VANESSA GOMES DA SILVA

IMUNOMODULADORES CONTRA BACTERIOSES EM TILÁPIAS:

AVALIAÇÃO DE ÁCIDOS ORGÂNICOS CONTRA *Francisella orientalis* E DETERMINAÇÃO DE POTENCIAL PROBIÓTICO DE CEPAS CONTRA *Streptococcus agalactiae*

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Orientador: Prof. Dr. Ulisses de Pádua Pereira

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RESUMO

A aquicultura tornou-se um dos setores mais proeminentes do agronegócio mundial. A produção brasileira de peixe de cultivo alcançou 860mil toneladas em 2022, e a tilápia permanece como a espécie mais cultivada no país. Porém, os sistemas intensivos trazem reveses como a alta incidência de doenças bacterianas, em especial a franciselose e estreptococose, que causam surtos com alta mortalidade nas pisciculturas. Para controlar esses surtos, vários antimicrobianos são utilizados tanto terapêutica quanto profilaticamente. O uso excessivo e indiscriminado implica no surgimento de cepas resistentes que se acumulam na água, nos sedimentos, no pescado comercializado e oferecem risco para o tratamento de infecções em animais e humanos. Assim, a aquicultura moderna tem visado práticas mais sustentáveis a fim de fornecer produtos seguros para o consumidor final. Nesse sentido, esta pesquisa buscou avaliar novos aditivos alimentares quanto à sua capacidade de promover ganhos à imunidade e maior resistência às enfermidades de maior ocorrência na tilapicultura nacional. No trabalho A, diferentes concentrações (3 g.kg-1 e 5 g.kg-1) de um aditivo composto por ácidos orgânicos foi incorporado à ração, fornecido durante 21 dias para juvenis de tilápia que, em seguida, foram desafiadas com Francisella orientalis. Os animais alimentados com 0,5% de Bacti-nil®Aqua apresentaram melhoras em parâmetros imunológicos, como lisozima e atividade antibacteriana do soro, e menor mortalidade quando comparados com grupos controles. A microbiota intestinal manteve características pré-desafio entre os grupos tratados, o que pode ser considerado um efeito benéfico do produto sobre o microbioma intestinal. No trabalho B, diferentes cepas de microrganismos tiveram seu potencial probiótico avaliado e, posteriormente foram testados quanto ao efeito imunomodulador e à resistência contra *Streptococcus agalactiae* sorotipo Ib. *Lactococcus lactis* apresentou boa tolerância a baixo pH, sais biliares e NaCl, assim como demonstrou antagonismo contra *Enterococcus faecium* e *S. agalactiae in vitro*. As cepas da levedura *Yarrowia lipolytica* demonstraram boa resistência nos testes in vitro. Na avaliação histomorfológica do fígado, os grupos alimentados com *Y. lipolytica* tiveram escores gerais mais baixos, indicando que a levedura ajudou a preservar a integridade do fígado. A adição de *L. lactis* e *Y. lipolytica* teve um efeito benéfico no sistema imune, com resultados significativos na atividade bactericida do soro e na sobrevivência após o desafio bacteriano. Os trabalhos desenvolvidos indicam que a adição de ácidos orgânicos e probióticos à ração são importantes ferramentas no estímulo da imunidade dos animais, assim como no combate a enfermidades, propagando uma aquicultura mais sustentável. Os microrganismos testados poderão ser amplamente utilizados como aditivos na ração e as técnicas desenvolvidas permitem a exploração de novas cepas probióticas, em especial, cepas autóctones que oferecem maior adaptação e segurança para os animais e para cadeia produtiva.

Palavras-chave: ácidos orgânicos; probióticos; aquicultura sustentável; aditivos alimentares; imunomoduladores.

SILVA, Vanessa Gomes da. **Immunomodulators against bacteriosis in tilapia**: evaluation of organic acids against *Francisella orientalis* and determination of the probiotic potential of strains against *Streptococcus agalactiae*. 2023. 117pp. Thesis (Doctorate's degree in 2023) – Universidade Estadual de Londrina, Londrina, 2023.

ABSTRACT

Aquaculture has become one of the most prominent sectors of the world's agribusiness. Brazilian production of farmed fish reached 860,000 tons in 2022, and tilapia remains the most cultivated species in the country. However, intensive systems bring setbacks such as the high incidence of bacterial diseases, especially francisellosis, and streptococcosis, which cause outbreaks with high mortality in fish farms. To control these outbreaks, various antimicrobials are used both therapeutically and prophylactically. Excessive and indiscriminate use implies the emergence of resistant strains that accumulate in the water, sediments, and commercialized fish, and poses a risk to the treatment of the community in animals and humans. Thus, modern aquaculture has more attractive targeted practices to provide safe products for the final consumer. In this sense, this research sought to evaluate new food additives regarding their ability to promote gains in immunity and greater resistance to the most common diseases in domestic tilapia farming. In work A, different concentrations (3 g.kg-1 and 5 g.kg-1) of an additive composed of organic were incorporated into the feed and supplied for 21 days to tilapia juveniles, which were then challenged with Francisella orientalis . Animals fed 0.5% Bacti-nil®Aqua showed improvements in immunological terms, such as lysozyme and serum antibacterial activity, and lower mortality when compared to controlled groups. The intestinal microbiota maintained pre-challenge characteristics among the treated groups, which can be considered a beneficial effect of the product on the intestinal microbiome. In work B, different strains of microorganisms had their probiotic potential evaluated and, subsequently, were tested for their immunomodulatory effect and resistance against *Streptococcus agalactiae* serotype Ib. *Lactococcus lactis* showed good tolerance to low pH, bile salts, and NaCl, as well as proven antagonism against *Enterococcus faecium* and *S. agalactiae in vitro*. The strains of *Yarrowia lipolytica* showed good resistance in testes *in vitro*. In the histomorphological evaluation of the liver, the groups fed with *Y. lipolytica* had lower overall scores, indicating that the yeast helped to preserve the integrity of the liver. The addition of *L. lactis* and *Y. lipolytica* had a beneficial effect on the immune system, with significant results in serum bactericidal activity and survival after bacterial challenge. The works demonstrated that the addition of organic and probiotics to the feed are important tools in stimulating the immunity of the animals, as well as in the fight against diseases, propagating a more sustainable aquaculture. The tested microorganisms could be widely used as feed additives and the advanced techniques will allow the exploration of new probiotic strains, in particular, autochthonous strains that offer greater adaptation and safety for the animals and for the production chain.

Keywords: organic acids; probiotics; sustainable aquaculture; food additives; immunomodulators.

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well diffusion method ……

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ARTIGO B

LISTA DE ABREVIATURAS E SIGLAS

ACH50	*Alternative complemente activity*; Atividade do sistema complemento
AMP	Peptídeos antimicrobianos
BHI	*Brain and heart infusion broth*; Caldo infusão cérebro e coração
CFCS	*Cell-free culture supernatant*; Sobrenadante livre de células
CFU	*Colony forming units*; Unidades formadora de colônia
CHAH	*Cystine heart agar supplemented with 1% bovine hemoglobin*; Ágar cistina-coração suplementado com 1% de hemoglobina bovina
DFI	*Daily feed intake*; Ingestão diária
dpi	*Days post-infection*; Dias pós-infecção
DWG	*Daily weight gain*; Ganho de peso diário
EF	*Enterococcus faecium*
EG	*Eosinophilic granulocytes*; Granulócitos eosinofílicos
FBW	*Final body weight*; Peso final
FCR	*Feed conversion ratio*; Taxa de conversão alimentar
GC	*Goblet cells*; Células caliceformes
GIFT	*Genetically improved farmed tilapia*; Tilápia geneticamente melhorada
IBW	*Initial body weight*; Peso inicial
Ig	Imunoglobulina
IP	Intra-peritonial
LAB	*Lactic acid bacteria*; Bactéria ácido lática
LABBEP	*Laboratory of Fish Microbiology;* Laboratório de bacteriologia em peixes
LL	*Lactococcus lactis*
LP	Lâmina própria
MALT	*Mucosa-associated lymphoid tissue*; Tecido linfoide associado à mucosa
MAMP	Padrões moleculares associados à micro-organismos

MBC	*Minimum bactericidal concentration*; Concentração bactericida mínima
MHBA	*Mueller Hinton Blood Agar*; Agar Muller Hinton sangue
MIC	*Minimal inhibitory concentration*; Concentração inibitória mínima
MRS	Meio de Man, Rogosa, and Sharpe
MV	*Mucosal villi*; Vilosidades da mucosa
NO	Óxido nítrico
OA	*Organic acids*; Ácidos orgânicos
PAMP	Padrões moleculares associados à patógenos
PB	*Poor broth*; Caldo pobre
PBS	*Phosphate-buffered saline*; Tampão fosfato-salino
PRR	Receptores de reconhecimento padrão
RaRBC	*Rabbit red blood cells*; Hemácias de coelho
ROS	Espécies reativas de oxigênio
SEM	*Scanning Electron Microscopy*; Microscopia eletrônica de varredura
SGR	*Specific growth rate*; Taxa de crescimento específico
Sisgen	*National System for the Management of Genetic Heritage and Associated Traditional Knowledge*; Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado
SM	*Subepithelial mucosa*; Mucosa subepitelial
SNV	Supranuclear vacuoles; Vacúolos supranuclear
TGI	Trato gastrointestinal
TNTC	*Too numerous to count*; Muito numeroso para contar
UEL	*State University of Londrina*; Universidade Estadual de Londrina
UFC	Unidades formadora de colônia
WG	*Weight gain*; Ganho de peso
YL	*Yarrowia lipolytica*
YPD	*Yeast peptone dextrose*; meio dextrose, peptona e extrato de levedura

SUMÁRIO

1 INTRODUÇÃO

A produção pesqueira mundial, proveniente da pesca e da aquicultura tem demonstrado franco progresso nos últimos anos e, em 2020, alcançou um recorde histórico de 214 milhões de toneladas (t), dos quais 178 milhões de toneladas foram de animais aquáticos e 36 milhões de toneladas de algas. Os países asiáticos foram os principais produtores de animais aquáticos, com 70% do total, seguidos pelas Américas (12%), Europa (10%), África (7%) e Oceania (1%). Somente a China foi responsável por 35% do total produzido (FAO, 2022).

A aquicultura produziu 122,6 milhões de toneladas, dos quais, 87,5 milhões de toneladas foram de animais aquáticos com estimado valor de venda de US$ 264,8 bilhões e 35,1 milhões de toneladas de algas no valor de US$ 16,5 bilhões (FAO, 2022).

Com uma das maiores bacias hidrográficas do mundo e cerca de 12% de toda água doce do planeta, além de represas artificiais e reservatórios de água, o Brasil apresenta excelentes condições para a produção de organismos aquáticos. Assim sendo, a Food and Agriculture Organization of the United States (FAO) estima que em 2030, a aquicultura brasileira produzirá cerca de 750 mil toneladas de pescado, o que representará um crescimento de 19,3% em relação a 2020. Esse aumento será possível, principalmente, pela intensificação e expansão da produção sustentável da aquicultura (FAO, 2022).

A tilápia do Nilo (*Oreochromis niloticus*) é a terceira espécie mais produzida no mundo e a primeira da piscicultura brasileira, representando 64,6% do total produzido em 2021. O Paraná configura como o maior produtor nacional com 144.8 mil toneladas (IBGE, 2022). Esse desempenho deve-se a sua taxa de crescimento rápido, tolerância à alta densidade de estocagem e alta resistência a doenças (NG; ROMANO, 2013). No entanto, como as tilápias são criadas em sistemas de produção intensiva, elas se tornam mais suscetíveis a doenças, especialmente aquelas causadas por bactérias, fungos e vírus.

Streptococcus agalactiae, *S. iniae*, *Aeromonas* móveis, *Flavobacterium columnare* e *Francisella orientalis* são responsáveis por grandes perdas na criação de tilápias e por alguns surtos nos últimos anos (DONG et al., 2015; JEFFERY et al., 2010; LEAL; TAVARES; FIGUEIREDO, 2014; LIN et al., 2016; RAJ et al., 2019). Tradicionalmente, antimicrobianos são utilizados no combate a esses

surtos e, também, como métodos profiláticos ou ainda como promotores de crescimento. Porém, essa conduta leva ao surgimento de cepas resistentes que podem colonizar o pescado destinado ao consumo, impactando assim a saúde humana. Como consequência, os antimicrobianos têm sido progressivamente banidos da aquicultura, em especial quando empregados para fins não terapêuticos; já o interesse por imunoestimulantes, como prebióticos e probióticos tem aumentado como estratégia profilática mais sustentável (EL-SAADONY *et al.*, 2021; PANDIYAN *et al.*, 2013).

Os probióticos atuam beneficamente no hospedeiro e no ambiente, ao incrementar a microbiota intestinal e a qualidade da água (CHEN *et al.*, 2019; CZECH *et al.*, 2018b; MOHAMMADIAN *et al.*, 2019; VAN DOAN *et al.*, 2016). Já os prebióticos são compostos não digeríveis que estimulam seletivamente a proliferação e atividade de microrganismos desejáveis no trato gastrointestinal (TGI) (DAVANI-DAVARI *et al.*, 2019; SAAD, 2006).

Este trabalho buscou: 1) avaliar o efeito de ácidos orgânicos na modulação do sistema imune, no desempenho zootécnico, na microbiota intestinal de tilápias e na resistência contra *Francisella orientalis* e; 2) avaliar o potencial probiótico de bactérias láticas e leveduras e seu efeito no desempenho zootécnico, nos parâmetros imunológicos e na sobrevivência de tilápia do Nilo após infecção experimental por *Streptococcus agalactiae*.

2 REFERENCIAL TEÓRICO

2.1 AQUICULTURA E TILAPICULTURA

A fim de promover pesca e aquicultura sustentáveis, algumas diretrizes são adotadas mundialmente, como a implementação de pesca baseada em princípios científicos, políticas de gerenciamento na aquicultura, aliadas a transparência no comércio internacional de pescado. Tais condutas levaram a uma produção mundial de pescado estimada em 178 milhões de toneladas em 2020. A pesca contribuiu com 90 milhões de toneladas (51%) e a aquicultura com 88 milhões de toneladas (49%). A China permanece como a maior produtora mundial de pescado (35%), seguida pela Índia (8%), Indonésia (7%), Vietnam (5%) e Peru (3%) (FAO, 2022).

Entre 2005 e 2020, a aquicultura brasileira cresceu 145%, passando de 257 mil para 630 mil toneladas produzidas, o que movimentou R$ 5,44 bilhões, registrando a 13ª maior produção aquícola do mundo (FAO, 2022). Em 2021, o país registrou 648,5 mil t produzidas, movimentando R$ 6,37 bilhões. A produção total de peixes para consumo atingiu 558,9 mil toneladas, um aumento de 0,92% em relação a 2020, e foi avaliada em R$ 4,7 bilhões (IBGE, 2022).

A tilápia do Nilo é a terceira espécie mais produzida mundialmente (9%), perdendo apenas para duas espécies de carpas, Carpa capim (*Ctenopharyngodon idellus*) e Carpa prateada (*Hypophthalmichthys molitrix*) (21,8%, juntas) (FAO, 2022). No Brasil, a tilápia manteve-se como a espécie mais cultivada, tendo sido despescadas 361,2 mil toneladas em 2021. O Paraná permanece como maior produtor nacional de tilápias, com 139,2 mil toneladas, fruto de um aumento anual de 3,5% (IBGE, 2022).

Embora o país seja referência na produção, o consumo de pescado pelo brasileiro é de aproximadamente 9 kg/habitante/ano, abaixo do recomendado pela FAO de 12 kg/hab/ano e muito aquém da média mundial de cerca de 20,5 kg/hab/ano (FAO, 2022; LOPES; OLIVEIRA; RAMOS, 2016).

A história da tilápia no Brasil é relativamente recente. Na década de 1950, a tilápia do Congo (*Tilapia rendalli*) foi introduzida para o repovoamento das represas com a finalidade de combater a proliferação de algas macrófitas aquáticas.

Porém, de hábito alimentar herbívoro, não se adaptou bem para a produção e tornou-se praga em açudes do Nordeste e no estado de São Paulo (ZIMMERMANN; FITZSIMMONS, 2004).

Em 1971, a tilápia do Nilo (*Oreochromis niloticus*) e a tilápia de Zanzibar (*Oreochromis hornorum*) foram introduzidas no Brasil pelo Departamento Nacional de Obras Contra a Seca, por apresentarem características importantes para a piscicultura nacional (SCHULTER; VIEIRA FILHO, 2018). Desde então, o setor passou por grandes transformações. Especialmente a partir da década de 1990, quando técnicas de inversão sexual, investimentos em tecnologia, insumos, beneficiamento e comercialização foram adotados (KUBITZA, 2003).

O bom desempenho da tilápia dá-se por sua própria biologia caracterizada pela rusticidade, adaptação a diferentes sistemas de cultivo, boa tolerância a variações de temperatura e salinidade, alta prolificidade, rápido crescimento e boa conversão alimentar (EL-SAYED; MANSOUR; EZZAT, 2005). Assim, sistemas semi-intensivos e intensivos, em tanques-rede ou viveiros escavados, são comumente empregados na tilapicultura brasileira, sendo as tilápias chitralada e a GIFT (genetically improved farmed tilapia) as principais linhagens cultivadas na atualidade (VALENTI *et al.*, 2021). Por outro lado, sistemas intensivos com alta densidade de estocagem, levam ao estresse crônico, um importante imunossupressor que, possivelmente, levará ao surgimento de enfermidades causadas por bactérias, fungos, parasitas e alterações nutricionais (TELLI *et al.*, 2014).

2.2 Respostas imunológicas e Doenças infecciosas em peixes

O sistema imunológico é uma rede de processos biológicos que visa proteger um organismo contra doenças, identificando e eliminando o patógeno e suprimindo o surgimento de tumores. Outro papel importante do sistema imunológico é manter a homeostase durante um processo inflamatório. Didaticamente, o sistema imune é dividido em inato e adaptativo, ambos divididos em defesa mediada por células e fatores humorais (substâncias solúveis), embora hoje se saiba que esses dois sistemas trabalhem juntos (MAGNADOTTIR, 2010).

A imunidade inata é o primeiro sistema de defesa e atua também como uma barreira mecânica, porém é incapaz de desenvolver memória após

exposição prévia. Esta inicia-se assim que ocorre a invasão por um patógeno, barreiras físicas e químicas são prontamente ativadas (muco, escama, pele e brânquias). As imunidades celular e humoral estão presentes nos peixes, porém não completamente desenvolvidas como em vertebrados superiores (ELLIS, 1998). Após o organismo entrar em contato com estruturas microbianas características, conhecidas como padrões moleculares associados a patógenos (PAMPs), por exemplo, lipopolissacarídeo bacteriano (LPS), peptideoglicano ou certos açucares, a resposta inata, mediada por sistema complemento, lisozima, proteínas da fase aguda, antimicrobianos, interferon, lectinas, proteases, monócitos, macrófagos, granulócitos, e células *natural killer*, é ativada (HOSEINIFAR *et al.*, 2015).

Com o desenvolvimento da infecção, o antígeno é processado e apresentado às células do sistema imune adaptativo, linfócitos B e T, que elaboram resposta específica por meio de imunoglobulinas (Ig) e linfócitos T citototóxicos (HOSEINIFAR *et al.*, 2015). Os anticorpos produzidos são detectáveis em até 10 dias após o estímulo inicial e compõem um instrumento crucial para a imunidade duradoura e o sucesso de programas vacinais (MAGNADOTTIR, 2010). Por muito tempo, acreditou-se que apenas IgM existisse em peixes, na forma de um tetrâmero com oito sítios de combinação antigênica. No entanto, várias outras Igs funcionais, como IgD, IgZ, IgZ-2 e IgT, foram descritas em peixes teleósteos, o que demonstra um sistema imunológico adaptativo mais complexo e organizado do que o conhecido anteriormente (RAJME-MANZUR et al., 2021; ZHU et al., 2013).

2.2.1 Imunidade inata em peixes ósseos

Peixes não possuem medula óssea e linfonodos como os mamíferos, e assim, os principais órgãos linfoides são os rins, timo, baço e tecido linfoide associado à mucosa (MALT) (PRESS; EVENSEN, 1999). Esses órgãos são responsáveis pela produção dos compostos celulares e humorais envolvidos na imunidade inata e adaptativa. O sistema imune reconhece os PAMPs e assim ativa vários mecanismos como fagocitose, produção de citocinas e, consequente, ativação do sistema imune adaptativo após apresentação de antígeno (SMITH; RISE; CHRISTIAN, 2019).

O rim é composto por duas regiões, anterior e posterior, ambas com função hematopoiética, porém a porção cranial atua na produção, diferenciação e

maturação de leucócitos, linfócitos B, monócitos, macrófagos e granulócitos (TORROBA; ZAPATA, 2003). O timo é um importante órgão responsável pelo desenvolvimento e maturação dos linfócitos T, sua involução ocorre em animais adultos, porém a fase é variável entre espécies e pode ainda estar relacionada às variações sazonais e ciclo hormonal (PRESS; EVENSEN, 1999). O baço é responsável pela hematopoiese e pelo descarte de células velhas ou defeituosas (BILLER-TAKAHASHI; URBINATI, 2014). MALT é composto pelo tecido linfoide encontrado nas vísceras que inclui a mucosa gastrointestinal, as brânquias e a pele. Nesses tecidos ocorre a produção de muco com compostos solúveis como lisozima, proteínas do sistema complemento e imunoglobulinas, atuando como primeira barreira contra patógenos. Podemos encontrar também uma grande quantidade de macrófagos, linfócitos, mastócitos e granulócitos capazes de fagocitar, processar e desenvolver memória imunológica contra o antígeno (LAZADO; CAIPANG, 2014). O fígado possui a mesma função que em mamíferos, produzindo proteínas do sistema complemento e proteínas da fase aguda da resposta inflamatória.

