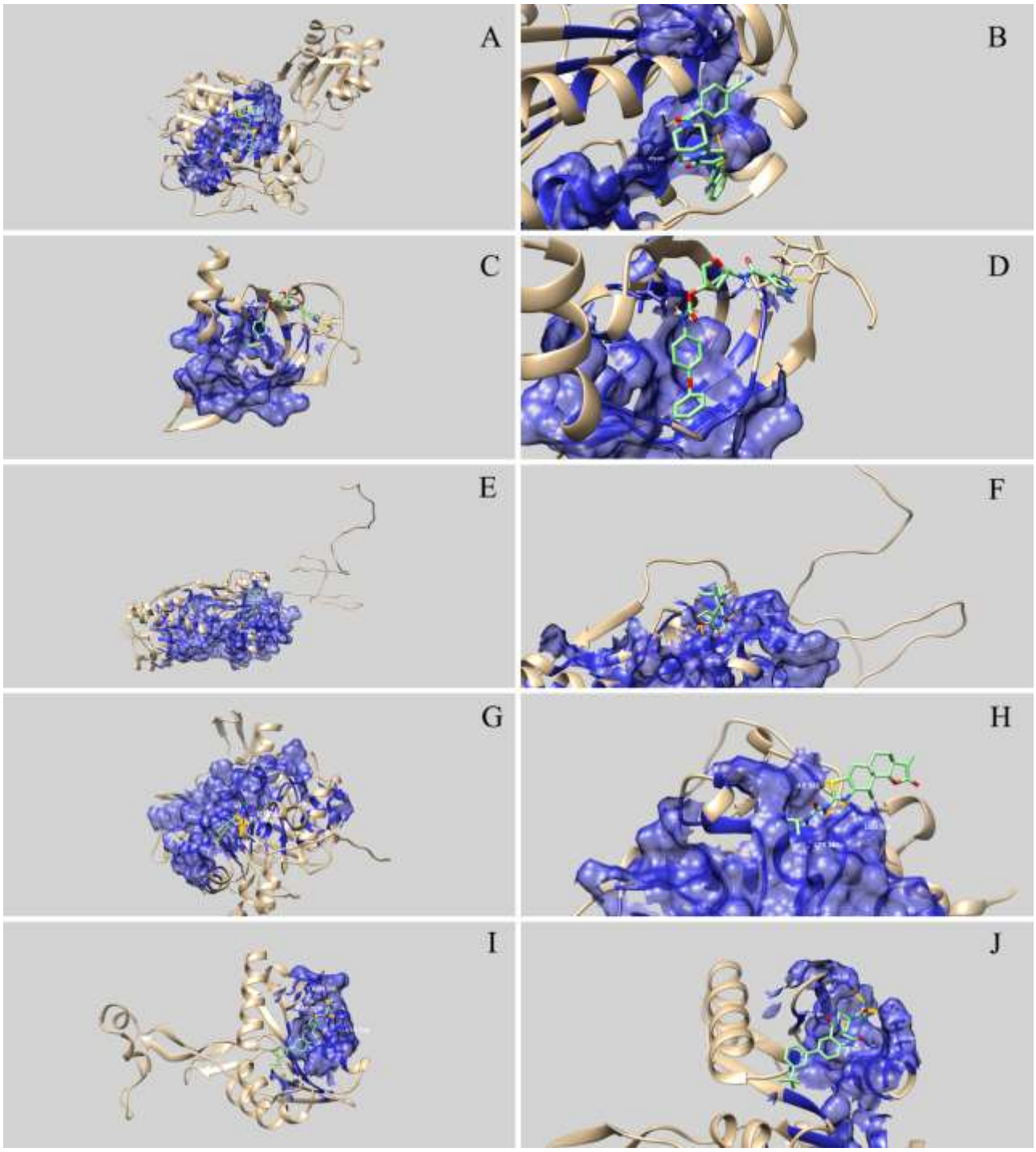

Table 1 Subtractive genomics steps to obtain potential drug target proteins within the core genome of 24 *Streptococcus agalactiae* serotype III genomes isolated from fish (n = 5) and humans (n = 19). The inclusion criterium of each step is underlined.

Steps (software or database)	Number of genes/proteins
Step 1: Strains' core genome (Orthofinder)	
Core genes	1473
Shared genes	131
Singletons	127
Step 2.1: Strains' core gene homology with hosts proteome (BLASTp)	
Core genes non-homologous to *Homo sapiens* proteome	857
Core genes non-homologous to *Oreochromis* spp. proteome	773
Step 2.2: Core genes non-homologous to both host genomes (Orthofinder)	
Core genes	666
Shared genes	176
Singletons	0
Step 3: Core protein subcellular localization prediction (SurfG+)	
Cytoplasmic (drug targets)	408
Membrane	154
Potentially surface exposed	80
Secreted	24
Step 4: Protein structure prediction (Mholline)	
G0: Non-aligned sequences	69
G1: e-value > 10e-5 or ID < 15 %	0
G2: e-value ≤ 10e-5 and ID ≥ 25 %	328
Very high quality sequences: ID ≥ 75 %	19
High quality sequences: ≥ 50 % ID < 75 %	52
Good quality sequences: ID > 50 %	5
Medium to good quality sequences: ≥ 35 % ID < 50 %	88
Medium to low quality sequences: ≥ 25 % ID < 35 %	93
Low quality sequences: ID ≥ 25 %	25
Very low quality sequences: ID ≥ 25 %	46
G3: e-value ≤ 10e-5 and 15 % ≥ ID < 25 %	11
Step 5: Protein essentiality (Database of Essential Genes)	
Bitscore ≥ 100 and e-value ≤ 10e-4	48
Step 6: Presence within genomic island (GIPSy, BRIG)	
Proteins encoded by genes present within genomic islands	3

Proteins encoded by genes present at the edge of genomic islands	3
Proteins encoded by genes present outside genomic islands	42
Step 7: Binding site/pocket detection (DogSiteScorer/ProteinPlus)	
Proteins having pockets with druggability score ≥ 0.8	5
Proteins having pockets with druggability score < 0.8	1

1849

1850

Table 2 Details of *Streptococcus agalactiae* serotype III core proteins with potential to serve as new drug targets

Protein	**WP_000077187**	**WP_001068667**	**WP_001090621**	**WP_001067088**	**WP_000282567**
Mholline structure prediction quality	High	High	High	High	High
Database of Essential Genes best hit					
e-value	9.78e-160	3.17e-79	0.0	6.59e-30	3.22e-95
Bit score	454	227	672	120	276
Protein position in strain S73 genome	comp(1908924..1910135)	604893..605240	704606..705610	comp(1051267..1052466)	1053435..1054136
Strain S73 genomic island (position)	MI10	RI2/SI3/PAI5	RI3	MI4	MI4
DogSiteScorer binding site prediction					
Pocket volume (Å^3)	577.92	404.54	2349.7	2320.06	456.38
Pocket surface (Å^2)	810.76	827.89	2977.87	25553.96	649.69
Druggability score	0.82	0.82	0.81	0.81	0.84
Uniprot database best hit					
Protein name	Phosphopentomutase	50S ribosomal protein L19	Transcriptional regulator (RegM)	Oxidoreductase, FMN-binding	Flavoprotein-related protein
Organism	*S. agalactiae* ser. III	*S. agalactiae* ser. III	*S. agalactiae* ser. V	*S. agalactiae* ser. V	*S. agalactiae* ser. V
ID	100 %	100 %	100 %	98.7 %	97.4 %
Uniprot code	Q8CMH7	Q8E6H6	Q8E0M3	Q8DZN9	Q8DZN7
Possible function	Phosphotransfer between C1 and C5 of a pentose	Joining of small and large ribosomal subunits	Related to carbon metabolism and virulence expression	Flavoprotein cofactor	Flavoprotein-related

Table 3 Characteristics of the bond between *S. agalactiae* serotype III drug target proteins and drug-like natural compounds. Amino acid residues present in the protein pocket are underlined.