Os tecidos linfoides são responsáveis pela produção de células de defesa como monócitos, macrófagos, neutrófilos e células dendríticas (SMITH; RISE; CHRISTIAN, 2019). Os monócitos são considerados células transitórias já que, durante o processo inflamatório, migram da circulação para o tecido e se tornam macrófagos, os quais tem ação fagocítica, produzem espécies reativas de oxigênio (ROS) e óxido nítrico (NO). Neutrófilos são os granulócitos mais abundantes encontrados no sangue e tecidos linfoides, com ação fagocítica que produzem ânions superóxido com ação bactericida e liberação de grânulos com ação citotóxica e enzimas antimicrobianas, além da produção de ROS e NO (SECOMBES; FLETCHER, 1992; SMITH; RISE; CHRISTIAN, 2019). Linfócitos B com atividade fagocítica já foram descritos em teleósteos como salmão do Atlântico (*Salmo salar L.*) e bacalhau do Atlântico (*Gadus morhua L.*) (ØVERLAND *et al.*, 2010).

Dentre os compostos da imunidade humoral mais estudados temos o sistema complemento, lisozima, peptídeos antimicrobianos e proteínas da fase aguda. Essas moléculas possuem efeitos bactericidas, além de promover a inflamação e a fagocitose.

O sistema complemento é uma cascata de proteínas séricas que culminam na formação do complexo de ataque a membrana plasmática da célula. São mais de 40 proteínas solúveis ligadas à membrana com atividade lítica, quimiotática,

pró-inflamatória e de opsonização (ELLIS, 1999). Tradicionalmente o sistema complemento possui três vias de ativação: a via clássica, ativada pelo complexo antígeno-anticorpo e que, portanto, atua nos sistemas inato e adaptativo; a via alternativa, independente de anticorpo; e a via das lectinas, a qual é ativada pela ligação da lectina ligadora de manose à manose presente na superfície dos patógenos (TIZARD, 2014). O sistema complemento em peixes ósseos apresenta maior diversidade de proteínas que os mamíferos, o que compensa pelo sistema imune adaptativo não tão bem desenvolvido. Algumas proteínas da cascata, como a C3 apresentam isotipos variados, sugerindo uma maior capacidade de reconhecer e destruir uma gama maior de patógenos (SMITH; RISE; CHRISTIAN, 2019).

Lisozima é uma enzima lítica que atua na camada de peptideoglicano da parede celular de bactérias Gram positivas e negativas, resultando na lise bacteriana (ELLIS, 1999). Está envolvida na opsonização, na fagocitose e na ativação do sistema complemento. Está presente no muco, tecido linfoide, plasma e sua atividade depende do sexo, idade, tamanho, estação, temperatura da água, pH, infecção e nível de estresse (SAURABH; SAHOO, 2008).

Peptídeos antimicrobianos (AMPs) já foram reportados em peixes, mas não em todas as espécies e atuam lesionando ou formando poros em membranas plasmáticas. Os AMPs mais frequentes em peixes são piscidinas, defensinas, hepcidinas, catelicidinas, β-defensinas e peptídeos derivados de histonas (MAKESH M., 2022). As β-defensinas apresentaram efeito inibitório sobre *Escherichia coli* DH5α e *S. agalactiae*, em tilápias do Nilo (DONG *et al.*, 2015). Foi demonstrada a atividade das catelicidinas contra *Streptococcus* do grupo A e *Escherichia coli* em análises *in vivo* (LAI; GALLO, 2009).

Em peixes e em mamíferos, lesão tecidual, infecção e inflamação induzem leucócitos a secretar citocinas, como IL-1, IL-6, IL-8 e TNF-α, que por sua vez estimulam os hepatócitos a produzir e liberar proteínas de fase aguda (proteína C reativa, proteínas séricas amilóides A, proteínas de ligação a metais). Estão envolvidas em vários processos como inativação de enzimas proteolíticas, neutralizam, previnem e recuperam danos teciduais, promovendo assim homeostase, também estão envolvidas com a resposta imune específica ao estimular citocinas pró-inflamatórias (CASTRO-OSSES *et al.*, 2017; ROY *et al.*, 2016).

2.2.2 Franciselose

Os primeiros relatos de *Francisella* spp. em tilápias (*Oreochromis* spp.) ocorreram em Taiwan em 1992 (CHEN *et al.*, 1994). Posteriormente, a bactéria foi identificada em vários países como: Estados Unidos (SOTO *et al.*, 2011), Noruega (OTTEM *et al.*, 2008), Reino Unido (JEFFERY *et al.*, 2010), China (LIN *et al.*, 2016), México (ORTEGA *et al.*, 2016), Honduras (SOTO *et al.*, 2019) e Brasil (LEAL; TAVARES; FIGUEIREDO, 2014).

Bactérias do gênero *Francisella* são cocobacilos Gram negativos imóveis, pleomórficos, parasitas intracelulares facultativos, anaeróbios facultativos, não formadores de esporos, variando de 300 a 700nm, catalase positiva, citocromo oxidase negativa e dependente de cisteína. O gênero faz parte da família *Francisellaceae*, ordem *Thiotrichales*, da subclasse *Gammaproteobacteria*. *F. tularensis* é a espécie clássica dessa família, causadora da tularemia, altamente infecciosa para mamíferos, incluindo humanos (BIRKBECK; FEIST; VERNER - JEFFREYS, 2011; COLQUHOUN; DUODU, 2011). No total, o gênero *Francisella* possui 18 espécies, das quais *Francisella noatunensis*, que afeta peixes de água fria, e *Francisella orientalis*, que afeta peixes de água quente (RAMIREZ-PAREDES *et al.*, 2020). Os genes *iglA, iglB, iglC* e *iglD* são importantes fatores de virulência, localizados em uma ilha de patogenicidade com 16-19 genes, necessários para o crescimento intracelular, que também participam no escape do fagolisossoma (NANO; SCHMERK, 2007).

A infecção causada por *F. noatunensis* foi descrita em bacalhau do Atlântico (ISACHSEN *et al.*, 2012), robalo (POUDYAL *et al.*, 2020), bagre e carpa (DONG *et al.*, 2016). A bactéria é altamente infecciosa em alevinos, podendo ser letal com apenas 23 unidades formadoras de colônias (UFC) (SOTO; FERNANDEZ; HAWKE, 2009). No Brasil, *F. orientalis* é endêmica e o primeiro surto de franciselose em alevinos e juvenis de tilápias do Nilo (*Oreochromis niloticus*) foi registrado no inverno de 2012 em Minas Gerais (LEAL; TAVARES; FIGUEIREDO, 2014).

Em tilápias, pode se manifestar de forma aguda com sinais clínicos inespecíficos como palidez, perda de escamas, anorexia, apatia, natação errática e melanose (JEFFERY *et al.*, 2010; SEBASTIÃO *et al.*, 2017). Esta enfermidade também se caracteriza por esplenomegalia, renomegalia e pela presença de granulomas multifocais em órgãos internos como baço, rins e fígado (FERNANDEZ-

ALARCON *et al.*, 2019; SOTO *et al.*, 2009). Estes granulomas se caracterizam pela presença de material necrótico, envolto por células epitelioides e, mais na periferia, uma zona de células vacuolizadas, pequenos vasos sanguíneos e linfócitos (BIRKBECK; FEIST; VERNER - JEFFREYS, 2011). Além da alta mortalidade em alevinos e juvenis, a infecção crônica em adultos leva a presença de melanomacrófagos no tecido muscular e condenação de carcaça (LEAL; QUEIRÓZ; FIGUEIREDO, 2018).

O tratamento é difícil por se tratar de uma bactéria intracelular facultativa e pela inapetência causada em peixes severamente infectados (COLQUHOUN; DUODU, 2011). Porém, bons resultados foram alcançados com o uso de oxitetraciclina via ração em dosagens de 100 mg.kg.peixe^{-1} e 200 mg.kg.peixe^{-1} foram consideradas seguras, quando usadas em momentos estratégicos do curso da doença (FAVERO *et al.*, 2021). Florfenicol, outro antimicrobiano licenciado no Brasil para a aquicultura, reduziu a mortalidade causada por *F. orientalis*, quando administrado em doses de 15 mg.kg.peixe^{-1} e 20 mg.kg.peixe^{-1} durante 10 dias, sem causar efeitos adversos (SOTO *et al.*, 2013).

Algumas vacinas experimentais apresentaram resultados promissores contra franciselose. Tilápias inoculadas com uma bacterina com adjuvante, administrada por via intraperitoneal (IP), revelaram boa taxa de sobrevivência quando desafiada com uma cepa homóloga, porém maior mortalidade quando desafiada com cepa heteróloga. Tal diferença pode ser correlacionada a produção de anticorpos específicos (SHAHIN *et al.*, 2019). Outro experimento, também com uma bacterina com adjuvante, nesse caso administrada por banho de imersão, mostrou-se promissor. Ainda que as taxas de sobrevivência não tenham sido muito expressivas, de 50 a 63%, certamente oferece mais uma alternativa a ser explorada (OLIVEIRA *et al.*, 2022).

A empresa Vaxxinova, disponibiliza uma vacina autógena na forma de uma bacterina com adjuvante oleoso, para ser administrada por via IP em dose única, a linha Govaxx®. A produção dessas vacinas leva em torno de 3 meses e por utilizar cepas oriundas das próprias fazendas, possui alta especificidade antigênica, diminuindo assim, a ocorrência de falhas vacinais. Segundo a empresa, a eficácia da vacina autógena pode chegar a 80% (VAXXINOVA, 2021).

2.2.3 Estreptococose

Streptococcus agalactiae, também conhecido como *Streptococococus* do grupo B (GBS), é responsável pela estreptococose, doença que acomete uma ampla gama de espécies, inclusive humanos (ZHANG, 2021). GBS fazem parte da microbiota do trato gastrointestinal e urinário de humanos, porém já foi descrito como patógeno oportunista em recém-nascidos e em pessoas com alguma comorbidade (ARMISTEAD *et al.*, 2019; LYHS *et al.*, 2016). A estreptococose é responsável por grandes perdas econômicas e, em 2011, trouxe prejuízos de US$ 40 milhões para tilapicultura na China, devido à alta morbidade e mortalidade, que pode chegar a até 80% em surtos (CHEN *et al.*, 2012).

Os GBS são cocos Gram positivos, hemolíticos ou não, catalase e oxidase negativos, anaeróbios facultativos, com capacidade de fermentar ácido lático e, baseado na presença e no tipo dos antígenos de superfície, pertencentes ao grupo B de Lancefield (ARMISTEAD *et al.*, 2019). Os antígenos polissacarídeos capsulares permitem a classificação em diferentes sorotipos, os já descritos são: Ia, Ib, II, III, IV, V, VI, VII, VIII e IX (SLOTVED *et al.*, 2007). Desses, os sorotipos Ia, Ib, II e III são os mais prevalentes na tilapicultura. No Brasil, o sorotipo Ib é o mais prevalente, porém cepas do sorotipo Ia já foram implicadas em surtos (GODOY *et al.*, 2013). Em 2016, um isolado de *S. agalactiae* sorotipo III com amplo perfil de resistência a antimicrobianos foi identificado pela primeira vez no país (CHIDEROLI *et al.*, 2017).

GBS apresentam uma vasta gama de fatores de virulência que contribuem para a infecção e a gravidade das lesões nos hospedeiros. Esses fatores são toxinas formadoras de poros, fatores para evasão imune, peptídeos de resistência antimicrobiana (AMPs), fatores de aderência e invasão à célula hospedeira, dentre outros. Assim temos, β-hemolisina/citolisina (β-H/C), fator Christie-Atkins-Munch-Peterson (CAMP), proteína de ligação ao fibrinogênio (FbsA/B/C), proteína de ligação à laminina (Lmb), adesina bacteriana imunogênica (Bib A), pili (Pil A/B/C), proteínas de repetição ricas em serina (srr), αC proteína, C5α peptidase (ScpB), superóxido dismutase (SodA), D-alanilação de ácidos lipoteicoicos, proteína de ligação à penicilina (PBPs), dentre outros (ARMISTEAD *et al.*, 2019; RAJAGOPAL, 2009). Elucidar o modo de ação desses fatores de virulência pode auxiliar no desenvolvimento de novas estratégias terapêuticas.

Os sinais clínicos mais comuns da estreptococose são: natação

errática, escoliose, exoftalmia uni ou bilateral, opacidade de córnea, anorexia, distensão abdominal, ascite, melanose, pontos hemorrágicos no tegumento, especialmente em base de nadadeiras, hepatomegalia, nefrite e meningite (EVANS *et al.*, 2002; PRADEEP *et al.*, 2016). A histopatologia revela necrose generalizada e inflamação granulomatosa de vários órgãos, como rim cranial e caudal (GOMES; AFONSO; GARTNER, 2006).

Estresse é o fator predisponente determinante para o surgimento da doença. Outros estressores relacionados aos surtos de estreptococose incluem alta densidade de estocagem, extremos de temperatura, baixo oxigênio dissolvido, alta salinidade ou alcalinidade, altas taxas de alimentação, e rotinas de manejo (AMAL *et al.*, 2015; IREGUI *et al.*, 2016).

A transmissão horizontal ocorre por lesões na epiderme, predação de animais doentes e, em menor grau, via fecal-oral, porém a transmissão vertical também já foi demonstrada em tilápia híbrida (*Oreochromis* spp.) (PRADEEP *et al.*, 2016).

As principais medidas preconizadas para a prevenção da doença incluem vacinação, boas práticas de manejo, uso de probióticos, e para controle, o uso de antimicrobianos e eliminação de animais doentes (MAULU *et al.*, 2021).

A utilização de antimicrobianos é uma prática recorrente não somente como forma de tratamento, mas também profilaticamente. Porém, o abuso desses fármacos para controlar estreptococose pode levar ao surgimento de cepas multirresistentes (ZHANG, 2021).

As vacinas disponíveis no mercado brasileiro são: vacina inativada contra *S. agalactiae*, sorotipo Ib (Aquavac® Strep SA, MSD); vacina inativada contra *S. agalactiae*, sorotipos Ia e III (Aquavac® Strep SA1, MSD); vacina bivalente inativada contra *S. agalactiae* (sorotipo Ib) e *S. iniae* (Aquavac® Strep SA-SI, MSD); vacina tetravalente contra *S. agalactiae* (Aquavac®Strep 4, MSD); vacina inativada contra *S. agalactiae*, sorotipo Ib (ALPHA JET ® micro 1 TiLa, Zoetis). Há ainda a popularização das vacinas autógenas, desenvolvidas a partir de cepas isoladas das próprias fazendas de criação de peixes, desenvolvendo assim produtos específicos.

Embora a vacinação seja um excelente método profilático, o seu efeito pode ser potencializado com a modulação da microbiota intestinal. Probióticos já descritos com ação imunomoduladora incluem *Bacillus licheniformis* e *Lactobacillus rhamnosus*. *Bacillus licheniformis* promoveu ganho zootécnico, melhoras na

imunidade e na resistência ao GBS (ABARIKE *et al*., 2018). Bem como, a suplementação com *Lactobacillus rhamnosus* em tilápias trouxe melhores índices zootécnicos, estimulou a microbiota intestinal, a resposta imune e a resistência à GBS (XIA *et al*., 2018).

2.3 USO DE SUPLEMENTOS ALIMENTARES FUNCIONAIS NA AQUICULTURA

A aquicultura foi o subsetor de produção de alimentos que mais cresceu nas últimas três décadas (FAO, 2022). Porém, a intensificação na produção impõe altos índices de estresse aos animais e, consequentemente, leva a surtos de bacterioses. A principal medida empregada para controlar esses surtos, são os antimicrobianos, que podem afetar negativamente o ambiente e a saúde humana, além de ocasionar o surgimento de cepas resistentes. Em resposta, a Europa e Estados Unidos tem imposto restrições ao uso desses medicamentos (CARBONE; FAGGIO, 2016). No Brasil, apenas dois produtos à base de oxitetraciclina e florfenicol são licenciados pelo Ministério da Agricultura, Pecuária e Abastecimento para uso na piscicultura (SINDAM, 2021). Essa situação tem suscitado o interesse em estratégias alternativas para o controle de doenças.

A aplicação de suplementos alimentares funcionais, principalmente imunomoduladores como, ácidos orgânicos, probióticos, prebióticos e simbióticos na aquicultura é considerada uma alternativa viável e segura, com capacidade de melhorar o desempenho de crescimento, a imunidade, a resistência a doenças, a sobrevivência contra patógenos e a qualidade da água (DAWOOD *et al.*, 2020; HE *et al.*, 2017; NIMRAT *et al.*, 2012; XU *et al.*, 2021; YAMASHITA *et al.*, 2017).

Os ácidos orgânicos são compostos que contêm em sua estrutura o grupamento carboxila. Os compostos relacionados à atividade antimicrobiana são os ácidos de cadeia curta (C1-C7) e monocarboxílicos simples, como os ácidos fórmico, acético, propiônico e butírico, ou ainda os ácidos carboxílicos com um grupo hidroxila (geralmente no carbono alfa), como os ácidos láctico, málico, tartárico e cítrico. Os ácidos fórmico e sórbico também possuem atividade antifúngica (RICKE, 2003). Cada ácido tem seu próprio espectro de atividade antimicrobiana, o que torna interessante o uso de misturas de ácidos orgânicos na alimentação animal, pela maior abrangência do espectro antimicrobiano e pelo possível efeito sinérgico como promotor de crescimento (DIBNER; BUTTIN, 2002).

Os ácidos orgânicos podem estimular a secreção de enzimas pancreáticas, diminuir o pH gástrico, atuar como fonte de energia, melhorar a utilização de minerais e aumentar a digestibilidade dos nutrientes, o que pode levar a um melhor desempenho de crescimento dos peixes (SARDAR; SHAMNA; SAHU, 2020). Ainda, inibem o crescimento de bactérias Gram negativas, penetrando na parede celular e liberando prótons no citoplasma, bem como a alteração das populações da microbiota intestinal em várias espécies de animais aquáticos (DAWOOD; KOSHIO; ESTEBAN, 2018).

Probióticos são micro-organismos vivos que, quando administrados em quantidades adequadas, conferem benefício à saúde do hospedeiro (FAO, 2016).

O modo de ação não está completamente elucidado, mas alguns mecanismos já foram sugeridos. Podem atuar por exclusão competitiva, disputando sítios de adesão e nutrientes com patógenos, o que justificaria a necessidade da ingestão continuada para alcançar os efeitos esperados (CHABRILLON *et al.*, 2005). Também secretam substâncias inibitórias com efeito antimicrobiano como bacteriocinas, sideróforos, lisozima, peróxido de hidrogênio, proteases, ácidos graxos voláteis e ácidos orgânicos (YAN; BOYD; GRANT BURGESS, 2002). Há uma melhor utilização da ração pelo aumento da palatabilidade, o que estimula o apetite e consequentemente, o ganho de peso (AL-DOHAIL; HASHIM; ALIYU-PAIKO, 2009; SÁENZ DE RODRIGÁÑEZ *et al.*, 2009). Ainda, os animais apresentam maior resistência ao estresse ambiental durante o manejo (FIROUZBAKHSH *et al.*, 2011; MUJEEB RAHIMAN *et al.*, 2010). Já foi demonstrado que probióticos podem alterar a morfologia intestinal aumentando a superfície de absorção, além de excretar diversos metabólitos capazes de suprimir a inflamação e alterar o pH, modificando a fisiologia e o metabolismo de nutrientes, moldando o microbioma intestinal (OPIYO *et al.*, 2019; PIRARAT *et al.*, 2011).

Certamente o principal modo de ação diz respeito à modulação do sistema imune. Cepas probióticas, como qualquer outro micro-organismo, possuem estruturas específicas e altamente conservadas chamadas de padrões moleculares associados à micro-organismos (MAMPs), os quais no hospedeiro são detectados pelos receptores de reconhecimento padrão (PRRs). Esses MAMPs são peptideoglicanos, ácido lipoteicoico, lipopolisacarídeos, exopolissacarídeos, ácido nucleico e flagelina. Com a ligação MAMP-PRR, uma cascata de sinalização é desencadeada estimulando fagocitose, aumento na atividade de explosão

respiratória, aumento na atividade de anti-protease e peroxidase, produção de lisozima, incremento na atividade do sistema complemento e produção de citocinas e quimiocinas, aumentando assim a resposta imune do hospedeiro (RINGØ *et al.*, 2010). A suplementação com *Lactiplantibacillus plantarum* à dieta de tilápias levou ao aumento da expressão gênica das citocinas, IL-10, IL7F, IL-1β e IL-8, o que foi relacionado à maior sobrevivência contra *Enterococcus faecalis* (FOYSAL et al., 2020). A suplementação da levedura *Y. lipolytica* aumentou a expressão gênica das citocinas pró-inflamatórias IL-1β, IL-6, IL-8, IL-12, TNF-α e fator de transcrição NF-κβ no intestino após 2 e 4 semanas (REYES-BECERRIL; ALAMILLO; ANGULO, 2021).

Para que sejam consideradas como cepas probióticas, os micro-organismos devem ser inócuos, manter-se viáveis por longo tempo durante a estocagem e transporte, tolerar o baixo pH do suco gástrico, resistir à ação da bile e das secreções pancreática e intestinal e não transportar genes transmissores de resistência a antibióticos (COPPOLA; TURNES, 2004).

Os aditivos imunomoduladores podem ser fornecidos diretamente na água ou via alimentação (NIMRAT *et al.*, 2012; XU *et al.*, 2021). Dentre os probióticos já descritos na aquicultura temos microalgas (*Nannochloropsis* e *Thalassiosira*) (HUANG *et al.*, 2022), leveduras (*Saccharomyces, Yarrowia*) (ADEL *et al.*, 2017; LICONA-JAIN *et al.*, 2020); bactérias gram positivas (*Bacillus, Lactococcus, Micrococcus, Carnobacterium, Weissella*) (ABARIKE *et al.*, 2018; ABD EL-RHMAN; KHATTAB; SHALABY, 2009; MOURIÑO *et al.*, 2012; REDA *et al.*, 2018; ROBERTSON *et al.*, 2000) e bactérias gram negativas (*Alteromonas* spp., *Pseudomonas* spp. *e Vibrio* spp.) (ABD EL-RHMAN; KHATTAB; SHALABY, 2009; KESARCODI-WATSON *et al.*, 2012; MOONSAMY *et al.*, 2020). Já foram descritas leveduras probióticas com características antifúngicas, antibacterianas, antitumorais e anti-inflamatórias (ŞANLIDERE ALOĞLU; DEMIR ÖZER; ÖNER, 2016).

Prebióticos são substratos não digeríveis, benéficos ao hospedeiro ao estimular seletivamente a proliferação ou atividade de populações microbianas desejáveis no intestino e, assim, melhoraram a performance de crescimento, a atividade de enzimas digestivas, a resposta imune e a resistência ao estresse (CHEN *et al.*, 2019; HOSEINIFAR *et al.*, 2015).

Alguns prebióticos mais comumente utilizados são provenientes de parede celular de leveduras como β-glucano, mananooligossacarídeo (MOS) (HISANO *et al.*, 2018; HOSEINIFAR *et al.*, 2015). Já os frutooligossacarídeos (FOS)

provém da fermentação microbiana da sacarose e a inulina que é encontrada em uma grande variedade de frutas, vegetais e cereais (CARBONE; FAGGIO, 2016; PASSOS; PARK, 2003). Outros foram descritos na aquicultura como: oligossacarídeos de arabinoxilano (AXOS), galactooligossacarídeo (GOS), e oligossacarídeos (DEVI *et al.*, 2019a; GERAYLOU *et al.*, 2012; HAHOR; THONGPRAJUKAEW; SUANYUK, 2019).

Finalmente, os simbióticos são produtos da combinação de ambos, nos quais os prebióticos fornecem o substrato, como vitaminas, aminoácidos e peptídeos para o bom desempenho de probióticos, que por sua vez, utilizam os produtos do metabolismo bacteriano como fonte de carbono (CZECH *et al.*, 2018a; MOHAMMADIAN *et al.*, 2019). Por exemplo, a inulina que ao ser fermentada por probióticos dos grupos Bifidobacteria, Bactérias ácido láticas e Clostrídio, ainda produz FOS como um dos substratos intermediários (CARBONE; FAGGIO, 2016). A adição de um simbiótico na alimentação de Carpa rohu (*Labeo rohita)* promoveu melhora na atividade antioxidante, na resposta imune inata e adaptativa, modulou a expressão gênica de citocinas e diminuiu a mortalidade por *Aeromonas hydrophila* (DEVI *et al.*, 2019b).

As bactérias ácido láticas (BAL) são um grupo vastamente estudado como um aditivo para estabelecer um manejo sustentável na aquicultura. E recentemente, a busca por cepas oriundas da microbiota dos peixes com potencial probiótico tem se tornado mais comum, e assim, aumentando o leque de cepas probióticas disponíveis e a segurança de empregá-las (KAKTCHAM *et al.*, 2018; KAVITHA; RAJA; PERUMAL, 2018; KUEBUTORNYE *et al.*, 2020a; MEDINA *et al.*, 2020; REDA *et al.*, 2018).

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3 HIPÓTESES

i. O fornecimento de ácidos orgânicos para tilápias estimula o sistema imune, modula a microbiota intestinal, melhora parâmetros zootécnicos e reduz a mortalidade após desafio com *F. orientalis*,

ii. A administração de probióticos para tilápias estimula o sistema imune, incrementa parâmetros zootécnicos e diminui a mortalidade após desafio com *S. agalactiae*.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Avaliar se o fornecimento de ácidos orgânicos para tilápias traz benefícios perante o desafio experimental com *Francisella orientalis* e avaliar a eficácia de um probiótico para tilápias contra o desafio experimental com *Streptococcus agalactiae.*

5.2 OBJETIVOS ESPECÍFICOS

i. Definir a melhor concentração de ácidos orgânicos como aditivo alimentar para que se obtenha melhores resultados no desempenho zootécnico de tilápias do Nilo;
ii. Caracterizar a microbiota intestinal de tilápias pré e pós suplementação com ácidos orgânicos;
iii. Avaliar a eficiência de ácidos orgânicos como imunomoduladores e seu efeito protetor frente ao desafio com *Francisella orientalis*;
iv. Avaliar potencial probiótico de *Enterococcus faecium, Lactococcus lactis* e *Yarrowia lipolytica*;
v. Avaliar a eficácia de distintas formulações de cepas probióticas como imunomoduladores para tilápias e no desempenho zootécnico;
vi. Avaliar o efeito protetor de distintas formulações de cepas probióticas frente ao desafio com *Streptococcus agalactiae*.

6 ARTIGO A – Effect of an organic acid blend in Nile tilapia growth performance, immunity, gut microbiota, and resistance to challenge against francisellosis

Artigo submetido para a revista “Research in Veterinary Science” em 27 de janeiro de 2023

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ABSTRACT

Organic acids (OAs) are a class of feed additives that have prophylactic and inhibitory properties against pathogenic bacteria. In this study, we investigated growth performance, innate immune response, gut microbiota, and disease resistance against *Francisella orientalis* F1 in Nile tilapia (*Oreochromis niloticus*) fed different doses of

Bacti-nil®Aqua, a blend of short- and medium-chain OAs. For 21 days, 680 juvenile tilapias were fed a control diet or diets supplemented with a 0.3% (D3) or 0.5% (D5) OA blend. The feed conversion rate of fish fed the 0.5% enriched diet was considerably lower ($p<0.05$) than that of the fish fed the basal diet. Lysozyme and serum bactericidal activities were significantly elevated following OA administration. After infection, no differences in the diversity and composition of gut microbiota were observed between the groups. After the bacterial challenge, the mortality was significantly lower in group D5 ($p<0.01$). The diet supplemented with Bacti-nil®Aqua improved the immune response and resistance of tilapia juveniles against *F. orientalis* infection. Thus, this OA blend could serve as a feed additive with good activity against franciselosis.

Keywords: organic acid, immune response, functional additive, disease resistance

1 Introduction

Over the years, global aquaculture has made substantial progress, most likely due to the implementation of science-based fisheries and aquaculture management policies (FAO, 2018). Nile tilapia (*Oreochromis niloticus*) is the second-largest freshwater species produced in the world, because of its fast growth rate, tolerance to high stocking density, and in good farming conditions show strong disease resistance (FAO, 2020; NG; ROMANO, 2013). In contrast, tilapias raised in intensive production systems may be more susceptible to diseases caused by bacteria, fungi, and viruses, due to high levels of stress triggered by high stocking density and the resulting poor water quality (Maulu et al., 2021).

Francisella orientalis, *Streptococcus agalactiae, S. iniae,* motile *Aeromonas* and *Flavobacterium oreochromis* have been responsible for major losses in tilapia farming owing to outbreaks in the past few years (Dong et al., 2015; Jeffery et al., 2010; Leal et al., 2014; Lin et al., 2016; Raj et al., 2019).

Francisellosis, an endemic granulomatous bacterial illness with acute to chronic onset caused by *F. orientalis*, has been found in various fish species (Maekawa et al., 2021; Ramirez-Paredes et al., 2020; Soto et al., 2009). However, no commercial vaccines are currently available, and the treatment is primarily based on antibiotics (Shahin et al., 2020).

The indiscriminate use of antimicrobials has become common in intensive aquaculture systems, affecting water and the environment, posing food safety risks to public health owing to antibiotic residues in fish, as well as chronic sub-dose exposure to these medications, which increases the development of antimicrobial resistance (Gastalho et al., 2014).

Considering the negative impact of this practice, the use of functional feed additives, such as probiotics, prebiotics, phytobiotics, and organic acids (OA), has been explored for stimulating the immune response and thus reducing dependence on antibiotics (Chen et al., 2019; Czech et al., 2018; Dibner and Buttin, 2002; Hoseinifar et al., 2016; Mohammadian et al., 2019).

Many feed additives have been introduced into fish diets in order to boost growth and health. In a previous study, rainbow trout (*Oncorhynchus mykiss*) had their serum and skin mucus lysozyme and glutathione peroxidase activities boosted by adding 0.25% malic acid to their diet; however, there were no significant changes in their growth performance, feed intake, weight increase, specific growth rate, or feed conversion ratio (Yousefi et al., 2023). In another study, dietary supplementation with 1% butyric acid mitigated the effects on intestinal damage by improving folds and villus height in giant groupers (*Epinephelus lanceolatus*), although the diet did not improve growth performance (Yong et al., 2020). Another study reported that 1% formic acid supplementation significantly improved white blood cell count, red blood cell count, hemoglobin, hematocrit, platelet count, and biochemical parameters compared to the control group, and tilapias challenged with *Aeromonas veronii* presented a lower mortality rate (20%) (Reda et al., 2022).

Organic acids show inhibitory activity and prophylactic properties, apparently by altering the cytoplasmic pH of pathogenic bacteria, as well as by reducing digesta pH, increasing pancreatic output, and exerting trophic effects on the gastrointestinal mucosa (Dibner and Buttin, 2002; Ng et al., 2015). Bacti-nil®Aqua is a synergistic mixture of medium- and short-chain OAs specifically prepared for aquatic species. Although Bacti-nil®Aqua has been described as a promising alternative for *Vibrio parahaemolyticus* control in shrimp (*Penaeus vannamei*) culture (Morales-Covarrubias et al., 2022), the product has not yet been validated for tilapias and its effect against *F. orientalis* has not been studied.

Therefore, in this study, we aimed to evaluate the effect of supplementing feed with different doses of Bacti-nil®Aqua on tilapia growth, immunity, gut microbiota, and

response to *F. orientalis* challenge post-supplementation.

2 Material and Methods

2.1 Minimum bactericidal concentration (MBC)

Aeromonas hydrophila and *Francisella orientalis* strain F1 were employed to determine the minimum inhibitory concentrations (MIC) of Bacti-nil®Aqua using a broth microdilution method adapted from the procedure reported by the Clinical Laboratory and Standards Institute (CLSI, 2018). *A. hydrophila* was grown in Muller Hinton agar (Kasvi, São José dos Pinhais, PR, Brazil) enriched with 5% of defibrinated sheep blood (MHBA) and incubated at 28 °C for 24 h. *F. orientalis* F1 was grown in Cystine heart agar supplemented with 1% of bovine hemoglobin (CHAH) (Kasvi), and incubated at 28 °C for 72 h. Then, three to five colonies were suspended in phosphate-buffered saline (PBS), the suspensions were adjusted to a 0.5 McFarland standard, and the inoculum was diluted to 10^5 CFU/mL^{-1}. The assay was carried out in 96-well microdilution plates at 28 °C for 24–48 h. Bacti-nil®Aqua was serially diluted in PBS by a factor of two, ranging from 0.375 to 6 g.mL^{-1}. Eugon broth (BD Difco^{TM}) was employed for testing *F. orientalis* F1. MIC was calculated as the lowest concentration of a substance that prevents visible development of a microorganism within a defined period; the experiment was performed in triplicate. However, because the product solution was turbid and interfered with visualization, the samples were streaked on CHAH plates to confirm complete inhibition; therefore, MBC was assessed instead of MIC. *A. hydrophila* was used as a comparative standard because it is also a Gram-negative pathogen but not a facultative intracellular lifestyle.

2.2 Electron microscopy

An inoculum of *F. orientalis* F1 at 4 McFarland's standard was incubated at 28 °C for 120 minutes with the product concentrations of 0.625 g.mL^{-1} (MBC), 3000 ppm, 5000 ppm, and control in PBS. Then, scanning electron microscopy (SEM) was performed in accordance with a method reported by Suphoronski et al. (2019).

2.3 Fish

A local producer in Paraná state, Brazil, provided 680 healthy Nile tilapias (initial weight, 9.31 ± 0.32 g). Twenty fish were sampled and submitted for microbiological diagnosis to confirm health status when aseptic samples from the eyes, brain, head, kidney, and liver were streaked on MHBA and CHAH. The trial began only after the negative culture results were confirmed. The trial was conducted in an indoor recirculating system containing dechlorinated water, with a 12:12 h light:dark photoperiod; water temperature, 26.17 ± 0.8 °C; pH, 6.8–7.6; dissolved oxygen (DO), 3.5–5.5 $mg.L^{-1}$, and total ammonia <0.4 $mg.L^{-1}$. The study was carried out following the Ethical Principles in Animal Research of the National Council for the Control of Animal Experimentation and approved by the State University of Londrina's Ethics Committee on the Use of Animals (approval number CEUA/UEL-14555.2019.64).

2.4 Diet

Following a 2-week acclimatization period, the fish were fed three times a day for 21 days until apparent satiation, resulting in approximately 6 ± 0.3% of biomass daily. The experimental diets were basal (D0) or supplemented with Bacti-nil®Aqua at 3 $g.kg^{-1}$ (D3) and 5 $g.kg^{-1}$ (D5). ADISSEO (Dendermonde, Belgium) provided the diets, and their nutrient composition is shown in Table 1. Bacti-nil®Aqua is a synergistic blend of short- and medium-chain OAs.

2.5 Experimental design and disease challenge

The diets were given to the groups for 21 days before the challenge and for at least 21 days after infection, or until no mortality was observed for three days. *F. orientalis* F1, isolated from an outbreak in Southeast Brazil (Facimoto et al., 2019), was used in all *in vitro* and *in vivo* tests. The fish were fasted for 24 h before the challenge and subsequently submitted to an immersion bath, which was prepared by diluting a suspension of *F. orientalis* F1 in 30 L of tank water. The final challenge dose was 9.5×10^6 CFU/mL^{-1}. The fish were kept in the bath for 3 hours before the water supply was restored. The negative control (NC) and high-dose control (HC) groups were treated similarly but were not exposed to the pathogen. The experimental design is described in detail below:

- NC: negative control group: fish that were fed a basal diet for 21 days and no bacterial challenge; two tanks (total: 110 fish).
- FC: positive control group: fish that were fed a basal diet for 21 days and challenged with *F. orientalis* F1; two tanks (total: 110 fish).
- HC: high-dose control: fish that were fed a diet supplemented with 0.5% Bacti-nil®Aqua for 21 days and no bacterial challenge; two tanks (total: 110 fish).
- D3: fish that were fed a diet supplemented with 0.3% Bacti-nil®Aqua for 21 days and challenged with *F. orientalis* F1; three tanks (total: 165 fish).
- D5: fish that were fed a diet supplemented with 0.5% Bacti-nil®Aqua for 21 days and challenged with *F. orientalis* F1; three tanks (total: 165 fish).

The HC group was employed as a safety assay to verify any intoxication or injury effect of the product's highest dose on fish in the absence of bacterial challenge, allowing for a longer observation period. Following infection, head kidney and spleen fragments from dead fish were grown on CHAH to confirm the presence of the pathogen.

2.6 Growth performance

Fish were individually counted and weighed at baseline (day zero) and before challenge (day 21) to assess growth performance, which was calculated before the bacterial challenge, using the following formulas: weight gain (WG) = final weight (g) − initial weight (g); daily feed intake = (total feed consumed / final number of individuals) / duration of the experiment; specific growth rate (SGR %) = 100 × (ln final weight – ln initial weight) / duration of the experiment; feed conversion ratio (FCR) = feed given / weight gain (van Doan et al., 2017). As weighing is a brief procedure, the animals were not anesthetized.

2.7 Innate immune analysis

For innate immune analysis, during the pre-challenge period, the groups were combined according to the type of feed administered into groups fed a basal diet (NC and FC), a diet supplemented with 0.3% (D3), and a diet supplemented with 0.5% (D5

and HC).

The groups were sampled twice, first at the end of the feeding trial (day 21) and subsequently 20 days after challenge with *F. orientalis* F1. After being anesthetized by immersion in 100 mg.L^{-1} benzocaine, six fish were sampled from each treatment group and the control group. Blood samples were collected from the caudal vein without anticoagulant, allowed to coagulate at 4 °C for 4 h, and centrifuged at 1400 × *g* for 10 min to obtain serum, which was stored at −80 °C until analysis.

The concentration of lysozyme in the blood was determined using a method adapted from a previous study (Ellis, 1990). Initially, a calibration curve was established using chicken egg lysozyme L6876 (Sigma, St. Louis, MO, USA) standard solutions. Then, a suspension of *Micrococcus lysodeikticus* M3770 (200 µL) (Sigma) and the test serum (150 µL) were placed into a tube. The serum lysozyme activity was determined by measuring the initial and final absorbances (at 492nm) after the lysis of *Micrococcus lysodeikticus*. The lysozyme standard curve linear regression equation was used to calculate serum lysozyme levels.

Alternative complement pathway activity (ACH50) was determined following a previously described method with modifications (Kumari and Sahoo, 2005). Briefly, the serum samples were serially diluted (1:5) four times, mixed with rabbit erythrocyte suspensions, and incubated at 22 °C for 2 h in an orbital shaker. Then, this mixture was centrifuged at 5000 rpm for 3 min. The optical density of the supernatant (at 450 nm) was used to evaluate the extent of hemolysis. Samples with hemolysis greater than 90% or less than 15% were excluded from the final calculation, and samples with 50% erythrocytes lysis were represented as % hem.mL^{-1}.

Serum bactericidal activity was measured in a flat-bottom 96-well microplate, an adaptation of a previously described method (Silva et al., 2009). Fish serum was tested for antimicrobial activity against *Aeromonas hydrophila*. *A. hydrophila* suspension was prepared at 0.5 McFarland standard and diluted 100,000 times in Poor broth (PB). Following that, eight serial dilutions of serum in PB medium were performed at a 1:2 ratio. As a negative control, a saline solution diluted in PB was used. Twenty microliters of the final *Aeromonas* inoculum were added to each well containing the serum and positive control. The microplates were incubated at 28 °C for 24 h. The reciprocal of the last dilution that showed bactericidal activity was used to calculate serum bactericidal activity.

2.8 Intestine histology and morphology

After 21 days of feeding trial and 20 days after bacterial challenge, the animals were euthanized by benzocaine overdose at 250 mg/mL, and the foregut was collected for histopathological analysis. Initially, the material was fixed in 10% buffered formalin for 48 hours and stored in 70% ethanol. Subsequently, the material was serially dehydrated in ethanol and embedded in paraffin wax, then cut into sections of 5 µm, and stained with hematoxylin-eosin (HE) for histopathological evaluation (Souza et al., 2020b). Histomorphological analysis was performed for the following samples: NC (n=12), FC (n=12), HC (n=12), D3 (n=18), and D5 (n=18).

A semi-quantitative score was applied according to a modification of a previously reported method (Souza et al., 2020b) as well as considering the findings of Pirarat et al. (2011) and Wolf et al. (2015). Changes in intestinal histomorphology and the presence of inflammation were scored from 1 to 3 according to severity, and the parameters considered are listed in Table 5.

2.9 Microbiome analysis

Groups NC, FC, and D5 were sampled at the end of the feeding trial to determine the impact of product supplementation on the bacterial microbiome, and 20 days after bacterial challenge to analyze the gut microbiome following infection to study the effects of Bacti-nil®Aqua on tilapia. At the first sampling, the NC and FC groups were merged into a single group based on the feeding regime and identified as Basal diet. Stool and adherent intestinal microbiota samples from the control (NC and FC) and treated groups (D5) were analyzed separately.

Two parallel analyses were performed, one for the stool microbiome and the other for the adherent intestinal microbiota. The intestinal loops were aseptically removed from the abdominal cavity and placed in a sterile Petri dish. Next, the feces were expelled and stored in sterile microtubes, and the intestinal loops were stored in 15 mL conical centrifuge tubes. The QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to extract DNA according to the manufacturer's instructions. The samples were grouped into sequencing libraries, and the amplicons were sequenced using the paired-end method on an Illumina MiSeq platform with a 250-cycle V3 MiSeq Reagent Kit (Souza et al., 2020a).

The following stages were carried out using the MOTHUR v.1.36.1 program, as reported by Suphoronski et al. (2019). For taxonomic comparison, sequences were classified into operational taxonomic units (OTUs). A subsample of 3711 sequences per sample was chosen to reduce bias, and the Chao index was used to estimate richness and the Shannon and Simpson indices to estimate diversity, and their mean values were subjected to t-test, with a significance level of 5%.

2.10 Statistical analyses

All statistical analyses were performed using R software. Data were subjected to the Bartlett test and Shapiro–Wilk test to verify the homogeneity of variances and normality of residuals. Growth performance and immune system results were analyzed using ANOVA, followed by Tukey's test, with a significance level of 5% ($p<0.05$). Data that did not meet the homogeneity and normality criteria were analyzed using the non-parametric Kruskal–Wallis test, followed by Dunn's test with a significance level of 5%. Data were presented as the mean ± standard error. The survival rate was analyzed using Fisher's exact test with a significance level of 5%.

3 Results

3.1 Minimum bactericidal concentration (MBC)

Bacti-nil®Aqua at 0.625 $\mu g.mL^{-1}$ or higher inactivated *F. orientalis* F1 cells (Table 2).

3.2 Electron microscopy

Scanning electron microscopy (SEM) analysis revealed a decrease in the number of *F. orientalis* F1 cells after 120 min of exposure to different concentrations of the product, indicating that the product, by coating the cells, led to a loss in their integrity (Figure 1). The greatest reduction occurred with MBC (0.625 $\mu g.mL^{-1}$), and the effects of 3000 ppm and 5000 ppm Bacti-nil®Aqua were not considerably different.

3.3 Disease challenge

Mortality started two days post-infection in the FC group and three days post-infection in the treated groups (D3 and D5). Fish in the D3 and FC groups did not show any differences in mortality and presented the lowest survival rates at the end of the trial, 26.90%, and 23.96%, respectively. The D5 group showed a significantly higher survival rate (43.45%, $p<0.01$) than the FC and D3 groups (Figure 2). *F. orientalis* was isolated from all the dead fish, and the strains were recovered and verified by identification tests (colony morphology and Gram staining).

3.4 Growth performance

Growth performance was assessed after 21 days of the feeding trial, and the groups were organized according to the type of feed: basal diet (NC and FC), diet supplemented with 0.3% (D3), and diet supplemented with 0.5% (D5 and HC). The results are presented in Table 3.

3.5 Innate immune analysis

Serum lysozyme, alternative complement, and serum bactericidal activities are shown in Table 4. Before the *F. orientalis* challenge, serum lysozyme activity in D5/HC group was significantly higher ($p<0.05$) than that in the non-treated NC/FC group. After the challenge, the diet supplemented at D5 was significantly higher than that in the basal diet ($p<0.05$). Before infection, both supplemented diets, D5/HC and D3, resulted in significantly higher serum bactericidal activity ($p<0.05$) than the basal diets. After the challenge, D3 and D5 had higher serum bactericidal activities ($p<0.05$) than the basal diets. However, no significant difference ($p>0.05$) was observed between the serum ACH50 levels in the treated fish and the control fish, before or after infection (Table 4).