Protein		Compound		Nº H-bonds	Binding affinity (kcal·mol^{-1})	Amino Acid Residues interacting
Annotation in S73 strain genome	**Uniprot best match (gene)**	**ZINC ID**	**IUPAC name**			
WP_000077187	Phosphopentomutase (*deoB1*)	ZINC05410520	4-[(1R,9S)-5-(1-benzothiophen-2-yl)-6-oxo-7,11-diazatricyclo[7.3.1.02,7]trideca-2,4-diene-11-carbonyl]benzonitrile	3	−6	SER270, ARG320, ARG320
WP_001068667	50S ribosomal protein L19 (*rplS*)	ZINC03838587	[(3S,3aR,6R,6aS)-3-(pyrimidine-5-carbonylamino)-2,3,3a,5,6,6a-hexahydrofuro[3,2-b]furan-6-yl] N-(4-phenoxyphenyl)carbamate	4	−5.2	ARG21, ARG21, GLU57, GLU57
WP_001090621	RegM trancriptional regulator (*sag0707*) / Catabolite control protein A (*ccpA*)	ZINC04236030	(4aS)-3-(furan-2-carbonyl)-9-[3-(trifluoromethyl)phenyl]-2,4,4a,6-tetrahydro-1H-pyrazino[2,1-c][1,4]benzodiazepine-5,11-dione	3	−8.4	ARG136, ARG136, ARG143
WP_001067088	Oxidoreductase, FMN-binding (*sag1061*)	ZINC03839958	1-[(3S,3aS,5aS,10S,10aS,10bS)-3,5a,10-trimethyl-2-oxo-3,3a,4,5,6,10,10a,10b-octahydro-[1]benzofuro[7,6-f][1,3]benzothiazol-8-yl]-3-propan-2-ylurea	3	−6.4	LEU350, ILE352, LYS382
WP_000282567	Flavoprotein-related protein (*sag1063*)	ZINC04222225	N-[(6aS,8S)-6,12-dioxo-2-[3-(trifluoromethyl)phenyl]-5,6a,7,8,9,10-hexahydropyrido[2,1-c][1,4]benzodiazepin-8-yl]pyrazine-2-carboxamide	3	−7.9	ASP203, ASN206, LYS226

Supplementary Material

Supplementary Table 1 Information on the genomes used in this work

SPECIE	STRAIN	ACCESSION NUMBER	SERO TYPE	ST	HOST	ISOLATION SOURCE	ORIGIN	SIZE (bp)	GENE NUMBER	PROTEIN NUMBER	SEQUENCING TECHNOLOGY
S. agalactiae	SGEHI2015-25	NZ_CP025029.1	III	283	fish	muscle (fresh fish)	Singapore	2054713	2173	2005	PacBio
S. agalactiae	SGEHI2015-113	NZ_CP025026.1	III	283	fish	muscle (fresh fish)	Singapore	2043851	2156	1975	PacBio
S. agalactiae	SGEHI2015-107	NZ_CP025027.1	III	283	fish	muscle (fresh fish)	Singapore	2016805	2119	1948	PacBio
S. agalactiae	SGEHI2015-95	NZ_CP025028.1	III	283	fish	muscle (fresh fish)	Singapore	2116769	2248	2050	PacBio
S. agalactiae	S73	NZ_CP030845.1	III	na	fish	Brain	Brazil	2059915	2089	1953	Illumina
S. agalactiae	32790-3A	NZ_CP029561.1	III	17	human	blood	China	2148904	2249	2022	PacBio
S. agalactiae	874391	NZ_CP022537.1	III	17	human	vagina	Japan	2153937	2224	2034	PacBio
S. agalactiae	CU_GBS_08	NZ_CP010874.1	III	283	human	blood	Hong Kong	2084511	2132	1989	Illumina MiSeq
S. agalactiae	CU_GBS_98	NZ_CP010875.1	III	283	human	cerebrospinal fluid	Hong Kong	2029669	2043	1908	Illumina MiSeq
S. agalactiae	CUGBS591	NZ_CP021862.1	III	12	human	joint aspirate	Hong Kong	2227680	2362	2161	PacBio
S. agalactiae	H002	NZ_CP011329.1	III	736	human	vagina	China	2147416	2143	1975	Illumina Hiseq
S. agalactiae	HU-GS5823	NZ_AP018935.1	III	335	human	blood	Japan	2231314	2286	2109	MinION; Illumina Miseq
S. agalactiae	NEM316	NC_004368.1	III	23	human	blood	Europa	2211485	2217	2083	na
S. agalactiae	NGBS128	NZ_CP012480.1	III	17	human	blood	Canada	2074179	2069	1863	PacBio; Illumina
S. agalactiae	Sag158	NZ_CP019979.1	III	19	human	blood	China	2096882	2186	2000	PacBio
S. agalactiae	Sag27	NZ_CP031556.1	III	19	human	perianal	China	2205229	2256	2075	PacBio
S. agalactiae	SG-M1	NZ_CP012419.2	III	283	human	meningitis	Singapore	2116811	2161	2009	PacBio
S. agalactiae	SG-M158	NZ_CP021864.1	III	283	human	blood	Singapore	2116811	2248	2075	PacBio
S. agalactiae	SG-M163	NZ_CP021863.1	III	283	human	blood	Singapore	2116810	2246	2072	PacBio
S. agalactiae	SG-M25	NZ_CP021867.1	III	19	human	blood	Singapore	2208337	2331	2146	PacBio
S. agalactiae	SG-M29	NZ_CP021866.1	III	283	human	blood	Singapore	2116773	2247	2072	PacBio
S. agalactiae	SG-M4	NZ_CP021870.1	III	23	human	blood	Singapore	2072007	2153	1991	PacBio