3.6 Intestine histology and morphology

After 21 days of treatment with the product, the histomorphological analysis did not reveal any pronounced differences between the pre- and post-challenge groups (Table 6). In FC, D3, and D5, mucosal villi (MV) were of normal length and shape; however, autolysis was observed at the top of the intestinal villi, which prevented the

complete evaluation of MV length in the other groups. The width of the lamina propria (LP), abundance of goblet cells (GC), and presence of apoptotic enterocytes maintained basal values post-challenge in the D5 group (Figure 3). The NC group had a higher SNV score (strong reduction). The SNV in the treated groups (D3, D5, and HC) and FC demonstrated size reduction, and they were less regularly aligned. Some lymphocytes were present pre- and post-challenge in the treated groups but not in the NC group.

3.7 Microbiome analysis

Metagenomic analysis generated 895,168 sequences, of which 702,858 were obtained after quality control. A subsample of 3,711 reads per sample was used to normalize the data, following which a rarefaction curve was designed (Supplementary Figures 1 and 2). Subsampling yielded coverage higher than 99%, implying that it was representative of the total population. A total of 489 operational taxonomic units (OTUs) were retrieved from all samples and taxonomically grouped into 27 phyla, 66 classes, 122 orders, 226 families, and 489 genera.

In stool samples (Table 7), no significant differences were found in Chao richness and Shannon and Simpson diversity indices between the treatment groups ($p>0.05$). Although no significant difference was observed in microbiome diversity, the Simpson index was higher in the D5 group (0.77 and 0.72) than in the NC group (0.66 and 0.54) in stool samples and adherent intestinal microbiota samples, respectively. In stool samples, before infection, the NC and D5 groups demonstrated a low level of species diversity by the Chao index; however, after infection, greater diversity was observed among groups, especially the D5 group.

After the bacterial challenge, in adherent intestinal microbiota samples, the Shannon index for FC (1.70) was significantly different ($p<0.05$) from NC (0.71) (Table 8).

The relative averages of the most abundant taxonomic groups were *Fusobacteria* (73.75%), *Proteobacteria* (12.85%), and *Firmicutes* (8.25%) at the phylum level; *Fusobacteria* (74.99%), *Gammaproteobacteria* (9.36%), *Bacilli* (4.56%), and *Clostridia* (3.11%), at the class level; *Fusobacteriaceae* (74.98%), *Enterobacteriaceae* (6.58%), and *Bacillaceae* (3.59%), at the family level; and *Cetobacterium* (74.89%), *Plesiomonas* (5.15%), *Romboutsia* (3.09%), and *Bacillus*

(2.28%), at the genus level. Other groups were present at a lower frequency. The mean relative abundances of the main genera in stool and adherent intestinal microbiota are presented in Figures 4 and 5, respectively. Similarly, Supplementary Tables 1 and 2 display the relative frequencies of the most abundant genera in stool and adherent intestinal samples, respectively. After infection, the D5 and FC groups showed a decrease in the *Fusobacteria* phyla and an increase in the *Proteobacteria* and *Firmicutes* phyla in stool samples (Supplementary Figure 3).

In stool samples, the composition of the gut microbiome was dominated by *Cetobacterium*, *Plesiomonas*, and *Romboutsia* in all the groups (Figure 4). In stool samples, before infection, the D5 group showed a reduction in the abundance of *Vibrionaceae* compared with the NC group, as *Cetobacterium* became more expressive. In addition, following *F. orientalis* challenge, the genus *Bacillus* rose in the FC and D5 groups.

In adherent intestinal samples, *Cetobacterium* and *Plesiomonas* were the most abundant genera, whereas *Romboutsia* was not abundant. Before the challenge, the treated group D5 showed decreased *Vibrionaceae* (Figure 5) compared to the NC group. Following the challenge, FC and D5 also displayed increases in *Bacillus* and *Bacillaceae*_1_unclassified.

4 Discussion

In this study, we evaluated the efficacy of a blend of short- and medium-chain OAs and the resistance against francisellosis, an important disease that affects tilapia. Dietary acidifiers benefit aquacultural output by improving feed utilization, growth efficiency, and disease resistance, as well as being described as potential antibiotic replacements in aquafeeds (das Neves et al., 2021; Reda et al., 2016). The MBC of Bacti-nil®Aqua against *A. hydrophila* and *F. orientalis* F1 was 5 $\mu g.mL^{-1}$ and 0.625 $\mu g.mL^{-1}$, respectively. These results were expected since facultative intracellular bacteria often have low value for antimicrobial testing (Lehar et al., 2015). Thus, this assay demonstrated that this blend of organic acids has an antimicrobial effect against pathogenic bacteria in Nile tilapia. Previous research has demonstrated that sodium butyrate has an antimicrobial effect against *A. hydrophila* and *S. agalactiae* (Jesus et al., 2019).

Scanning electron microscopy (SEM) was performed after 120min of exposure

to different concentrations of Bacti-nil®Aqua (0.625 μg.mL^{-1}, 3000 ppm, and 5000 ppm), and a decrease in cell count and a loss in structural integrity were observed. In vitro antibacterial effects of an acidifying mixture of formic acid, propionic acid, and calcium propionate at doses of 1 or 2 g/kg were seen against *A. sobria*, as well as improved growth performance, hemogram parameters, and body chemical composition of *O. niloticus* fingerlings (Reda et al., 2016).

Francisellosis causes important economic loss in tilapia production. As no commercial vaccinations are available, oral antibiotic therapy is the preferred technique for outbreak control. The better survival rate in the D5 group ($p<0.01$) implies that dietary organic acids may help protect tilapia against *F. orientalis* infection. In tilapia, OA/salt and oxytetracycline treatments had comparable effects on preventing *A. sobria* infection (Reda et al., 2016). Rainbow trout (*Oncorhynchus mykiss*) fed prebiotics, including a mixture of β-glucan and OAs, exhibited a significantly lower relative risk of mortality (> 30%) than the control group after experimental infection with *Yersinia ruckeri* (Rømer Villumsen et al., 2020). Nile tilapia fed a 0.5% OA diet (formic acid, lactic acid, malic acid, tartaric acid, and citric acid) were more resistant to *S. agalactiae* than those fed non-supplemented diets, implying that OA diets could potentially reduce the dependence on oxytetracycline for growth promotion and inhibition of pathogenic bacteria (Koh et al., 2016).

Tilapia fed a non-supplemented diet demonstrated increased body weight gain compared with fish fed organic acid-supplemented diets, but these results were not significantly different. However, D5 demonstrated a significantly different SGR compared to D3, as well as a significantly lower FCR compared to the Basal diet ($p<0.05$). In Nile tilapias, Suphoronski et al. (2019) reported better FCR and SGR, as well as better survival rates after bacterial challenge 21 days after supplementation with different combinations of an OA (potassium diformate) and phytogenic compound. There was no significant difference in rainbow trout (*Oncorhynchus mykiss*) after 40 days of a supplemented diet (citric and sorbic acid, thymol, and vanillin); however, SGR and FCR were improved only after long-term exposure (82 days) to OA (Pelusio et al., 2020). In contrast, giant groupers fed a control diet showed significantly higher FCR, SGR, and WG than did fish fed a diet supplemented with 1% butyric acid; therefore, the OA-supplemented diet did not improve growth (Yong et al., 2020). The growth-promoting benefits of organic acids may vary depending on the fish species, type of organic acid utilized, and dosage, but also on the period of supplementation.

Fish lack a highly developed adaptive immune system and, therefore, depend mostly on innate immunity. Lysozyme is a bactericidal enzyme that acts in infections by breaking the bacterial cell wall, and high levels have been detected in infected fish because of the inflammatory response (Fei et al., 2018; Zhou et al., 2011). In the present study, the dietary organic acid-supplemented groups showed significantly higher lysozyme and serum bactericidal activities. Similarly, a sodium butyrate-supplemented diet improved lysozyme activity in Nile tilapia (Dawood et al., 2020). Tilapia fed a diet supplemented with a mixture of short-chain OAs, including formic acid, propionic acid, and calcium propionate (2 g/kg), showed enhanced serum bactericidal, lysozyme, and nitric oxide activities compared to the basal diet, implying that high doses of this blend are required to sustain an improved immunological status (Reda et al., 2016).

The alternative complement pathway consists of plasma proteins from the innate immune system that participates in the primary defense against infection. The assay result represents the volume of serum inducing 50% hemolysis, expressed in ACH50 units.mL^{-1} (Fei et al., 2018). No significant difference ($p>0.05$) was observed either before or after infection. Nile tilapias fed a diet with low molecular weight sodium alginate showed significantly increased serum lysozyme and complement activity compared to tilapias in the control group (van Doan et al., 2016). It is possible to infer that the inclusion of Bacti-nil®Aqua could stimulate the fish immune system, which could support a better survival rate. Acidifiers influence the intestinal tract by lowering the pH in the stomach and small intestine, which increases the action of digestive enzymes as well as nutrient digestibility, and by acidic dissociation in the bacterial cell and the accumulation of free radicals leads to a bactericidal or bacteriostatic effect (das Neves et al., 2021; Huan et al., 2018). In the present study, both dosages of 0.3 and 0.5% of Bactinil reduced the Francisella bacterial count in vitro, as well as stimulated the immune parameters, lysozyme, and bactericidal activity of the serum.

The thin lamina propria and hyperplasia of goblet cells on D5 may indicate that the intestine was preserved following exposure to the pathogen, even if no significant differences were observed in the intestinal histomorphological analysis. The higher SNV score in the NC group suggests extracellular digestion, as SNVs are responsible for pinocytic absorption of macromolecules (Teles et al., 2015). These results support previous research showing that adding organic acids to a meal enhances tilapia's ability to produce mucus due to a greater number of goblet cells and absorption of

nutrients (Abd El-Naby et al., 2019; Addam et al., 2019). The presence of histomorphological intestinal features similar to those present before the challenge showed that TGI was conserved and Bacti-nil®Aqua helped maintain the intestinal balance of tilapia. Furthermore, OAs have protective effects on the intestinal epithelium (Shah et al., 2015), which explains the preservation of intestinal histomorphology. The intestine displayed a mild inflammatory response, suggesting that both dosages may have prevented a more severe enteritis, which would have negatively impacted the tilapia's ability to grow and survive. In addition, a preserved intestinal microbiota indicated that there was no damage and the animals remained stable.

Fusobacteria, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were the most abundant phyla in the gut microbiota before and after the *F. orientalis* F1 challenge suggesting that despite the pathogen challenge, the microbiome was balanced and not drastically altered (Supplementary Figures 3 and 4). This finding is consistent with previous reports on the Nile tilapia gut microbiota (Souza et al., 2020a; Suphoronski et al., 2019) and zebrafish fed a sodium-acetate-supplemented diet (Zhang et al., 2020). Organic acids are more effective in killing gram-negative bacteria (He et al., 2017), as shown in a study with *Vibrio* spp. (da Silva et al., 2013). This inhibitory potential helps to explain the effect of the product in reducing *Vibrionaceae* in stool samples before infection (Figure 4). This reduction possibly enabled an increase in *Cetobacterium* (*Fusobacteria*). *Cetobacterium* is a core genus in tilapia and can produce vitamin B12, which is important for digestion and nutrition processes in fishes (Tsuchiya et al., 2007), and as a result, these fish do not require dietary vitamin B12 incorporation (Sugita et al., 1991). Regarding the genus results of the microbiome analysis, after infection, in the D5 group, *Bacillus* comprised 8.6% and 3.8% of the stool and adherent intestine samples, respectively (Supplementary Tables 1 and 2). *Bacillus* spp. are often used as probiotics and confer increased immune parameters and greater resistance to diseases (Kuebutornye et al., 2020). *Romboutsia* (*Firmicutes*), another genus found in stool samples, but less frequently in adherent intestinal samples, is thought to be less important in terms of delivering vitamins and cofactors because it shows a poor capacity for vitamin and amino acid synthesis (Ramírez et al., 2018).

Although the phylum *Bacteroidetes* was less abundant, it may have predisposed the presence of important fish pathogens, such as *Flavobacterium*. Before the bacterial challenge, *Flavobacterium* was absent in the treated group (Supplementary Tables 1 and 2) but was present in greater amounts in the D5 and FC groups after infection. As

opportunistic infections are more likely to infect fish with francisellosis (Suphoronski et al., 2019), Bacti-nil®Aqua may have been successful in altering the microbiome and preventing the appearance of this pathogen before infection.

Francisella orientalis is a member of the *Gammaproteobacteria* group, however, its relative frequency in FC (0.01%) and D5 (0.05%) (Supplementary Tables 1 and 2) did not contribute to the rise in *Proteobacteria* phylum in stool samples, suggesting that other causes may have been involved. The dominance of *Gammaproteobacteria* has been related to vegetable diets (Desai et al., 2012), and the inclusion of sodium butyrate, a short-chain OA, in the diet of juvenile turbot significantly elevated the relative abundance of *Proteobacteria* and *Actinobacteria* compared to the control diet (Liu et al., 2019). In addition, it has been suggested that a sudden increase in abundance of this phylum is related to an imbalance in gut microbiota, which may represent dysbiosis or inflammatory disorders in dogs and cats (Moon et al., 2018; Shin et al., 2015). In adherent intestinal samples, *Francisella* was also identified in FC and D5, at 0.34% and 0.01%, respectively, which did not alter the abundance of *Proteobacteria*.

Nutrition, immune system improvement, disease resistance, and survival are highly influenced by intestinal microbiota (Burr et al., 2005; Nayak, 2010). Diet is a key component that influences the gut microbiota. Some studies have demonstrated that the addition of organic acids to fish diets can successfully alter intestinal microbiota and, enhance fish growth performance, immunity, and illness resistance (Addam et al., 2019; Ng et al., 2009; Pelusio et al., 2020). Although D5 showed no significant difference ($p>0.05$) in diversity compared to FC and NC, it demonstrated a significantly higher survival rate and better FCR compared to the non-treated groups, which indicates a beneficial effect of OAs supplementation.

5 Conclusion

The utilization of a combination of short and medium organic acids rather than a single molecule may have contributed to the favorable effects of Bacti-nil®Aqua observed in the present study, which had a synergistic effect on tilapia. This study demonstrated that Bacti-nil®Aqua at 5 $g.kg^{-1}$ has antibacterial effects, and dietary supplementation could improve nutrient utilization, immunological parameters, and resistance of Nile tilapia to *F. orientalis* infection. Furthermore, gut microbiota diversity

and composition revealed the maintenance of pre-challenge characteristics among the treated groups, which could be related to the protective effect of the product on the gut microbiome. Bacti-nil®Aqua is a promising feed additive given the need to limit antibiotic misuse and promote sustainable aquaculture.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Vanessa Gomes da Silva: Methodology, Writing - original draft, Writing - review & editing, Data curation, Investigation; Leonardo Mantovani Favero: Investigation, Writing - review & editing; Raffaella Menegheti Mainardi: Investigation, Writing - review & editing; Natália Amoroso Ferrari: Investigation, Writing - review & editing; Arthur Roberto da Costa: Investigation, Writing - review & editing; Roberta Torres Chideroli: Methodology, run static and others Software; Giovana Wingeter Di Santis, Felipe Pinheiro de Souza, Nelson M. Lopera-Barrero, Daniela Dib Gonçalves, Admilton G. de Oliveira: Methodology, Software, Formal analysis; Waldo G. Nuez-Ortin: Conceptualization, Methodology, Supervision,Validation, Resources; Maria Mercè Isern-Subich: Conceptualization, Methodology, Supervision,Validation, Resources; Nelson Mauricio Lopera-Barrero, Admilton G. de Oliveira: Conceptualization, Methodology; Ulisses de Pádua Pereira: Conceptualization, Methodology, Supervision, Validation, Project administration, Resources, Writing -review & editing.

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Table 1: Dietary formulations and composition of the experimental diets and Bacti-nil®Aqua.

Ingredient (%)	**Experimental diets**		
	Basal Diet	**D3**	**D5**
Feather meal	3	3	3
Corn	33.54	33.54	33.54
Offal meal	20	20	20
Soybean meal 45% PB	9.58	9.58	9.58
Wheat bran 16% PB	12	11.7	11.5
Bacti-nil®Aqua blend	0	0.3	0.5
Meat and bone meal 46% PB	10.97	10.97	10.97
Hemoglobin	8	8	8
Salt (NaCl)	0.8	0.8	0.8
Poultry oil	1.36	1.36	1.36
DL-Methionine	0.15	0.15	0.15
Vitamin–mineral premix	0.6	0.6	0.6
Total	100	100	100
Composition of Bacti-nil®Aqua (%):			
Short chain fatty acids (Formic, Propionic and Sorbic acids)			27
Medium chain fatty acids (Caprylic and Capric Acids)			10
Excipient (Silicic acid)			63
Total			100

Formulation and estimated composition of the basal diet: digestible energy 3000 Mj. kg^{-1}; digestible protein 30%; crude protein 36%; fat 7%; crude fiber 3%; ash 9.29%; calcium 2,31%; phosphorus 1.33%; starch 26%; arginine 1.91%; lysine 2.54%; threonine 1.37%; tryptophan 0.35%; methionine 0.65%; vitamin C 700 mg. Abbreviations: D3, 0.3% supplemented diet; D5, 0.5% supplemented diet.

Table 2: Minimum bactericidal concentration (MBC) of Bacti-nil®Aqua against *F. orientalis* (F1) and *A. hydrophila* (REF 11).

$\mu g.mL^{-1}$	6	5	4	3	2.5	2	1.5	1.25	1	0.75	0.625	0.5	0.375
REF11	-	-	+	+	+	+	+	+	+	+	+	+	+
F1	-	-	-	-	-	-	-	-	-	-	-	+	+

MBC was determined as the lowest concentration of Bacti-nil®Aqua that showed no growth on a blood agar plate. (+): visible growth after plating; (−): no visible growth after plating.

Table 3: Growth performance of Nile tilapia fed a control diet and diets supplemented with Bacti-nil®Aqua at different concentrations for 21 days, before *F. orientalis* F1 infection.

Parameters	**Basal Diet (NC+FC)**	**0.5% diet (D5+HC)**	**0.3% diet (D3)**
Initial weight (g)	33.95±1.16	32.41±1.01	32.94±1.03
Final weight (g)	46.51±1.79	44.48±1.28	44.72±1.26
Weight gain (g)	0.60±0.03	0.57±0.04	0.56±0.01
Daily feed intake (g)	0.62±0.05	0.56±0.03	0.57±0.02
Feed Conversion Ratio (FCR)	1.03 ± 0.06^{a}	0.98 ± 0.03^{b}	1.01 ± 0.04^{ab}
Specific Growth Rate (SGR)	1.50 ± 0.04^{ab}	1.51 ± 0.09^{a}	1.46 ± 0.03^{b}

Data in the same row with different letters are significantly different ($P<0.05$) among the treatments. Values are presented as the mean ± standard error.

Abbreviations: NC, negative control group; FC, positive control group; D5, 0.5% supplemented diet; HC, 0.3% supplemented diet control group; D3, 0.3% supplemented diet.

Table 4: Serum lysozyme activity, alternative complement pathway activity (ACH50), and serum bactericidal activity (mean ± standard error) in fish administered different treatments before and after bacterial challenge with *F. orientalis* strain F1. Different letters in the column indicate treatments were significantly different (P<0.05).

	Groups	Lysozyme ($\mu g.mL^{-1}$)	ACH50 (% hem.mL^{-1})	Serum bactericidal activity (log 2 + 1)
Pre-challenge	NC FC	8.27±2.55^{b}	8.39±3.18	2.90±2.91^{b}
	HC D5	10.49±1.93^{a}	8.49±3.17	5.61±1.93^{a}
	D3	9.78±2.50^{ab}	7.26±6.52	5.60±1.46^{a}
Post-challenge	NC	9.70±1.81^{c}	7.62±3.24	4.41±0.77^{b}
	FC	10.50±1.88^{bc}	6.48±1.07	3.06±2.69^{b}
	HC	14.06±4.00^{ab}	5.95±2.52	5.54±1.20^{ab}
	D5	16.19±3.79^{a}	6.17±2.61	5.70±0.84^{a}
	D3	16.35±10.31^{ab}	5.93±2.07	5.59±0.71^{a}

Abbreviations: ACH50, alternative complement pathway activity; NC, negative control group; FC, positive control group; D5, 0.5% supplemented diet; HC, 0.3% supplemented diet control group; D3, 0.3% supplemented diet.

Table 5: Semiquantitative scores used for evaluating histological parameters in the intestine of Nile tilapia

Parameter	Condition	Score
Flattening of mucosal villi	Basal length	1
	Diffused shrinkage and onset of tissue disruption	2
	Diffused or total tissue disruption	3
Width of lamina propria	Normal size, LP with a thin and delicate core of cells	1
	Increased size of LP	2
	Largest LP	3
Presence of supranuclear vacuoles	Basal SNV size, normally aligned	1
	Diffuse size reduction, non-aligned	2
	Onset of extinction or no SNV	3
Abundance of goblet cells	Scattered cells, in a normal amount	1
	Diffused numbers, widely spread out, GC increased	2
	Highly abundant, densely grouped cells	3
Degree of infiltration of eosinophilic granulocytes into LP and SM	Few in the SM, basal, some migration into LP	1
	Diffuse number in SM increased migration into LP	2
	Dense EG in LP and SM	3
Intraepithelial lymphocytes[1]	Absent or rare	1
	Small amount	2
	Large amount	3

Apoptotic enterocytes (McKnight cells)[2]	Absent or rare	1
	Small amount	2
	Large amount	3

Adapted from the score used by Souza et al. (2020a) for Nile tilapia; [1]Pirarat et al., 2011; [2]Wolf et al., 2014).
Abbreviations: LP, lamina propria; SNV, supranuclear vacuoles; GC, goblet cells; SM, submucosa; EG, eosinophilic granulocytes.

Table 6: Histological scores for the intestinal morphology of Nile tilapia fed a control diet and diets supplemented with Bacti-nil®Aqua at different concentrations for 21 days, pre- and post-challenge with *F. orientalis* F1.

	GROUPS									
	NC		FC		HC		D3		D5	
Parameter	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Flattening of mucosal villi	X	X	1	1	2	X	1	2	1	1
Width of lamina propria	1	1	1	1	1	1	1	1	1	1
Presence of supranuclear vacuoles	3	3	2	2	2	3	2	2	2	2
Abundance of goblet cells	1	1	1	1	1	1	1	1	2	1
Degree of infiltration of eosinophilic granulocytes into LP and SM	1	1	1	2	1	2	1	1	1	1
Intraepithelial lymphocytes	1	1	2	2	1	2	2	2	2	2
Apoptotic enterocytes (McKnight cells)	1	1	1	1	1	1	1	1	2	1

X: samples presenting autolysis; Pre: pre-challenge; Post: post-challenge.
Abbreviations: LP, lamina propria; SM, subepithelial mucosa; NC, negative control group; FC, positive control group; D5, 0.5% supplemented diet; HC, 0.3% supplemented diet control group; D3, 0.3% supplemented diet.

Table 7: Analysis of the alpha diversity indices, Chao, Simpson, Sobs, and Shannon indices, of the stool sample microbiome of Nile tilapia fed different feeding treatments before and after bacterial challenge with *F. orientalis* F1 (mean ± standard error).

	Group	**Chao index**	**Simpson index**	**Sobs index**	**Shannon index**
PRE	1NC/FC	42.46±7.34	0.66±0.04	20.58±1.61	0.74±0.05
	1D5	29.74±3.73	0.77±0.04	18.50±1.48	0.55±0.08
POST	2NC	59.96±17.92	0.63±0.11	24.67±6.61	0.87±0.21
	2FC	68.20±17.23	0.51±0.12	47.33±12.66	1.38±0.37
	2D5	90.90±30.25	0.55±0.18	66.80±25.50	1.43±0.61

Different letters indicate treatments were significantly different (P<0.05).
Abbreviations: PRE, pre-challenge; POST, post-challenge; NC, negative control group; FC, positive control group; D5, 0.5% supplemented diet.

Table 8: Analysis of the alpha diversity indices, Chao, Simpson, Sobs, and Shannon indices, of the adherent intestinal microbiota samples from Nile tilapia fed different feeding treatments before and after bacterial challenge with *F. orientalis* F1 (mean ± standard error)

	Group	**Chao index**	**Simpson index**	**Sobs index**	**Shannon index**
PRE	1NC/FC	71.65±14.81	0.54±0.09	48±12.29	1.27±0.31
	1D5	44.08±13.99	0.72±0.09	23.83±3.65	0.68±0.19

	2NC	50.15±9.45	0.70±0.03	29.50±5.00	0.71±0.08[b]
POST	2FC	100.27±19.96	0.43±0.13	72,50±13.97	1.70±0.41[a]
	2D5	68.63±17.95	0.65±0.06	47.33±13.41	0.95±0.18[ab]

Different letters indicate treatments were significantly different (P<0.05).

Figure 1: Scanning electron microscopy of *F. orientalis* F1 (A) control, the bacteria showed normal morphological structure and (B-D) after exposure to Bacti-nil®Aqua at (B) 0.625 µg.mL^{-1} (MBC), (C) 3000 ppm, and (D) 5000 ppm for 120 min. Size bars indicate 5 µm.

Abbreviation: MBC, minimum bactericidal concentration.

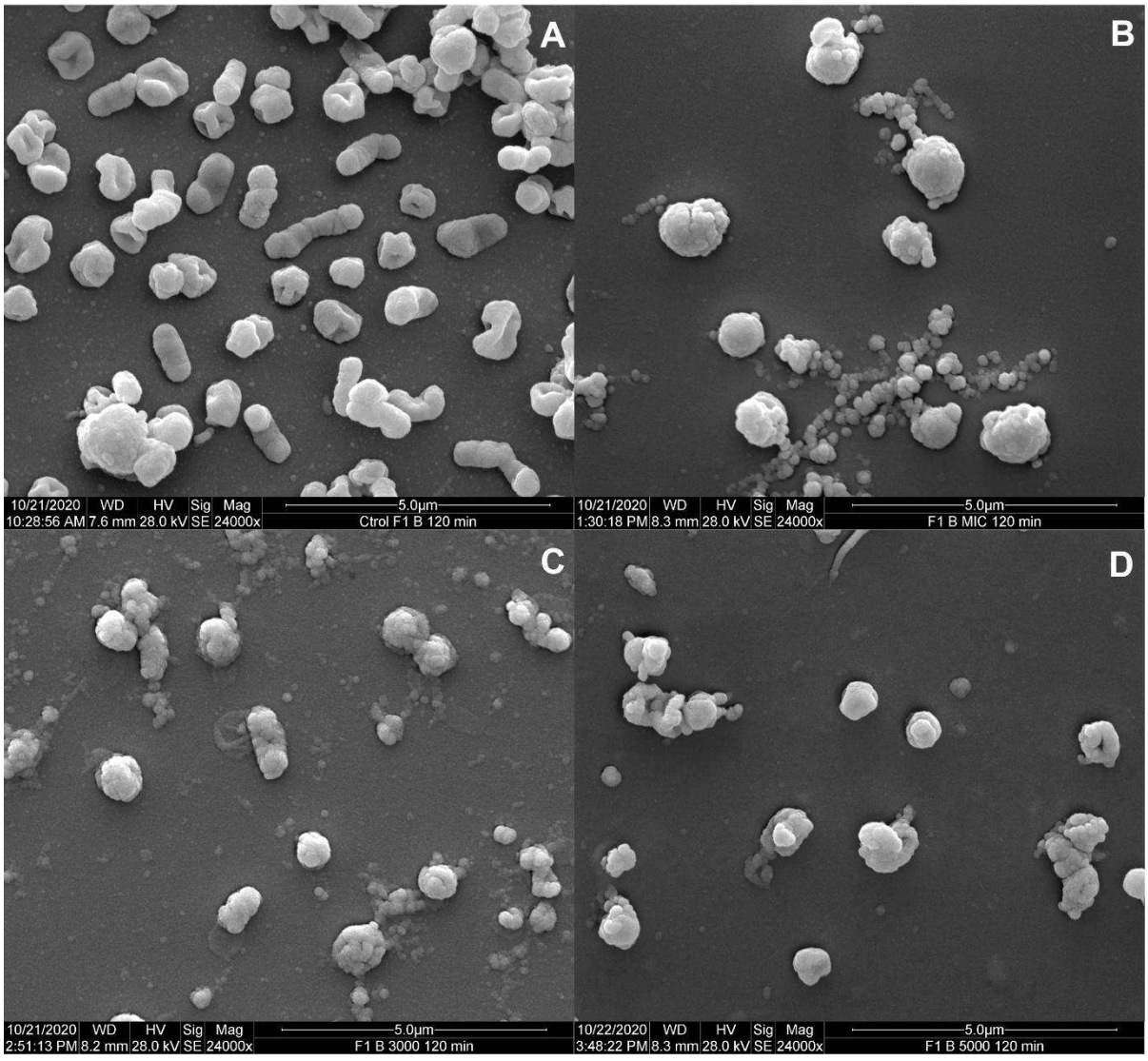
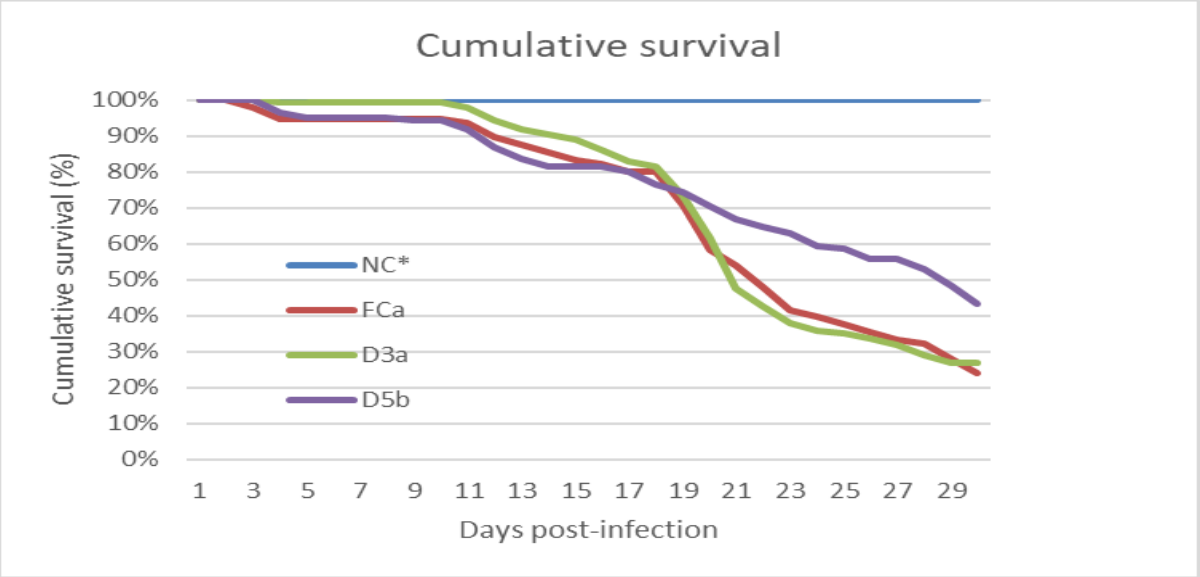


Figure 2: Cumulative survival following an immersion bath challenge with *F. orientalis* F1 in Nile tilapia (*Oreochromis niloticus*) fed basal diets (NC and FC), 0.5% supplemented diet (D5 and HC), or 0.3% supplemented diet (D3) for 21 days. Different letters indicate treatments were significantly different ($p<0.01$).



*NC and HC demonstrated the same survival rate; therefore, HC was omitted for clarification of data. Different letters indicate a significant difference between treatments according to Fisher's exact test ($p<0.01$).

Figure 3: Histomorphology of proximal intestine of tilapias after supplementation with Bacti-nil®Aqua for 21 days and post-infection with *F. orientalis* F1 (a) D5 group: the presence of GC in normal amount (∗), thin and delicate LP (HE, 40×); (b) D3 group: the presence of EG in LP (HE, 40×); (c) D3 group: flattening of MF and widening of LP (HE, 20×).
Abbreviations: MV, mucosal villi; GC, goblet cells; LP, lamina propria; EG, eosinophilic granulocytes; D5, 0.5% supplemented diet; D3, 0.3% supplemented diet; HE, hematoxylin-eosin.

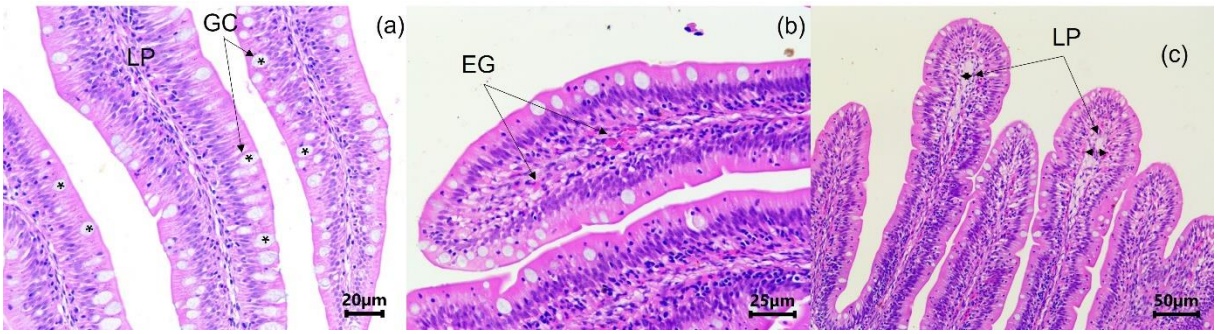
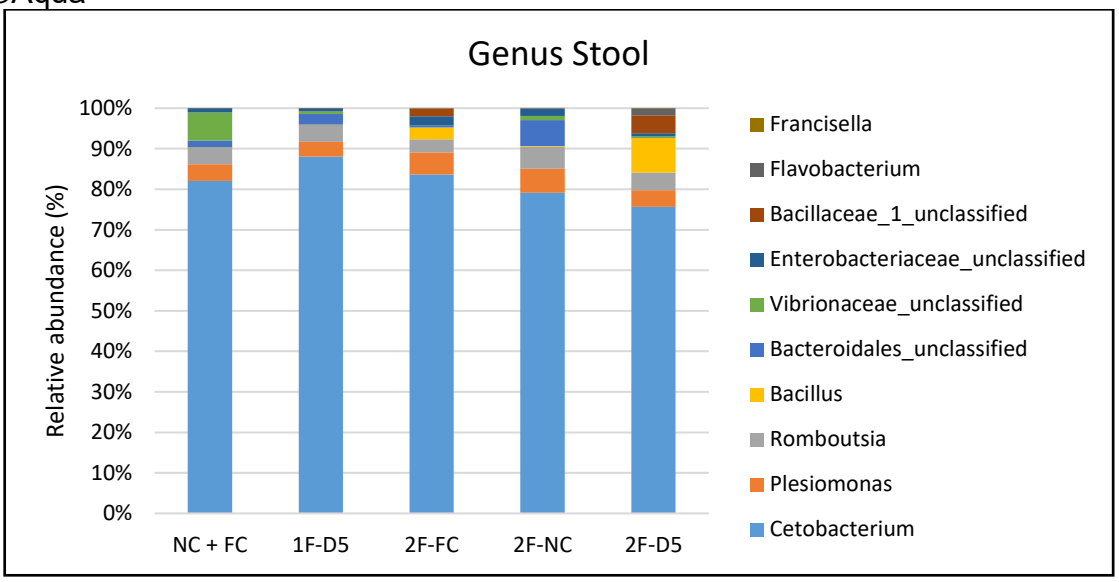
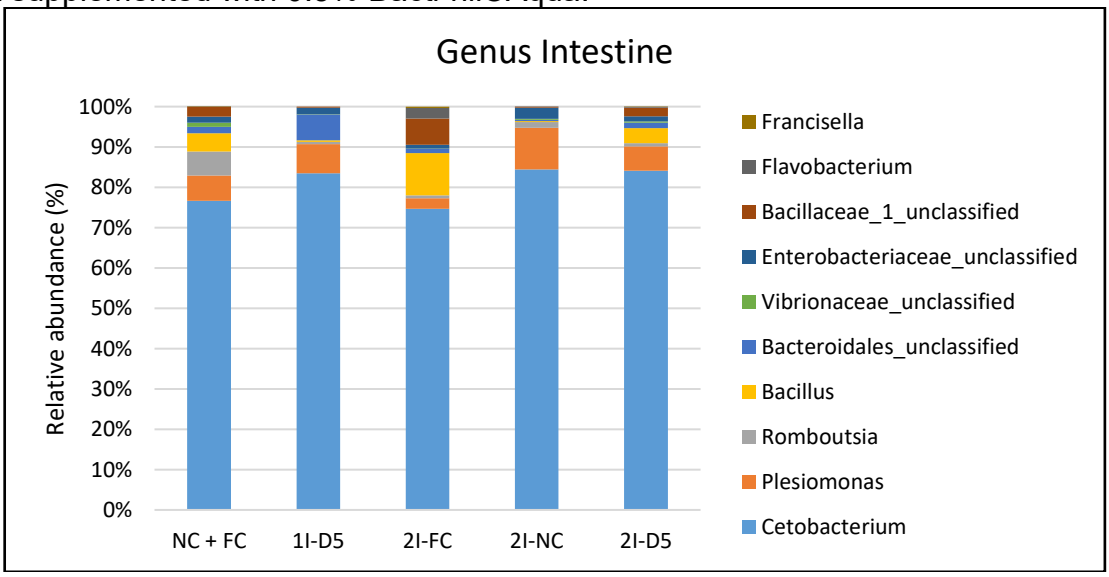


Figure 4: Mean relative abundance at genus level in stool samples of Nile tilapia (*Oreochromis niloticus*) treated with basal diet and diet supplemented with 0.5% Bacti-nil®Aqua



Abbreviations: NC, negative control group; FC, positive control group; 1F-D5, first sampling of feces in the 0.5% supplemented diet; 2F-FC, second sampling of feces in the positive control group; 2F-NC, second sampling of feces in the negative control group; 2F-D5, second sampling of feces in the 0.5% supplemented diet.

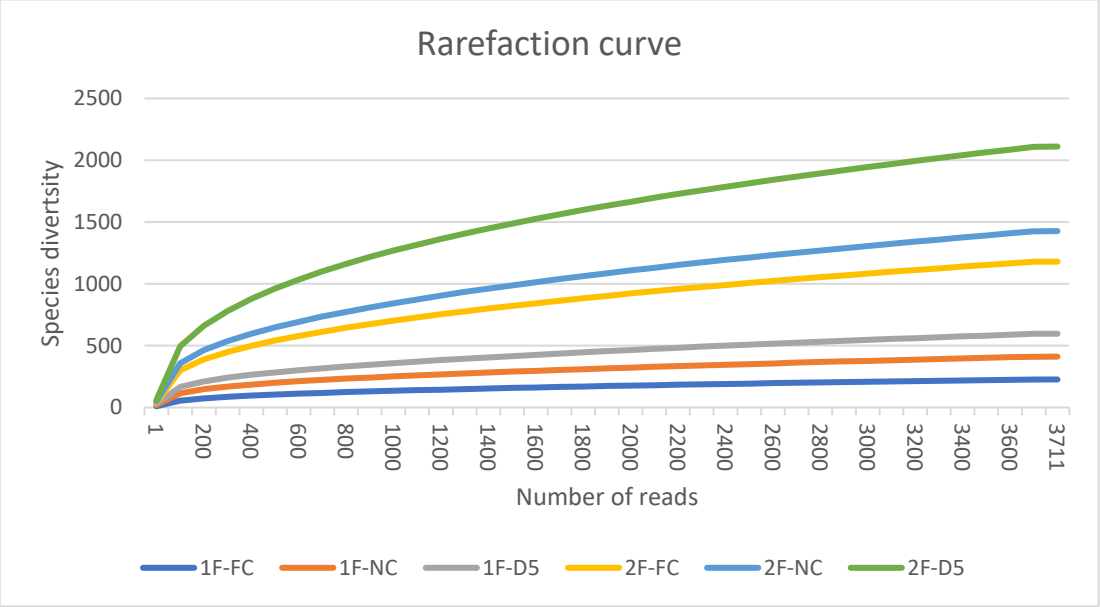
Figure 5: Mean relative abundance at genus level found in adherent intestinal microbiota samples of Nile tilapia (*Oreochromis niloticus*) treated with basal diet and diet supplemented with 0.5% Bacti-nil®Aqua.



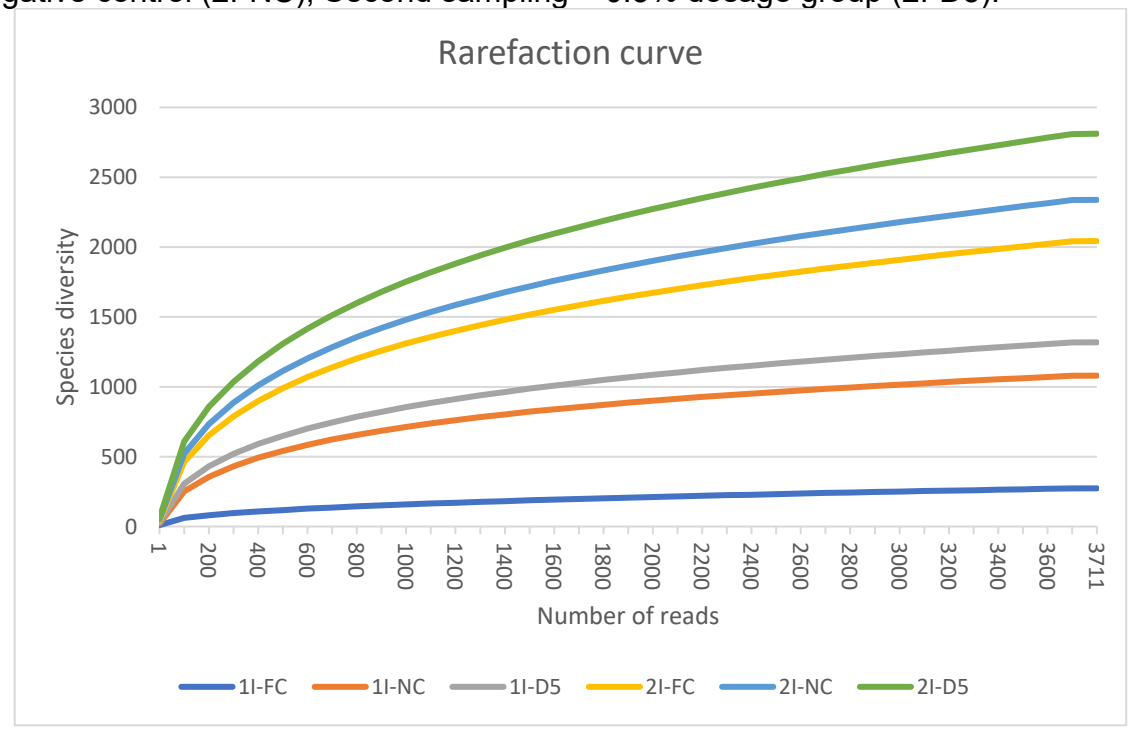
Abbreviations: NC, negative control group; FC, positive control group; 1I-D5, first sampling of intestinal microbiota in the 0.5% supplemented diet; 2I-FC, second sampling of intestinal microbiota in the positive control group; 2I-NC, second sampling of intestinal microbiota in the negative control group; 2I-D5, second sampling of intestinal microbiota in the 0.5% supplemented diet.