S. agalactiae	SG-M50	NZ_CP021865.1	III	283	human	blood	Singapore	2116810	2248	2074	PacBio
S. agalactiae	SG-M6	NZ_CP021869.1	III	17	human	blood	Singapore	2106018	2216	2013	PacBio
S. thermophilus	JIM8232	NC_017581.1	na	na	na	Milk	France	1929905	2029	1727	Sanger, SOLiD

Supplementary Table 2 Strain S73 genomic islands position

Island ID	Start	Stop
PAI1	68244	120420
PAI2	139689	167554
PAI3	224027	245806
PAI4	255259	294247
PAI5	605240	617168
PAI6	1140604	1153487
PAI7	1884594	1903430
MI1	139689	167554
MI2	300326	310257
MI3	523178	530912
MI4	1034591	1053450
MI5	1066892	1079788
MI6	1087956	1095663
MI7	1360368	1367874
MI8	1557569	1565371
MI9	1639836	1673580
MI10	1907089	1914209
RI1	139689	167554
RI2	605240	617168
RI3	630788	707253
RI4	999679	1029365
RI5	1828986	1880468
RI6	1884594	1903430
SI1	139689	167554
SI2	523178	530912
SI3	605240	617168
SI4	1884594	1903430
SI5	1949253	1980497
GI1 (PAI, MI, RI e SI)	494057	517583
GI2 (PAI, MI, RI e SI)	1199007	1239807
GI3 (RI e SI)	1760247	1773004
GI4 (PAI, MI, RI e SI)	1987317	2001811

PAI: pathogenicity island; MI: metabolic island; RI: resistance island; SI: symbiotic island; GI: genomic island.

Supplementary Table 3 Information on the druggable pockets identified in *S. agalactiae* serotype III selected drug target proteins