SUPPLEMENTARY FIGURES

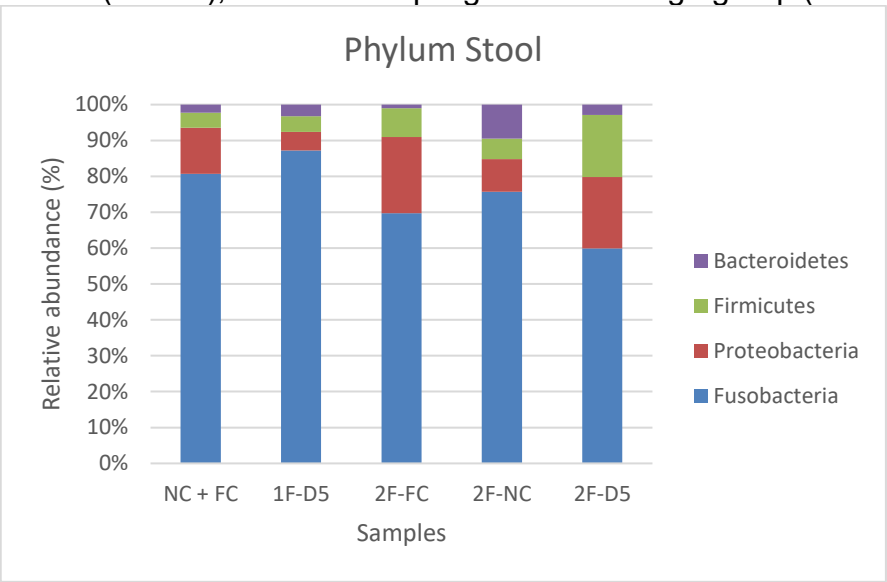
Supplementary Figure 1: Rarefaction curve showing increasing species along with the number of reads in stool samples. First sampling – positive control (1F-FC); First sampling – negative control (1F-NC); First sampling – 0.5% dosage group (1F-D5); Second sampling – positive control (2F-FC); Second sampling – negative control (2F-NC); Second sampling – 0.5% dosage group (2F-D5).



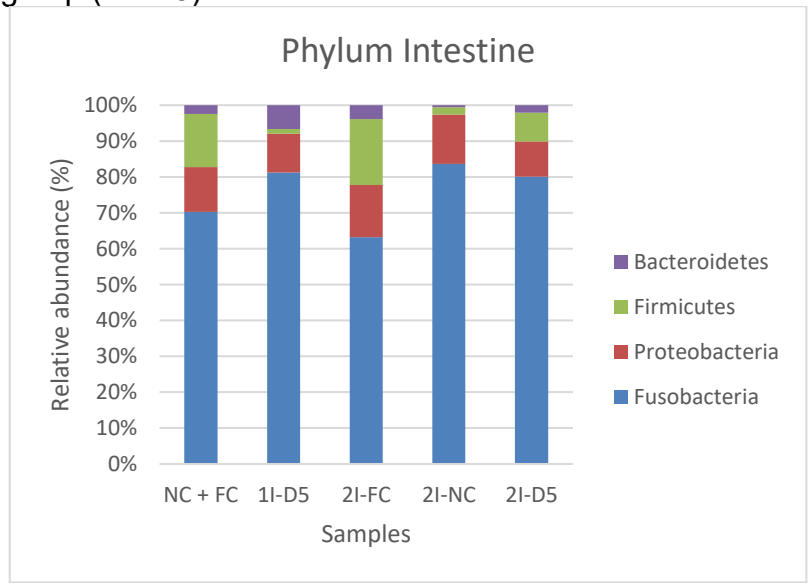
Supplementary Figure 2: Rarefaction curve showing increasing species along with the number of reads in adherent intestinal microbiota samples. First sampling – positive control (1I-FC); First sampling – negative control (1I-NC); First sampling – 0.5% dosage group (1I-D5); Second sampling – positive control (2I-FC); Second sampling – negative control (2I-NC); Second sampling – 0.5% dosage group (2I-D5).



Supplementary Figure 3: Relative abundance at phylum level found in stool samples of Nile tilapia treated with Basal diet and 0.5% dosage supplemented diet with Bactinil® Aqua, at the phylum level. First sampling – Basal diet (NC+FC); First sampling – 0.5% dosage group (1F-D5); Second sampling – positive control (2F-FC); Second sampling – negative control (2F-NC); Second sampling – 0.5% dosage group (2F-D5).



Supplementary Figure 4: Relative abundance at phylum level found in adherent intestinal microbiota samples of Nile tilapia treated with Basal diet and 0.5% dosage supplemented diet with Bactinil® Aqua, at the phylum level. First sampling – Basal diet (NC+FC); First sampling – 0.5% dosage group (1F-D5); Second sampling – positive control (2F-FC); Second sampling – negative control (2F-NC); Second sampling – 0.5% dosage group (2F-D5).



Supplementary Table 1: Relative frequency of the most abundant genera present in stool samples. Pre-challenge: Control diet (NC + FC) and 0.5% supplemented diet (D5). Post challenge: Positive control (2F-FC); Negative control (2F-NC); 0.5% supplemented diet (2F-D5).

	Pre-challenge (%)		Post challenge (%)		
	NC + FC	1F-D5	2F-FC	2F-NC	2F-D5
Cetobacterium	7606 (82.02)	9379 (88.08)	6705 (83.67)	7741 (79.10)	9056 (75.66)
Plesiomonas	384 (4.14)	380 (3.57)	435 (5.43)	581 (5.94)	490 (4.09)
Romboutsia	387 (4.17)	454 (4.27)	258 (3.22)	538 (5.50)	525 (4.38)
Bacillus	4 (0.04)	5 (0.05)	234 (2.92)	9 (0.09)	1017 (8.49)
*Bacteroidales*_unclassified	151 (1.63)	278 (2.61)	40 (0.49)	632 (6.46)	2 (0.02)
*Vibrionaceae*_unclassified	645 (6.96)	69 (0.64)	0 (0.00)	98 (1.00)	46 (0.39)
*Enterobacteriaceae*_unclassified	91 (0.98)	80 (0.75)	186 (2.32)	177 (1.81)	97 (0.81)
*Bacillaceae*_1_unclassified	5 (0.05)	3 (0.03)	151 (1.89)	9 (0.09)	527 (4.41)
Flavobacterium	0 (0.00)	0 (0.00)	4 (0.04)	1 (0.01)	205 (1.71)
Francisella	0 (0.00)	0 (0.00)	1 (0.01)	0 (0.00)	5 (0.05)
Total of reads	9273	10648	8014	9786	11969

Supplementary Table 2: Relative frequency of the most abundant genera present in adherent intestinal microbiota samples. Pre-challenge: Control diet (NC + FC) and 0.5% supplemented diet (D5). Post challenge: Positive control (2I-FC); Negative control (2I-NC); 0.5% supplemented diet (2I-D5).

	Pre-challenge (%)		Post challenge (%)		
	NC + FC	1I-D5	2I-FC	2I-NC	2I-D5
Cetobacterium	6119 (76.66)	9529 (83.46)	3895 (74.65)	8437 (84.42)	7697 (84.07)
Plesiomonas	497 (6.23)	825 (7.23)	137 (2.62)	1034 (10.35)	558 (6.10)
Romboutsia	475 (5.95)	67 (0.58)	36 (0.69)	137 (1.37)	68 (0.74)
Bacillus	361 (4.53)	39 (0.34)	547 (10.49)	29 (0.29)	344 (3.76)
*Bacteroidales*_unclassified	126 (1.58)	729 (6.39)	56 (1.07)	33 (0.33)	120 (1.31)
*Vibrionaceae*_unclassified	85 (1.07)	12 (0.10)	3 (0.06)	17 (0.17)	31 (0.34)
*Enterobacteriaceae*_unclassified	121 (1.52)	190 (1.66)	51 (0.97)	279 (2.80)	116 (1.27)
*Bacillaceae*_1_unclassified	195 (2.44)	27 (0.24)	338 (6.48)	26 (0.26)	199 (2.18)
Flavobacterium	2 (0.02)	0 (0.00)	137 (2.63)	1 (0.01)	20 (0,22)
Francisella	0 (0.00)	0 (0.00)	18 (0.34)	0 (0.00)	1 (0.01)
Total of reads	7982	11417	5218	9994	9154

7 ARTIGO B – Screening of potential probiotics, their effects on different combinations of probiotic bacteria and *Yarrowia lipolytica* strains on growth, immune response and disease resistance of Nile tilapia

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ABSTRACT

Probiotics are live beneficial bacteria that are consumed through food or drink and help to maintain a healthy microbial balance in the gastrointestinal tract. To assess probiotic potential, the lactic acid bacteria *Enterococcus faecium* (EF)*, Lactococcus lactis* (LL) and the yeast *Yarrowia lipolytica* (YL) were tested for tolerance to pH, salinity, bile resistance, antibiotic resistance, antagonism against fish pathogens, and a biosafety trial. Afterwards, an *in vivo* trial with juvenile Nile tilapias was conducted to evaluate growth performance, immunological response, and resistance to bacterial challenge. The experiment was conducted with nine treatments: (G1) Negative control; (G2) Positive control; (G3) fish fed commercial feed plus EF; (G4) fish fed commercial feed plus LL; (G5) fish fed commercial feed plus YL; (G6) fish fed commercial feed plus EF and LL; (G7) fish fed commercial feed plus EF and YL; (G8) fish fed commercial feed plus LL and YL; (G9) fish fed commercial feed plus EF, LL and YL. Groups G2-G9 were challenged with *Streptococcus agalactiae* serotype Ib. After 45 days of feeding trial, G8 showed a positive effect on growth, the immune system and survival after bacterial challenge. Groups G4 and G9 showed a statistical difference for serum bactericidal activity, but no difference in lysozyme and alternative complement was

detected. After bacterial challenge, G8 and G4 had the highest survival rates, with 53.85% and 47.37%, respectively. G3 presented a strong immunological response but the higher mortality rate, 14.29%. Groups fed *L. lactis* alone or in combination with *Y. lipolytica* had a good performance on growth metrics, immunological response, and survival rates, suggesting that it could be used as an important symbiotic additive for Nile tilapias in Brazil.
KEYWORDS: Multi-strain Probiotics; *Streptococcus agalactiae*; sustainable aquaculture

1. INTRODUCTION

Aquaculture is a rapidly expanding industry, and the global production of aquatic animals was estimated at 88 million tons in 2020 (FAO, 2022). This intense production, combined with failures in biosecurity measures, may facilitate emergence of bacterial disease outbreaks. This fact represents a persistent challenge for the industry, as it allows for the overuse of antibiotics, which can lead to the selection of resistant bacteria, and worsen environmental and human health issues (ALVES JESUS *et al.*, 2021).

In this regard, probiotics are becoming a popular choice as a prophylactic measure to prevent disease and improve the health and production of farmed fish, particularly tilapia (STANDEN *et al.*, 2016). These feed additives have been reported to improve nutrient absorption (DAWOOD *et al.*, 2019), boost immune response and intestinal immunological status by increasing the number of epithelial leukocyte and abundance of goblet cells (STANDEN *et al.*, 2016), increase disease resistance (LI et al., 2019), reduce environmental stress (HOSEINIFAR; SOLEIMANI; RINGØ, 2014). Lactic acid bacteria stand out among the bacteria used as probiotics because they are simple to work with, produce antimicrobial substances (organic acids, lactic acid, bacteriocins, and hydrogen peroxide), and induce a generalized immunological response in hosts (GATESOUPE, 2008).

Prior to use, it is crucial that the possible probiotic strains are thoroughly characterized. Tolerance to bile toxicity and gastric acidity, adherence to the intestinal epithelium, and the capacity to control the host's immunological response are among the important selection requirements for probiotics (DE MELO PEREIRA *et al.*, 2018; GIRI; SUKUMARAN; DANGI, 2012). Based on bile tolerance, mucus

adherence, mucus penetration, and suppression of fish pathogen growth, human lactic acid bacteria (LAB) strains of *Lactobacillus rhamnosus* ATCC 53103 and *Lactobacillus bulgaricus* were regarded as a promising treatment in aquaculture (NIKOSKELAINEN *et al.*, 2001). Bacterial isolates from the intestine of *Labeo rohita* were sensitive to the tested antibiotic and tolerated low pH (2.0 to 5.0) and high bile concentrations (2-6%) (GIRI; SUKUMARAN; DANGI, 2012).

There are currently a few commercially available probiotic compositions for fish. *Enterococcus faecium* and *Lactococcus lactis* are well-known strains that have been described as probiotics for several animal species (AMACHAWADI *et al.*, 2018; ASMARA *et al.*, 2021; ZHENG *et al.*, 2016), and recently in Nile tilapia (TACHIBANA *et al.*, 2020; WON *et al.*, 2020), ornamental fish (DIAS *et al.*, 2019), pirarucu (COSTA SOUSA *et al.*, 2019), and trout (MORTEZAEI *et al.*, 2020). *Yarrowia lipolytica* is a yeast model for the study of dimorphism, hydrophobic substrate degradation, lipid metabolism, protein secretion, and peroxisome biogenesis (NICAUD, 2012). The yeast has been shown to improve zootechnical parameters, immune and metabolic status, and reticular rumen function in turkeys (CZECH; MERSKA; OGNIK, 2014), piglets (CZECH *et al.*, 2016) and dairy calves (STEFAŃSKA *et al.*, 2018). Thus, it could be a new alternative for aquaculture as a promising probiotic yeast (ALVAREZ-SANCHEZ *et al.*, 2018). Multi-strain probiotics administered through the feed can increase productivity significantly by enhancing their effects on the host in a symbiotic, additive, and consistent manner (DIAS *et al.*, 2022). When given in doses of 3.36–6.72 g/kg, a commercial multi-strain probiotic (*Bacillus velezensis*, *Bacillus cereus* and *Lactobacillus casei*) improved growth, immune–antioxidative status, and gut health (CHEN *et al.*, 2020). To the best of our knowledge, however, there are no studies on the use of *E. faecium*, *L. lactis*, and *Y. lipolytica* in Nile tilapia (*Oreochromis niloticus*), in a single or combined probiotic formulation.

This study aimed to evaluate the probiotic potential of *E. faecium, L. lactis,* and *Y. lipolytica* considering their tolerance to pH, bile salts, NaCl, antibiotic resistance, and biosafety assays. In addition, we also evaluated the effects of probiotics supplementation and different feeding regimens on growth performance, innate immune responses of Nile tilapia (*O. niloticus*), and its resistance against *S. agalactiae* serotype Ib experimental infection.

2. MATERIAL AND METHODS

2.1 Ethic declaration

The study was conducted according to the Ethical Principles in Animal Research of the National Council for the Control of Animal Experimentation and approved by the State University of Londrina's Ethics Committee on the Use of Animals (Approval number CEUA/UEL-096.2020).

2.2 Strains

E. faecium LAC7.2 was isolated from the gastrointestinal tract of tilapias, from a local producer in the state of Paraná, Brazil, in 2017. *L. lactis* LAC9 was isolated from the gastrointestinal tract of healthy pirarucus (*Arapaima gigas*), from the state of Bahia, Brazil, in 2019. Lactic acid bacteria (LAB) were grown in the de Man, Rogosa, and Sharpe (MRS) culture medium at 28 °C for 48 h. Both strains are registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), under nº AA99F1F.

Y. lipolytica yeasts (QU29, QU31, QU36, QU69, QU123) were isolated from artisanal cheese in Southern Brazil. The yeasts are part of the Collection of Yeast Cultures from the Department of Microbiology, Immunology, and Parasitology at the Universidade Federal do Rio Grande do Sul (UFRGS), and registered in SisGen, under nº C09556C. Yeasts were cultured in yeast peptone dextrose (YPD) culture medium at 28 °C for 48 h.

Because the yeasts tested in this study performed similarly in every *in vitro* assay, the strain used in the *in vivo* analysis was determined by the one with the best culture and growing characteristics. As a result, YL29 was elected for the *in vivo* study.

2.3 Determination of probiotics activity

2.3.1 Effect of pH, bile salts, and sodium chloride

A modified protocol was used to test resistance to different pH levels (GULUARTE *et al.*, 2019). Briefly, bacteria and yeasts were cultured in MRS and YPD broth respectively, for 48 h at 28°C. Then, each microorganism strain was adjusted to turbidity compatible with the 0.5 McFarland standard ($1{,}5\times10^{8}$ UFC.ml^{-1}) in a Phosphate-buffered saline (PBS) solution (pH 7.4) and inoculated in a PBS solution adjusted with 2M HCl in acid conditions 2.1 and 3.2, and 7.4 as control of neutral

condition). The cultures were incubated in triplicates for 3 h at 28 °C, without agitation. Finally, serial dilutions were performed before inoculating the microorganisms on MRS or YPD agar for 48 h at 28 °C to determine viable colony forming units (CFU.mL^{-1}).

Bile tolerance was carried out according to the method used by Reda et al. (2018). Bile was collected from Nile tilapias after 24 hours of starvation, by aseptically puncturing the gall bladder, then filtered with a 0.22 µm Millipore filter and stored at -20°C until use. After adjusting to a 0.5 McFarland standard, the isolates were inoculated in triplicate in a PBS solution supplemented with 1%, 3%, and 5% bile then incubated for 6 h at 28°C. As a control, a tube without bile supplementation was used. After incubation, viable cell counts (CFU.mL^{-1}) were determined using a plate count method on MRS or YPD agar for 48 h at 28°C.

For determination of NaCl tolerance, bacteria and yeasts were cultured in MRS and YPD broth supplemented with NaCl (0, 1.5, 4.5 e 6.5%) and incubated for 48 h at 28°C. As a control, non-supplemented NaCl broth was used. Finally, serial dilutions in PBS solution were performed before plating onto MRS or YPD agar for 48 h at 28°C to determine viable colony forming units (CFU.mL^{-1}) (GULUARTE *et al.*, 2019).

2.3.2 Antagonistic activity of probiotic strains

Cross Streak Method

Each LAB isolate was inoculated on Muller Hinton agar (Kasvi, São José dos Pinhais, PR, Brazil) enriched with 5% of defibrinated sheep blood (MHBA) as a single straight line and incubated for 48 h at 28°C. Next, the plates were inoculated with the test organisms (*S. agalactiae*, yeasts, and remaining bacteria) by a single streak at an angle of 90° in relation to the LAB isolates, without touching the original streak, and incubated at 28°C for 24-48 h. The antagonistic effect of the LAB over the test strain was detected by an inhibition zone (HOSSAIN; RAHMAN, 2014).

Agar Well Diffusion Method using cell-free culture supernatants (CFCS)

The antimicrobial activity of LAB and yeasts was assessed using a modified protocol from a previous study (ABBASILIASI *et al.*, 2017). Bacteria and yeasts were grown in MRS and YPD broth, respectively, for 24 h at 28°C, and then adjusted to the 0.5 McFarland standard. *E. coli* ATCC 25922 was used as control and the fish pathogens tested were: *Aeromonas hydrophila, Streptococcus agalactiae*

serotype Ib, *Streptococcus agalactiae* serotype III, *Edwardsiella anguillarum* and *Edwardsiella ictaluri.* To obtain the CFCS, this inoculum was centrifuged at 6.000 rpm for 10 minutes and then filtered with a 0.22 µm Millipore filter. The CFCS (100µL) was poured in 6-mm wells of agar plates that had previously been seeded with the probiotic isolates and incubated at 28°C for 48-72h. The antimicrobial activity of the probiotic samples was determined by developing inhibition zones around the wells.

2.3.3 Antimicrobial susceptibility testing (AST)

The AST was carried out in accordance with the Clinical Laboratory Standards Institute (CLSI) protocol for disk diffusion assay (CLSI, 2006). The LAB isolates were cultured in MRS broth overnight before being suspended in PBS to achieve a 0.5 McFarland standard, and 35 (thirty-five) antimicrobials were tested. The plates were inoculated with a swab, the antibiotic disks placed, and incubated for 48 h at 28 °C. The diameter of the inhibition zones was measured and compared to CLSI standards for antimicrobial disk susceptibility tests (CLSI, 2020).

2.3.4 Biosafety

A total of 80 Nile tilapia (3-5 g) were randomly distributed into eight groups, in duplicate, each with 10 fish. The fish were acclimatized for 7 days. The probiotics were grown in MRS and YPD broth, for 48 h at 28°C. For the challenge, the samples were adjusted to the 0.5 McFarland standard in PBS (pH 7.4). Experimental fish were intraperitoneally injected with 100 µL, while the group control received the same volume of sterile PBS. For 10 days, fish were fed with a commercial diet and monitored for any clinical signs and mortality. After 10 days, the fish were euthanized, and examined for lesions. To re-isolate the strains of bacteria and yeasts used in the challenge, a liver sample from each fish was grown on MHBA for 48 hours at 28 °C.

2.4 Animals, Experimental Design, Feeding Protocol

A total of 470 healthy juvenile Nile tilapias with an initial weight of 16.62 ± 2.02 were randomly distributed in 9 150L tanks, 50 fish per tank. The experiment was carried out in an indoor recirculating system containing dechlorinated water, with a 12:12h light:dark photoperiod, the water temperature of 26,8 ± 0,8°C, pH of 6,8-7,6, dissolved oxygen (DO) of 3,5-5,5 $mg.L^{-1}$, and total ammonia <0.4 $mg.L^{-1}$. During acclimatization, fish were fed a commercial diet (Presence Nutripiscis TR 36% CP)

three times a day. Before the feeding trial, the fish were acclimatized for 7 days for observation in order to guarantee the state of health (absence of clinical signs suggestive of disease and monitoring for exophthalmia, erratic swimming, skin lesions, and others). Next, a sample of 20 fish was collected for microbiological diagnosis. For this, fish were randomly euthanized with benzocaine (250 mg/mL), and fragments of brain, liver, cranial kidney, and spleen were cultured aseptically on Mueller Hinton agar (Kasvi, São José dos Pinhais, PR, Brazil) enriched with 5% defibrinated sheep blood and Cystine heart agar supplemented with 1% of bovine hemoglobin (CHAH). The plates were incubated at 28°C for 5 days to ensure the fish's health (no bacterial growth in the plates).

The fish were divided into nine groups at random (G1, G2, G3, G4, G5, G6, G7, G8, and G9). G1 (Negative Control group) and G2 (Positive Control group) were fed a basal diet without supplementation. G3 through G9 were fed a basal diet supplemented with probiotics (Table 1). Food was given to the fish three times a day at a rate of 3-6% of their body weight. Fish were weighed every two weeks, and the amount of feed was adjusted accordingly.

To prepare the diets, approximately three bacterial colonies of *E. faecium* (EF), *L. lactis* (LL), and *Y. lipolytica* (YL) were cultured in 1L of MRS or YPD broth, and incubated in a rotation shaker at a speed of 180 rpm for 48 h at 28°C. Then, 100 mL was sprayed onto 1kg of feed plus 5mL of universal vehicle (Vansi ®) and allowed to dry for 8-12 hours at 28°C. A proportion of each strain was calculated to constitute 100 mL of the multi-strain probiotic feed. The final average concentrations of each probiotic were: *E. faecium* (3.3×10^{9} CF./mL^{-1}), *L. lactis* (2.8×10^{10} CFU.mL^{-1}), and *Y. lipolytica* (2.6×10^{8} CFU.mL^{-1}). Total bacterial and yeast counts in feed were performed on the day of preparation and after one week of storage to evaluate feed shelf life. The feed with probiotics was produced every seven days.

Table 1 – Groups division and feeding regimens. *E. faecium* (EF), *L. lactis* (LL), and *Y. lipolytica* (YL).

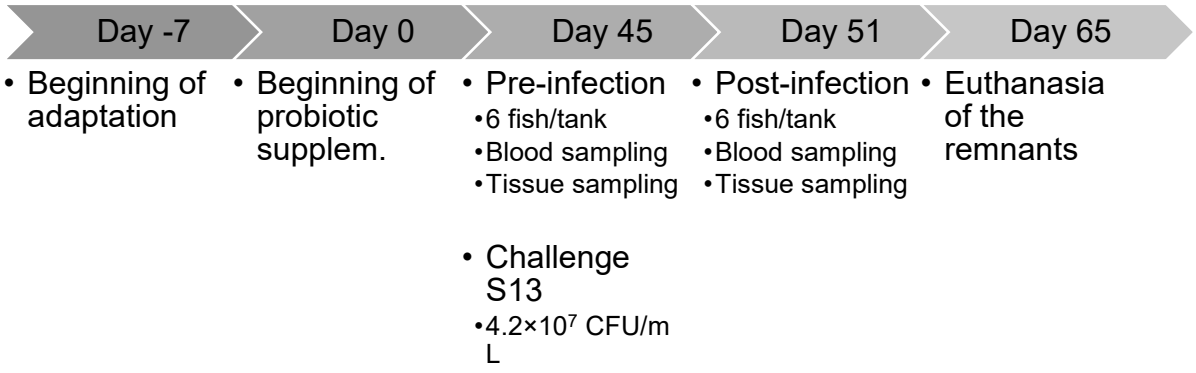
GROUP	DIET
G1	Negative control group. Basal diet. No bacterial challenge
G2	Positive control group. Basal diet and challenged with *S. agalactiae*
G3	Basal diet supplemented with EF and challenged with *S. agalactiae*

G4	Basal diet supplemented with LL and challenged with *S. agalactiae*
G5	Basal diet supplemented with YL and challenged with *S. agalactiae*
G6	Basal diet supplemented with EF + LL and challenged with *S. agalactiae*
G7	Basal diet supplemented with EF + YL and challenged with *S. agalactiae*
G8	Basal diet supplemented with LL + YL and challenged with *S. agalactiae*
G9	Basal diet supplemented with EF + LL + YL and challenged with *S. agalactiae*

2.5 Pathogen challenge

For the challenge, *S. agalactiae* serotype Ib (strain S13) was cultured in Brain and Heart Infusion Broth (BHI) and incubated overnight at 28 °C. Fish were given an intraperitoneal injection of 0.1 mL containing 4.2×10^7 CFU.mL^{-1} of *S. agalactiae* S13. The control group (G1) received an intraperitoneal injection of 0.1 mL of 0.85% saline solution. For 20 days, challenged fish were monitored for clinical signs, postmortem lesions, and daily mortalities. Dead fish were daily removed from the tanks, and the strain was reisolated by culturing the brain and kidney on Mueller Hinton agar enriched with 5% defibrinated sheep blood for 48 h at 28 °C (Figure 1).

Figure 1 – Schedule of the *in vivo* experiment with probiotic supplementation



2.6 Growth performance

Fish were counted and weighed individually at the start and end of the feeding trial, used the following formulae: Weight gain (WG) = final weight (g) - initial weight (g); Specific growth rate (SGR %) = 100 × (ln final weight - ln initial weight) / Duration of experiment; Feed conversion ratio (FCR) = feed given (dried weight) / weight gain (wet weight); Survival rate (%) = (final fish number/initial fish number) × 100 (VAN DOAN *et al.*, 2018).

2.7 Innate immune analyses

At the beginning and the end of the feeding trial (45d), six fish from each group were anesthetized and sampled to determine immune parameters. Blood was drawn from caudal blood vessels without anticoagulant, allowed to coagulate at room temperature to obtain serum, then centrifuged at 1400 × g for 10 minutes, and finally stored at -20 °C.

Serum lysozyme activity was determined using a methodology modified from that described previously (DEMERS; BAYNE, 1997). In summary, lysis of *Micrococcus lysodeikticus* (Sigma-Aldrich Chemical Co.) was evaluated at 492nm and the difference between initial and final absorbances was used to calculate lysozyme concentration (ug.mL^{-1}).

Alternative complement pathway activity (ACH50) was determined using rabbit red blood cells (RaRBCs) as target cells for hemolysis, following a previously described methodology (ORIOL SUNYER; TORT, 1995). Briefly, serially diluted sera were mixed with rabbit erythrocyte suspension and incubated at for 2 h 22°C with occasional shaking. The extent of hemolysis was estimated by measuring the optical density of the supernatant at 450 nm. Serum dilutions with greater than 90% or less than 15% lysis were excluded from the calculation, and the serum dilution with 50% lysis of RaRBC was represented as ACH5O %hem/mL^{-1} (SUPHORONSKI *et al.*, 2021).

Serum bactericidal activity was measured in a flat-bottom 96-well microplate, according to the method used in a previous work (SILVA *et al.*, 2009). Fish serum was tested for its antimicrobial activity against *A. hydrophila* prepared at a concentration of 0.5 McFarland standard and diluted 100,000 times in Poor broth (PB). Following that, the bacterial suspension was serially diluted (1:2) with PBS for 8 dilutions. Saline solution diluted in PB was used as a negative control. Twenty microliters of the diluted bacteria were added to each well containing the serum and positive control. The microplates were incubated for 24 h at 28°C. The serum bactericidal activity was the reciprocal of the least dilution that showed bactericidal activity.

2.8 Liver histology and morphology

Ten days after the challenge, six fish from each treatment were

euthanized by benzocaine overdose (250 $mg.mL^{-1}$). The liver was collected for histopathological analysis. Following 48 hours in 10% neutral buffered formalin, and storage in 70% ethanol, tissues were dehydrated in a graded ethanol series and embedded in a paraffin block. Serial sections (5 µm thick) of each tissue block were cut and stained with hematoxylin and eosin (HE) for histopathological evaluation (SOUZA *et al.*, 2020).

The following characteristics were regarded as liver alterations: glycogen accumulation, hepatocellular and pancreatic epithelial necrosis, inflammation, pigmented macrophage aggregates, congestion in large vessels, and eosinophilic globules in hepatocytes. The liver was examined and graded using an ordinal ranking system: 0 (absence of change), 1 (mild alteration), 2 (moderate alteration), and 3 (severe alteration). On the other hand, glycogen accumulation was scored as 1 (large accumulation of glycogen), 2 (mild depletion), 3 (moderate depletion), and 4 (marked depletion). The cumulative assessment was based on summing the average of individual lesion scores, where the highest total average score indicated worse condition of the fish (LEHMAN *et al.*, 2010).

2.9 Statistical analysis

Data were presented as means ± standard error. Analysis of variance (ANOVA) was performed, followed by Tukey's test if significant differences were found ($P<0.05$). Analyses that did not meet the assumptions of residual normality or homogeneity of variance were subjected to nonparametric Kruskal–Wallis analysis, followed by Dunn's test if significant differences were found ($P<0.05$). These statistical analyses were performed using R version 3.3.3 (R CORE TEAM, 2017). The survival rate was analyzed using Fisher's exact test, with a significance level of 5%, using OpenEpi v. 3.01 (DEAN AG; SULLIVAN KM; SOE MM, 2013).

3 RESULTS AND DISCUSSION

3.1 Determination of probiotics activity

3.1.1 Tolerance to low pH, high bile salts, and sodium chloride

Based on the bacterial viable count (mean log $CFU.ml^{-1}$), all isolated strains demonstrated good tolerance to various NaCl and bile salt concentrations ($P<0.05$), however some strains demonstrated greater tolerance than others. LL

presented significantly better tolerance in high concentrations of fish bile salts (3% and 5%) after 6 h and pH 3.2 after 1.5 h. Among the seven strains, EF was the most sensitive, unable to tolerate pH 2.1 for 3 h. Hence, this pH value can limit the growth of this LAB strain. Overall, yeasts tolerated well bile salts, pH, and NaCl, although strain YL 123 presented the highest tolerance, in the lowest pH 2.1 (Tables 2, 3 and 4) (Supplementary Figure 1). EF and YL29 showed the greatest growth at high concentrations of NaCl (4.5% and 6.5%). Osmotic tolerance is an important characteristic of a probiotic because high salt concentrations affect microorganisms by causing turgor pressure loss, allowing water leakage, and impairing its metabolism (COULIBALY *et al.*, 2008).

Table 2: Total viable counts (log CFU.mL^{-1}; mean ± SE) of potential probiotic strains at different pH for 3h at 28 °C.

	pH			
Strains	**7.4/0h**	**2.1/3h**	**3.2/3h**	**7.4/3h**
EF (LAC7.2)	6.68±0.02^{a}	0^{c}	6.69±0.09^{ab}	6.48±0.01^{ab}
LL (LAC9)	6.60±0.01^{a}	4.59±0.11^{bc}	6.82±0.08^{a}	6.85±0.08^{a}
YL 29	4.97±0.01^{b}	4.93±0.15^{ab}	5.23±0.05^{bc}	4.77±0.07^{cd}
YL 31	4.95±0.05^{b}	4.68±0.13^{bc}	5.16±0.05^{c}	4.24±0.06^{d}
YL 36	5.24±0.01^{b}	4.94±0.06^{ab}	5.19±0.04^{c}	4.61±0.13^{cd}
YL 69	5.27±0.13^{ab}	4.91±0.13^{ab}	5.12±0.08^{c}	4.82±0.16b^{cd}
YL 123	5.83±0.09^{a}	5.87±0.17^{a}	5.93±0.05^{abc}	5.60±0.01^{abc}

Different letters within the same column indicate significant difference among strains ($p<0,05$)

Table 3: Total viable counts (log CFU.mL^{-1}; mean ± SE) of potential probiotic strains at different fish bile concentrations (0% control, 1%, 3%, 5%) at 28 °C for 6 h and control at 0 h.

	Bile salts				
Strains	0%/0h	0%/6h	1%/6h	3%/6h	5%/6h
EF (LAC7.2)	6.76±0.02^{a}	6.86±0.01^{a}	7.81±0.27^{a}	7.31±0.03^{ab}	7.25±0.02^{ab}
LL (LAC9)	6.80±0.10^{a}	6.39±0.21^{ab}	7.72±0.18^{ac}	8.10±0.02^{a}	8.17±0.21^{a}
YL 29	5.50±0.10^{abc}	5.24±0.06^{abcd}	5.81±0.01^{abcd}	5.48±0.18^{cd}	5.48±0.18^{abcd}
YL 31	5.63±0.03^{ab}	5.09±0.09^{bcd}	5.42±0.42^{cd}	5.71±0.11^{abcd}	5.18±0.01^{cd}
YL 36	4.85±0.19^{c}	4.70±0.04^{d}	5.01±0.17^{d}	5.06±0.02^{d}	5.00±0.15^{d}

YL 69	5.34±0.01^{bc}	4.78±0.04^{cd}	5.47±0.07^{bcd}	5.61±0.13^{bcd}	5.23±0.09^{bcd}
YL 123	5.42±0.12^{bc}	5.53±0.13^{abc}	5.84±0.14^{abcd}	5.89±0.19^{abc}	5.81±0.07^{abc}

Different letters within the same column indicate significant difference among strains ($p<0,05$)

Table 4: Total viable counts (log CFU.mL^{-1}; mean ± SE) of potential probiotic strains at different sodium chloride concentration at 28 °C for 24 h and control at 0 h

	NaCl				
Strains	0%/0h	0%/24h	1,5%/24h	4,5%/24h	6,5%/24h
EF (LAC7.2)	6.76±0.02^{a}	TNTC	8.40±0.01^{ab}	8.48±0.01^{a}	TNTC
LL (LAC9)	6.80±0.10^{a}	TNTC	9.56±0.06^{a}	7.65±0.01^{bc}	7.86±0.01^{ab}
YL 29	5.50±0.10^{ab}	7.27±0.13^{c}	7.52±0.22^{c}	8.51±0.39^{a}	8.16±0.16^{a}
YL 31	5.63±0.03^{ab}	7.87±0.17^{bc}	8.04±0.16^{bc}	8.17±0.09^{abc}	6.89±0.01^{bc}
YL 36	4.85±0.19^{b}	8.01±0.11^{abc}	8.16±0.05^{bc}	7.39±0.09^{c}	6.29±0.11^{c}
YL 69	4.84±0.50^{b}	8.10±0.02^{ab}	8.01±0.11^{bc}	8.28±0.38^{ab}	7.49±0.32^{abc}
YL 123	5.42±0.12^{b}	8.24±0.10^{a}	8.46±0.07^{ab}	8.42±0.02^{ab}	7.93±0.19^{ab}

Different letters within the same column indicate significant difference among strains ($p<0,05$); TNTC: Too numerous to count.

Although most LAB strains are acidophilic, EF could not survive at pH 2.1 and LL showed an important loss in cell viability. As a result, they would not reach the gastrointestinal tract in adequate amounts to provide the required protection. This sensitivity to pH 2.1 may contribute to explain the poor response of EF-supplemented groups after *S. agalactiae* exposure. There is no agreement on the concentrations to which the selected strains should be tolerant. The average stomachal pH value in *O. niloticus* is 1.4–1.5, however ranges from 0.9 to 7.0 have been measured and several factors such as diet, age, and enzymes activity may influence gastric pH (GETACHEW, 1989; MORTEZAEI *et al.*, 2020).

Bile salt tolerance is a critical property required for probiotic to survive, grow, and act in the small intestine. Additionally, the concentration of bile salts in the small intestine varies between 0.2 and 2% depending on the individual and the type and amount of food consumed (KRISTOFFERSEN *et al.*, 2007). All isolates in the current investigation were able to tolerate a wide range of bile concentrations, up to 5%, indicating that when our isolates reached the small intestines, they would be able to survive, grow, and adhere to the fish gut epithelium. Mortezaei et al. (2020)

demonstrated that *L. lactis* isolated from rainbow trout could tolerate pH 3 and 8, but not pH 2.5, and could survive exposure to 10% fish bile juice after 1.5 h. All the yeasts tolerated different pH levels, which could be attributed to the cell wall structure allowing for greater tolerance under adverse conditions (OLAJUGBAGBE; ELUGBADEBO; OMAFUVBE, 2020). *Kluyveromyces lactis*, a marine extremophile yeast, was inhibited in growth at pH 2.5 and 3.5, as well as at 4.5% and 6.5% NaCl concentrations, but it still elicited an immune response in gilthead seabream (*Sparus aurata*) (GULUARTE *et al.*, 2019). Like our results, Kaktcham et al. (2018) demonstrated that two *Lactobacillus* strains isolated from Nile tilapia and common carp showed resistance to pH 2.0 after 5 hours, and that bovine bile salts (0.3% oxgall) had no effect in bacterial growing in any of the strains.

3.2.2 Antagonistic activity of probiotic strains

The cross-streak assay was employed to assess any possible antagonism between the potential probiotic strains under study, bacteria and yeasts. Antagonism was observed between LL with EF and *S. agalactiae* serotype III. The LAB strains did not inhibit any of the yeasts (Supplementary Figure 2). In addition to the antagonistic behavior that probiotics must exhibit toward pathogens, antagonistic behavior between two probiotic strains is also regarded as a crucial factor in the development of multi-strain probiotics. A functional or effective multi-strain probiotics consists of strains that do not inhibit or oppose one another but can cooperate to benefit the host (PUVANASUNDRAM *et al.*, 2021).

According to the agar well diffusion assay, *L. lactis* and *E. faecium* inhibited the growth of *S. agalactiae* serotype Ib and *E. anguillarum* (Table 5). Competitive exclusion is an important mode of action for probiotics, and the inhibitory activity of LAB is essential in preventing pathogen colonization of the GIT (VIECO-SAIZ *et al.*, 2019). The inhibitory impact of LAB against common fish pathogens in the current investigation demonstrated an antagonistic effect on the growth of both Gram-positive and Gram-negative pathogenic microorganisms.

None of the yeasts could inhibit any of the pathogens. In nature, antagonism between microorganisms is common, as they compete for space and resources (HIBBING *et al.*, 2010). Some studies have demonstrated the antagonistic activity of potential probiotics against pathogenic bacteria. For example, *L. lactis* isolated from the intestinal microbiota of rainbow trout inhibited the four fish pathogens

tested, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Yersinia ruckeri,* and *Vibrio anguillarum* (BALCÁZAR et al., 2008). In addition, *Psychrobacter* spp. and *Shewanella* spp. probiotic strains from the gut microbiota of Atlantic cod (*Gadus morhua*) showed antagonistic activity against *Vibrio anguillarum* and *Aeromonas salmonicida*, two of the most common bacterial pathogens in cod aquaculture, with stronger antagonism at the higher incubation temperature (CAIPANG; BRINCHMANN; KIRON, 2010). In contrast to our findings, some *S. cerevisiae* strains exhibited some, albeit weak, antimicrobial activity against *S. agalactiae* (PINPIMAI *et al.*, 2015). The antagonistic activity of LAB strains may be due to several antimicrobial compounds produced by these bacteria, such as organic acids, bacteriocins, and peptide compounds, which modulate the cellular immune response (SERVIN, 2004). A bacteriocin, enterocin A, was identified as the primary molecule in the *E. faecium* LAC7.2 strain by an *in silico* investigation. (SUPHORONSKI *et al.*, 2021), and this bacteriocin is known for its bactericidal effect (AYMERICH *et al.*, 1996)

Table 5: Antagonistic activity of potential probiotics against fish pathogens. *E. coli* ATCC 25922 used as control. (EF) *E. faecium* (LAC7.2); (LL) *L. lactis* (LAC9); (29) *Y. lipolytica* (QU29); (31) *Y. lipolytica* (QU31); (36) *Y. lipolytica* (QU36); (69) *Y. lipolytica* (QU69); (123) *Y. lipolytica* (QU123)

	Agar well diffusion (mm)					
Strains	*E. coli* ATCC 25922	*A. hydrophila*	*S. agalactiae* serotype Ib (S13)	*S. agalactiae* serotype III (S73)	*E. anguillarum* (BEP282)	*E. ictaluri* (BEP194)
EF	0	0	7	0	13	0
LL	0	7	10	0	15	0
29	0	0	0	0	0	0
31	0	0	0	0	0	0
36	0	0	0	0	0	0
69	0	0	0	0	0	0
123	0	0	0	0	0	0

3.1.3 Antimicrobial susceptibility testing (AST)

An essential criterion for analyzing the safety of potential probiotic bacteria is the evaluation of antibiotic susceptibility and the absence of transferable resistance genes in order to avoid the introduction harmful strains. *E. faecium* and *L. lactis* were tested for antibiotic sensitivity and a complete opposite profile emerged

(Table 6). Both LAB strains were resistant to tetracycline *in vitro* but susceptible to florfenicol. Florfenicol and tetracycline are the only two antibiotics authorized for use in fish farming in Brazil (SINDAM, 2021). Florfenicol is a widely used antibiotic in Brazil to treat streptococcal infection in tilapia. It is well tolerated by tilapia when administered at the recommended dose (10-20 mg/kg/day of antibacterial molecule) for 10 days, with lower doses being ineffective and higher doses being toxic and causing tissue injuries (DE OLIVEIRA *et al.*, 2018). Antibiotics were tested against *Lactococcus* and *Weissela* isolated from rainbow trout, and all *L. lactis* strains showed resistance to tetracycline, but no virulence genes were detected (MORTEZAEI *et al.*, 2020). Resistance genes for aminoglycosides and macrolides were discovered in one of the plasmids in *E. faecium* LAC7.2 (SUPHORONSKI *et al.*, 2021), which might explain the resistance to antibiotics such as amikacin, gentamicin, streptomycin, and tobramycin. Commercial probiotics for cattle and swine containing *E. faecium* demonstrated resistance to several antimicrobials, but none contained virulence genes (AMACHAWADI *et al.*, 2018). To be considered as a probiotic, a microorganism would ideally be sensitive to all tested antimicrobials, so as not to introduce elements that confer this phenotype into a new ecosystem (FAO, 2016).

Table 6 – Antimicrobial susceptibility test of *E. faecium* (LAC7.2) and *L. lactis* (LAC9).