Protein ID	Pocket ID	Volume (Å³)	Surface (Å²)	Druggability score	Amino acid residues
WP_000077187	P_0	577,92	810,76	0,82	VAL10, LEU12, ASN52, MET53, LYS55, ILE56, SER77, VAL247, GLY248, LYS249, ASN266, LYS267, SER268, ASN269, SER270, GLY272, ILE273, LEU276, THR292, ASN293, LEU294, VAL295, ASP296, PHE297, CYS313, GLU316, PHE317, ASP318, ARG320, LEU321, GLU323, ILE324, ILE325, MET328, LEU334, ILE336, LEU359, TYR361
WP_001090621	P_0	2349,7	2977,87	0,81	GLY68, VAL69, VAL70, ILE71, PRO72, ASN73, ALA75, ASN76, SER77, TYR78, PHE79, LEU82, VAL98, LEU99, ALA100, SER101, SER102, ASP103, GLU104, ASP105, ASP106, LYS108, GLU109, ASN111, VAL112, VAL113, ASN114, THR115, LEU116, PHE117, LYS119, GLN120, VAL121, ASP122, ILE124, PHE126, MET127, GLY128, HIS129, HIS130, LEU131, THA132, GLU133, ILE135, ARG136, GLU138, PHE139, SER140, ARG141, SER142, ARG143, THR144, PRO145, ILE146, VAL147, LEU148, ALA149, GLY150, THR151, VAL152, ASP153, LEU154, LEU158, PRO159, ASN162, ILE163, ASP164, TYR165, ALA168, ALA169, VAL172, ILE173,VAL186, SE187, GLY188, PRO189, LEU190, ILE191, ASP192, ASP193, ASN195, GLY196, ARG199, LEU200, TYR203, VAL219, PHE220, GLU221, TYR224, ARG225, TYR245, VAL246, ALA247, GLU248, ASP249, GLU250, SER275, ASN276, SER278, ILE280, ILE291, GLN293, LEU298, ILE309
WP_001067088	P_0	2320,06	2553,96	0,81	LEU26, SER27, PRO28, MET29, VAL30, THR31, ASN32, SER33, SER34, VAL40, ASP44, ILE45, TYR47, ALA48, ARG51, GLN58, ILE59, THR60, GLY61, ALA62, TYR64, GLY69, GLN70, LEU71, PHE72, TYR74, GLY75, PHE76, GLY86, LEU87, LYS89, LEU90, ALA93, LEU103, GLN104, THR106, HIS107, ALA108, PHE165, SER171, ALA173, GLN174, ARG175, LEU176, ARG229, THR231, PRO232, GLU233, GLU234, THR235, ALA264, ILE265, ALA266, AER267, TRP268, GLY169, HIS270, ASP271, VAL272, PHE273, ASN275, THR276, ARG278, LEU287, VAL288, ASN289, MET303, ALA304, THR305, GLY306, GLY307, ILE308, ASN309, LYS313, GLU316, ALA317, HIS320, ALA326, SER327, THR328, PRO329, EU330, VAL331, VAL332, LEU350, ILE352, ILE362, PRO363, LYS364, PHE367, ASP369, ILE370, VAL371, PRO372, LEU373, MET374, ASP375, TYR376, GLY377, GLU378, SER379, LEU380, PRO381, LYS 382
WP_000282567	P_1	456,38	649,69	0,84	ILE3, LEU4, ILE5, ILE32, ALA33, TYR36, VAL43, ILE84, LEU85, ILE86, ILE153, LEU154, VAL155, THR182, PHE183, LEU201, LEU202, ASP203, ASN206, VAL207, TYR208, LEU219, ILE220, LYS223, VAL224, LYS226, TYR227
WP_001068667	P_1	404,54	827,89	0,82	GLN12, LEU13, ARG14, ASP16, ILE17, PRO18, PHE20, ASP24, VAL26, VAL28, LYS31, VAL47, ILE55, GLU57, TYR59, THR60 VAL61, PHE74, PRO75, ILE76, HIS77, THR78, PRO79, ARG80, VAL81, ASO82, LYS83, ILE84, GLU85

Supplementary Table 4 BLASTp homology of drug target proteins from *Streptococcus agalactiae* serotype III to other serotypes

Strain (Accession number)	Serotype	Host	WP_000077187		WP_001068667		WP_001090621		WP_001067088		WP_000282567	
			Coverage (%)	ID (%)	Coverage (%)	ID (%)	Coverage (%)	ID (%)	Coverage (%)	ID (%)	Coverage (%)	ID (%)
S. agalactiae A909 (NC_007432)	Ia	Human	100	100	100	100	99	99	99	99	99	98
S. agalactiae 515 (NZ_CP051004)	Ia	Human	100	99.75	100	100	100	100	99	99	100	98.68
S. agalactiae GD201008-001 (NC_018646)	Ia	Fish	100	100	100	100	100	99.70	100	99	100	98.25
S. agalactiae ZQ0910 (NZ_CP049938)	Ia	Fish	100	99.75	100	100	100	99.70	100	99	100	98.25
S. agalactiae H36B (GCA_000167795)	Ib	Human	100	100	100	100	100	100	99	99	99	98
S. agalactiae 138P (CP007482)	Ib	Fish	75	98.62	100	100	100	100	98	98	99	98
S. agalactiae 18RS21 (GCA_000167715)	II	Human	100	100	100	100	100	100	100	100	100	99
S. agalactiae GBS1-NY (NZ_CP007570)	II	Human	100	100	100	100	100	96.11	100	100	100	99.56
S. agalactiae NEM316 (NC_004368)	III	Human	100	100	100	100	100	100	100	100	100	100
S. agalactiae CCUG 19094 (GCA_000288675)	III	Human	99	99	100	100	100	100	100	98.75	100	97.37
S. agalactiae COH1 (NZ_HG939456)	III	Human	100	99.75	100	100	100	100	100	98.75	99	99
S. agalactiae NGBS061 (NZ_CP007631)	IV	Human	100	100	100	100	100	96.41	100	100	100	99.56
S. agalactiae NGBS572 (NZ_CP007632)	IV	Human	100	100	100	100	100	96.41	100	98.75	99	98.67
S. agalactiae 2603V/R (NC_004116)	V	Human	100	100	100	100	100	100	99	99	98	97
S. agalactiae CJB111 (GCA_000167755)	V	Human	100	100	100	100	94	100	100	100	100	99.56
S. agalactiae 09mas018883 (NC_021485)	V	Bovine	100	100	100	100	100	100	100	100	100	99.56
S. agalactiae ILRI112 (HF952106)	VI	Dromedary	100	99	100	100	99	99	99	99	98	97
S. agalactiae GBS-M002 (NZ_CP013908)	VI	Human	100	100	100	100	100	96.41	100	99.25	100	98.25

Supplementary Figure 1 Similarity heatmap based on whole genomes of *Streptococcus agalactiae* serotype III isolates from human and fish hosts

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1: Strain 32790/Human/China	100	96	92	96	86	88	87	88	88	88	85	89	85	85	85	85	85	85	85	85	84	84	84	84	4
2: Strain 874391/Human/Japan	95	100	93	92	85	88	87	88	87	88	85	86	86	86	86	86	86	86	87	85	85	85	86	84	4
3: Strain SG-M6/Human/Singapore	94	96	100	95	88	89	89	89	88	89	88	89	88	88	88	88	88	88	89	87	87	88	88	87	4
4: Strain NGBS128/Human/Canada	99	96	95	100	89	89	90	90	90	90	88	92	88	88	88	88	88	88	89	88	88	88	88	88	4
5: Strain SG-M4/Human/Singapore	88	88	88	88	100	89	89	89	89	90	99	89	90	90	90	90	90	90	90	89	89	89	89	89	4
6: Strain Sag158/Human/China	89	90	88	88	88	100	97	100	96	95	88	89	89	89	89	89	89	89	90	88	88	89	89	88	4
7: Strain H002/Human/China	86	87	86	86	86	94	100	97	94	98	86	87	86	86	86	86	86	86	87	85	85	86	86	85	5
8: Strain Sag27/Human/China	85	86	84	83	84	95	95	100	93	94	84	86	85	85	85	85	85	85	85	84	84	84	85	84	5
9: Strain SG-M25/Human/Singapore	85	85	83	84	84	92	92	93	100	92	84	86	85	85	85	85	85	85	85	84	84	84	84	84	5
10: Strain HU-GS5823/Human/Japan	84	85	83	84	84	90	95	93	91	100	84	85	84	84	84	84	84	84	85	84	84	84	85	84	5
11: Strain NEM316/Human/Europe	82	83	83	82	93	83	83	84	84	85	100	84	85	85	85	85	85	85	85	84	84	84	84	84	4
12: Strain CUGBS591/Human/HongKong	85	82	83	84	83	83	84	85	85	85	83	100	88	88	88	88	88	88	88	88	88	88	88	88	5
13: Strain SGEHI2015-95/Fish/Singapore	86	87	87	86	88	88	87	88	88	88	89	93	100	100	100	100	100	100	99	97	98	96	96	95	4
14: Strain SG-M163/Human/Singapore	86	87	87	86	88	88	87	88	88	88	89	93	100	100	100	100	100	100	99	97	98	96	96	95	4
15: Strain SG-M1/Human/Singapore	86	87	87	86	88	88	87	88	88	88	89	93	100	100	100	100	100	100	99	97	98	96	96	95	4
16: Strain SG-M158/Human/Singapore	86	87	87	86	88	88	87	88	88	88	89	93	100	100	100	100	100	100	99	97	98	96	96	95	4
17: Strain SG-M50/Human/Singapore	86	87	87	86	88	88	87	88	88	88	89	93	100	100	100	100	100	100	99	97	98	96	96	95	4
18: Strain SG-M29/Human/Singapore	86	87	87	86	88	88	87	88	88	88	89	93	100	100	100	100	100	100	99	98	98	96	96	95	4
19: Strain CU_GBS_08/Human/HongKong	87	89	88	87	89	89	89	90	89	90	89	94	99	99	99	99	99	99	100	98	97	97	98	96	3
20: Strain S73/Fish/Brazil	87	88	88	88	90	89	89	90	90	90	90	94	99	99	99	99	99	99	99	100	99	97	98	98	4
21: Strain SGEHI2015-25/Fish/Singapore	88	89	88	88	90	90	89	90	90	90	90	95	100	100	100	100	100	100	99	99	100	97	98	98	4
22: Strain CU_GBS_98/Human/HongKong	88	90	90	89	91	91	90	91	90	92	91	95	99	99	99	99	99	99	100	99	98	100	99	98	3
23: Strain SGEHI2015-113/Fish/Singapore	88	90	90	89	91	91	90	91	90	92	91	96	99	99	99	99	99	99	100	99	99	99	100	99	4
24: Strain SGEHI2015-107/Fish/Singapore	89	90	90	89	91	91	91	91	91	92	92	96	100	100	100	100	100	100	100	100	100	99	100	100	4
25: *S. thermophilus* strain JIM8232	4	4	4	4	4	4	5	5	5	5	4	5	4	4	4	4	4	4	4	4	4	4	4	4	100

Supplementary Box 1 Top 10 ligands for each drug-target identified in *S. agalactiae* serotype III. Ligands identified in red were screened as the best docking to its target.

Protein	WP_000077187	
Best 10 ligands	ZINC05410520	ZINC04237087
	ZINC04235928	ZINC04259075
	ZINC04260398	ZINC04259499
	ZINC04277699	ZINC08300419
	ZINC04236005	ZINC04222225

Protein	WP_001068667	
Best 10 ligands	ZINC03838445	ZINC03838585
	ZINC03838485	ZINC03838587
	ZINC03838486	ZINC03838628
	ZINC03838531	ZINC03838631
	ZINC03838534	ZINC03838693

Protein	WP_001090621	
Best 10 ligands	ZINC04237100	ZINC04258935
	ZINC04235882	ZINC04222703
	ZINC04237087	ZINC04236304
	ZINC04277699	ZINC04237085
	ZINC04258917	ZINC04236030

Protein	WP_001067088	
Best 10 ligands	ZINC04237162	ZINC67897676
	ZINC03839958	ZINC04237238
	ZINC04259535	ZINC04259049
	ZINC05396545	ZINC08296267
	ZINC06137747	ZINC08300264

Protein	WP_000282567	
Best 10 ligands	ZINC08299978	ZINC04222225
	ZINC04236081	ZINC08300249
	ZINC04222703	ZINC04235880
	ZINC04236001	ZINC04235966
	ZINC04236036	ZINC04236020

7 CONSIDERAÇÕES FINAIS

A alta densidade de peixes em sistemas intensivos e a possibilidade de falhas vacinais favorecem a manutenção e disseminação de agentes como *Francisella orientalis* e *Streptococcus agalactiae*. O uso de antimicrobianos é ainda uma estratégia importante para o controle de surtos por doenças bacterianas na piscicultura. Buscar validar por métodos científicos os protocolos de antibioticoterapia empregados no campo é essencial para um uso eficiente e que evite efeitos colaterais a microbiomas, como seleção de microrganismos resistentes, ambiental, como o acúmulo dessas moléculas no solo e nos cursos d'água, e de saúde, como a contaminação de produtos cárneos destinado ao consumo humano. Ainda, o uso de ferramentas que fomentem o desenvolvimento mais rápido de novas moléculas antimicrobianas para uso em situações de infecções por linhagens multirresistentes deve ser estimulado. Os dados descritos nesta dissertação vão de encontro com essas premissas. Demonstrou-se que o uso da oxitetraciclina, molécula já empregada no campo, mas sem estudo formal de eficácia contra a franciselose, é capaz de controlar a evolução da doença e diminuir a mortalidade, sem causar efeitos deletérios graves nas tilápias, quando utilizada precocemente. A partir de estudos genômicos e ferramentas de bioinformática, 5 alvos para antimicrobianos foram previstos para o sorotipo III de *S. agalactiae*, com homologia para os outros sorotipos. Cinquenta compostos naturais são listados como possíveis inibidores destes alvos e podem facilmente ser testados em novos estudos *in vitro*. Finalmente, a popularização de estratégias de monitoramento constante da saúde de lotes é imprescindível para a detecção dos primeiros peixes com sinais clínicos de bacterioses e para iniciar precocemente a antibioticoterapia, possibilitando um melhor controle dessas enfermidades e diminuindo os possíveis riscos do uso de antimicrobianos.