Mode of action	**Antibiotics (concentrations)**	***E. faecium***	***L. lactis***
Cell wall inhibitors	Penicillin (10 µg)	R	I
	Bacitracin (10 µg)	R	I
	Ampicillin (10 µg)	R	S
	Ceftriaxone (30 µg)	R	S
	Amoxicilin-clavulanate (30 µg)	R	S
	Amoxicillin (10 µg)	R	S
	Aztreonam (30 µg)	R	R
	Cephalexin (30 µg)	R	S
	Cephalothin (30 µg)	R	S
	Cefotaxime (30 µg)	R	S
	Cefoperazone (75 µg)	R	S
	Cefovecin (30 µg)	R	S
	Ceftazidime (300 µg)	R	S
	Ceftiofur (30 µg)	R	S
	Cefuroxime (30 µg)	R	S
	Imipenem (10 µg)	R	S

	Oxacillin (1 µg)	R	R
Protein synthesis inhibitors	Amikacin (30 µg)	R	S
	Gentamicin (10 µg)	R	S
	Neomycin (30 µg)	R	S
	Streptomycin (10 µg)	R	S
	Tetracycline (30 µg)	R	R
	Chloramphenicol (30 µg)	S	S
	Lincomycin (2 µg)	R	S
	Clindamycin (2 µg)	R	S
	Doxycycline (30 µg)	S	S
	Florfenicol (30 µg)	S	S
	Tobramycin (10 µg)	R	S
DNA synthesis inhibitors	Ciprofloxacin (5 µg)	I	S
	Norfloxacin (10 µg)	R	I
	Enrofloxacin (5 µg)	R	S
	Marbofloxacin (5 µg)	R	S
Folic acid synthesis inhibitors	Trimethoprim-sulfamethoxazole (25 µg)	S	S
Multiple mechanisms	Nitrofurantoin (300 µg)	S	S
	Polymyxin (300 µg)	R	R

S: susceptible; R: resistant; I: intermediate

3.1.4 Biosafety

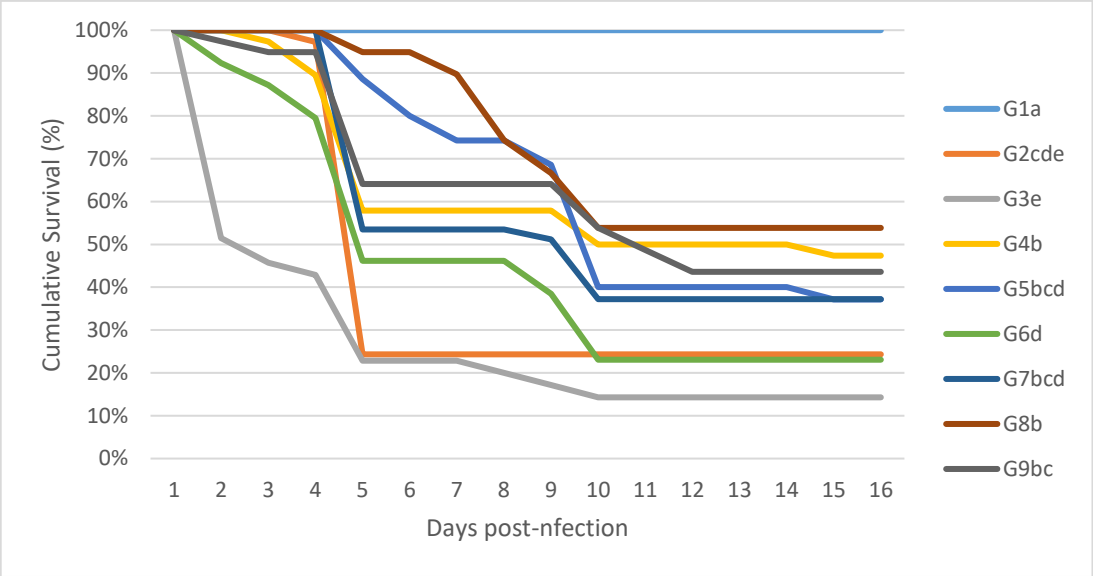
There were no disease symptoms, such as behavioral changes, edema, hemorrhage, or skin lesions, and no mortalities occurred during 10 days following infection. At the end of the experiment, the liver was sampled and inoculated on MHBA. All strains were recovered and confirmed by identification tests (colony morphology and Gram staining), confirming that the fish had been colonized but were non-pathogenic. In the control group, a PBS solution was injected, and no microorganism was recovered from the liver. In previous research, a 20-day biosafety assay conducted by oral and intraperitoneal infection with *Bacillus amyloliquefaciens* and *Paenibacillus* spp. revealed no pathogenic effect on rainbow trout. At the end, spleen and kidney microbiological analyses verified the lack of microorganisms (MEDINA *et al.*, 2020).

3.2 Pathogen challenge

After 45 days of the feeding trial, fish were challenged with *S. agalactiae* serotype Ib (Figure 2). Diseased fish exhibited erratic swimming, lethargy,

skin darkening, ocular opacity, exophthalmia, anorexia, and ascites. The survival curve of tilapia challenged with *S. agalactiae* revealed two mortality peaks on the 5th and 10th days. The highest survival rate was observed in the G8 group (53.85%) followed by the G4 group (47.37%). Although the G3 group presented a solid immune response, the survival was the lowest (14.29%), even lower than the positive control group (G2 - 24.32%), which may be an indication of the opportunistic pathogenic effect of this strain in animals stressed after bacterial exposure. The longer exposure time may have helped to manifest the pathogenic effect of *E. faecium*, which could not be observed in the biosafety assay. The antagonistic effect demonstrated in the cross-streak assay between *L. lactis* and *E. faecium in vitro* may explain the low survival rate of G6 (23.08%). Possibly, the dietary supplementation of *L. lactis*, which is part of fish intestinal microbiota coupled with *Y. lipolytica*, had a synergistic probiotic effect, and provided better survival rates in G8 (53.85%). The Nile tilapia's resistance to *S. agalactiae* infection was significantly increased by a single or combined supplementation of *Lactobacillus plantarum* or *Bacillus velezensis* compared to the control diet. Fish fed the mixed diet had the highest relative survival rate (58.33%), followed by treatments with *L. plantarum* (54.17%) and *B. velezensis* (41.67%) (VAN DOAN *et al.*, 2018). Suphoronski et al. (2021) challenged Nile tilapia with *S. agalactiae* serotype Ib and observed a statistically significant difference in mortality between the groups that received *E. faecium* in feed (75.56%), in water (73.33%), and in feed and water (73.33%), compared to the positive control (88.29%).

Figure 2: Cumulative survival rates of Nile tilapia challenged intraperitoneally with *S. agalactiae* after feeding a basal diet or a diet supplemented with probiotics for six weeks. Different letters indicate significant differences between treatments ($P<0.05$).



3.3 Diet probiotic viability and Growth performance

In the present study, probiotic counts on the diets were 5.2×10^{8} CFU.g^{-1} for *E. faecium*, 3.1×10^{9} CFU.g^{-1} for *L. lactis*, and 1.0×10^{7} CFU.g^{-1} for *Y. lipolytica*. One week later, diets were analyzed again to evaluate bacterial, and yeast viable counts, and the results were 4.3×10^{8} CFU.g^{-1} for *E. faecium*, 1.8×10^{9} CFU.g^{-1} for *L. lactis*, and 4.9×10^{6} CFU.g^{-1} for *Y. lipolytica*. These results indicate that storage for one week had little effect on microorganisms and they remained viable and stable. Supplemented diets at 10^{6} CFU.g^{-1} and 10^{8} CFU.g^{-1} for *E. faecium* and 10^{8} CFU.mL^{-1} for *Lactobacillus* were recorded (DIAS *et al.*, 2022; JATOBÁ *et al.*, 2008). Also, diets supplemented with 1.0×10^{6} CFU.g^{-1} *Y. lipolylica* were supplied to Juvenile Pacific red snapper (*Lutjanus peru*) (REYES-BECERRIL; ALAMILLO; ANGULO, 2021).

Table 7 shows data on growth performance. At the end of the feeding trial IBW, DWG and SGR revealed no significant difference (P>0.05) between the treatment groups and the control. Similar results have been previously reported in some fish fed different probiotic strains. After 30 days, fish fed a *Bacillus amyloliquefaciens* supplemented diet at concentration 1×10^{4} CFU.g^{-1} resulted in lower growth of tilapia than the fish fed 1×10^{6} CFU.g^{-1}, and it is suggested that the strain colonization is time and dose-dependent (REDA; SELIM, 2015). In contrast, a *L. lactis* supplemented diet increased the growth and feed utilization of tilapia, and it was more effective than *L. rhamnosus* or the mixture of the two concerning the immune status and disease resistance (XIA *et al.*, 2018).

Table 7: Growth performance and survival of Nile tilapia fed diets supplemented with *E. faecium* and/or *L. lactis* and/or *Y. lipolytica* for 6 weeks.

	Experimental diets								
	G1	G2	G3	G4	G5	G6	G7	G8	G9
IBW (g)	16,76	16,35	17,13	17,33	16,89	16,61	15,84	15,82	17,56
DWG (g)	1,25	1,14	1,16	1,13	1,06	1,13	1,11	1,08	1,15
SGR (%)	3,28	3,16	3,11	3,04	2,99	3,11	3,16	3,12	3,05
SR (%)	100^{a}	$24,32^{cde}$	$14,29^{e}$	$47,37^{b}$	$37,14^{bcd}$	$23,08^{d}$	$37,21^{bcd}$	$53,85^{b}$	$43,59^{bc}$

IBW: Initial body weight, DWG: daily weight gain; FCR: Feed conversion ratio, SGR: Specific growth rate, SR: Survival rate. Different letters in the same row indicate significant difference ($P<0.05$)

3.4 Innate immune analyses

The effect of the probiotic diets on tilapia's immune response is reported in Table 8. After the challenge, the highest serum bactericidal activities were found in G4 and G9 groups ($P<0.05$). Additionally, G2, G3 and G7 presented a minor but significant increase, compared to control. Serum bactericidal activity is well known for its capacity to suppress the growth and proliferation of pathogenic microorganisms during infection in host (YANO, 1996), which clearly contributed to the better survival rate of the G4 group (47.37%). Also, it is possible that the yeast *Y. lipolytica* acted as a prebiotic and aided to elicit a better performance of G9, since it is known that prebiotics are not digested but may act as immunostimulants (HOSEINIFAR *et al.*, 2015).

Pre-challenge, G3 showed an important increase in lysozyme and alternative complement pathway activity, followed by the multi-strain G8, even though no significant difference was observed. The serum concentration of complement in tilapia significantly increased after the addition of *E. faecium* (1×10^{7} CFU.mL^{-1}) to aquarium water, while lysozyme, total serum protein, albumin content, or concentration of glubulin did not change (WANG *et al.*, 2008). Lysozyme activity was significantly enhanced in *C. punctatus* fed *S. cerevisiae* and Galactooligosaccharide (GOS) after 6 weeks, but not at 2 or 4 weeks, indicating that a symbiotic diet could improve the nonspecific immune response (DEVI *et al.*, 2019a). In pacus (*Piaractus mesopotamicus*), a significant immune response was dependent on the amount and duration of *B. amyloliquefaciens* administration, and the serum bactericidal and

phagocytic activities required higher levels of probiotics, up to 10^6 CFU.g^{-1} (SELIM; REDA, 2015). Lysozyme can be found in mucus, serum, and tissues rich in leucocytes (ELLIS, 1999). The immune response of red sea bream (*Pagrus major*) was improved by supplementing *B. subtilis* to the diet at 1x10^8 and 1x10^{10} CFU.kg^{-1}, and serum bactericidal activity and serum lysozyme were significantly increased, but not mucus bactericidal activity or mucus lysozyme activity (ZAINELDIN *et al.*, 2018). According to Salinas et al. (2005), combining bacterial strains that complement one another and occupy distinct niches within the gut microflora environment may lead to an intensification or extension of the beneficial effects on the host immune system and general health.

Table 8: Serum lysozyme activity, alternative complement pathway activity (ACH50), and serum bactericidal activity (mean ± standard error) in tilapia administered different treatments before and after the *S. agalactiae* challenge. Different letters in the column indicate a significant difference between the treatments (P<0.05).

	Lysozyme (ug.mL^{-1})		**ACH50 (% hem.mL^{-1})**		**Serum bactericidal activity (log 2 + 1)**	
	PRE	**POST**	**PRE**	**POST**	**PRE**	**POST**
G1	14.60±2.67	19.49±2.64	5,90±2.19	13,89±0.42	3,78±0.31	2,89±0.28^{c}
G2	17.53±1.06	18.47±0.77	4,19±0.77	5,83±0.53	3,19±0.51	5,38±0.64^{ab}
G3	18.63±1.56	16.95±0.67	8,58±3.35	10,08±1.29	4,09±0.00	5,70±0.33^{ab}
G4	16.89±1.92	20.76±1.59	5,22±0.46	10,45±0.88	4,09±0.00	6,02±0.00^{a}
G5	16.62±2.06	20.51±1.53	7,06±2.11	10,90±0.38	3,19±0.51	4,73±0.32^{bc}
G6	16.62±2.34	21.78±0.99	6,58±2.22	7,41±0.33	3,19±0.51	4,73±0.32^{bc}
G7	16.52±0.81	16.95±0.48	6,48±0.39	8.16±0.46	3,79±0.62	5,70±0.33^{ab}
G8	18.35±2.47	18.03±0.66	7,68±1.81	8.70±0.71	3,78±0.31	4,73±0.32^{bc}
G9	14.51±3.25	18.79±1.38	6,54±0.83	9,57±0.25	3,78±0.31	6,02±0.00^{a}

PRE: pre-challenge; POST: post-challenge.

3.5 Liver histology and morphology

In fish, excess energy is normally stored as glycogen and/or lipid in the cytoplasm of hepatocytes, which is especially frequent in captive-raised fish (WOLF *et al.*, 2015). All groups experienced mild to moderate glycogen depletion in liver tissue (Table 9). The G3 and G8 received the highest and lowest overall score, respectively. Importantly, in G3, elevated lesion scores for hepatocellular and pancreatic epithelial necrosis, inflammation, and pigmented macrophage aggregates coincided with the highest cumulative score for lesions and elevated mortality. The better survival in G5,

G7 and G8 may be related to better liver health and integrity.

The groups composed of *Y. lipolytica* had lower overall scores, indicating that the yeast helped to preserve liver integrity. Since the deprivation of nucleotides greatly lowers the rate of hepatic protein synthesis, as demonstrated in a cirrhotic rat model, exogenous dietary nucleotides, such as those found in high concentrations in yeasts, play a crucial role in the repair and regeneration of liver damage (PÉREZ *et al.*, 2004). This property had previously been demonstrated, and because plasma alanine aminotransferase levels of catfish fed *S. cerevisiae* decreased, it was assumed that this supplement could help maintain the integrity of liver cell membranes and enhance liver function, as had the 3 g/kg and 6 g/kg treatments (ADEOYE *et al.*, 2020). Furthermore, the structure of the hepatocytes and villi remained essentially unchanged only in fish fed diets supplemented with the largest amount of yeast extract (15 g/kg), indicating that yeast nucleotides could improve hepatic function and promote liver and gut regeneration (HASSAAN *et al.*, 2018).

Table 9: Intensity of histological alteration in liver of Nile tilapia fed for 45 days, a control diet or a diet supplemented with *E. faecium*, *L. lactis* and *Y. lipolytica* alone or in different combinations.

Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**	**G9**
Glycogen and/or lipid storage	3.17	2.33	3.40	2.40	1.00	3.67	2.50	1.00	2.00
Hepatocellular necrosis	0.33	0.33	1.40	1.40	0.33	0.83	0.00	0.00	0.80
Pancreatic epithelial necrosis	1.00	1.50	1.60	1.80	0.67	1.33	0.50	0.00	1.20
Inflammation	1.50	1.17	2.20	2.00	0.67	2.00	1.25	0.75	1.60
Pigmented macrophages	0.83	0.67	1.40	0.60	0.67	1.00	0.25	0.50	0.60
Congestion in the vessels	0.33	0.00	0.80	0.80	0.50	0.83	0.50	0.75	0.00
Eosinophilic globules	0.83	0.67	1.60	1.80	1.00	2.00	0.00	0.00	1.20
Total	8.00	6.67	12.40	10.80	4.83	11.67	5.00	3.00	7.40

4 CONCLUSION

The inclusion of *L. lactis* and *Y. lipolytica* in the diet performed a synergistic and symbiotic effect in Nile tilapia, demonstrating that a mix of probiotic strains could grant an important tool for fish farming. The inclusion of *L. lactis* and *Y. lipolytica* as a symbiotic had a positive effect on immune response, survival after bacterial challenge and liver integrity. Further studies evaluating appropriate dosage and feeding regimens, as well as intestinal colonization should be considered.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Vanessa Gomes da Silva and Ulisses de Pádua Pereira conceived and designed the experiments. Vanessa Gomes da Silva, Leonardo Mantovani Favero, Raffaella Menegheti Mainardi, and Natália Amoroso Ferrari were involved in all *in vitro* and *in vivo* analyses of the study. Vanessa Gomes da Silva wrote the paper; Felipe Pinheiro de Souza: Methodology, Software, Formal analysis; Nelson Mauricio Lopera-Barrero: Conceptualization, Methodology; Ulisses de Pádua Pereira contributed to the methodology, supervision, validation, and project administration. Ulisses de Pádua Pereira coordinated all analyses of the project and proofread the manuscript. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

All additional information can be asked to corresponding author

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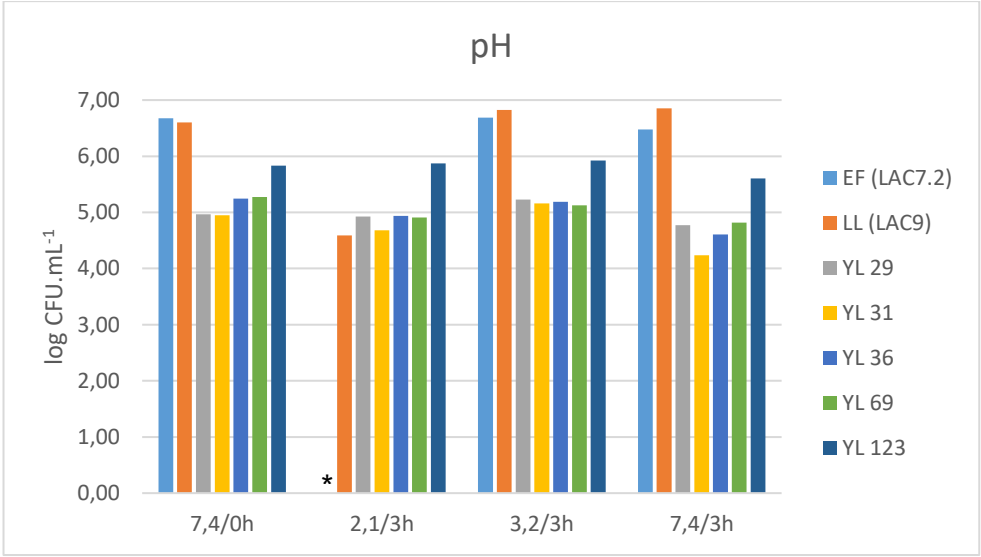
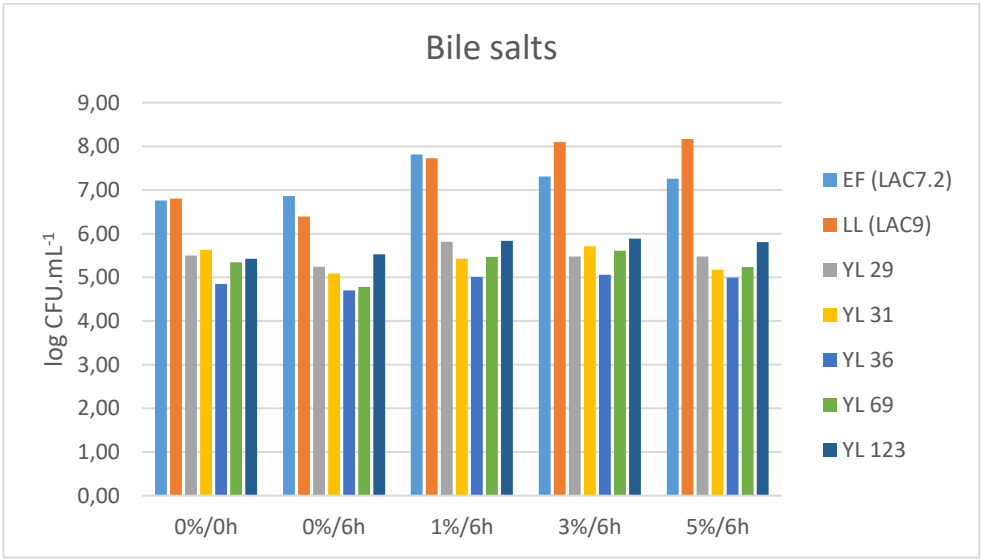
SUPLEMENTARY

Supplementary Figure 1: Probiotic characteristics of *E. faecium* (LAC7.2), *L. lactis* (LAC9) and *Y. lipolytica* (QU29, QU31, QU36, QU69, QU123). (A) Resistance of probiotic strains in PBS solution adjusted to different pH (2.1, 3.2, 7.4) for 3 h and pH 7.4/0h, at 28 °C. *E. faecium* was not viable at pH 2.1 (*); (B) Resistance of probiotic strains in PBS solution adjusted to different fish bile concentrations (0%, 1%, 3%, 5%) for 6 h and 0%/0h, at 28 °C; (C) Growth of probiotic strains in MRS and YPD medium containing different concentrations of NaCl (0%, 1.5%, 4.5%, 6.5%) for 24 h and 0%/0h, at 28 °C. Results were considered Too numerous to count (TNTC) and were not presented here (*).

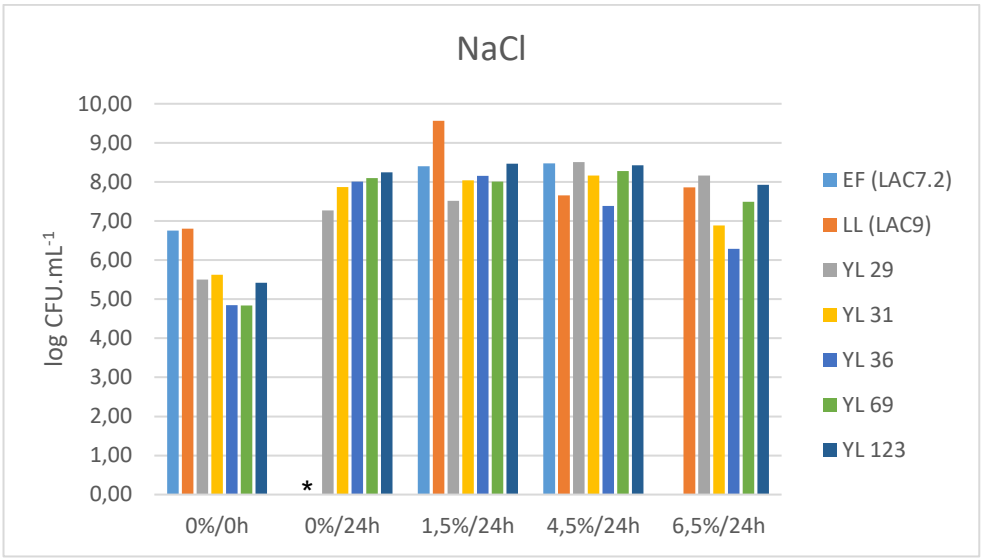
(A)



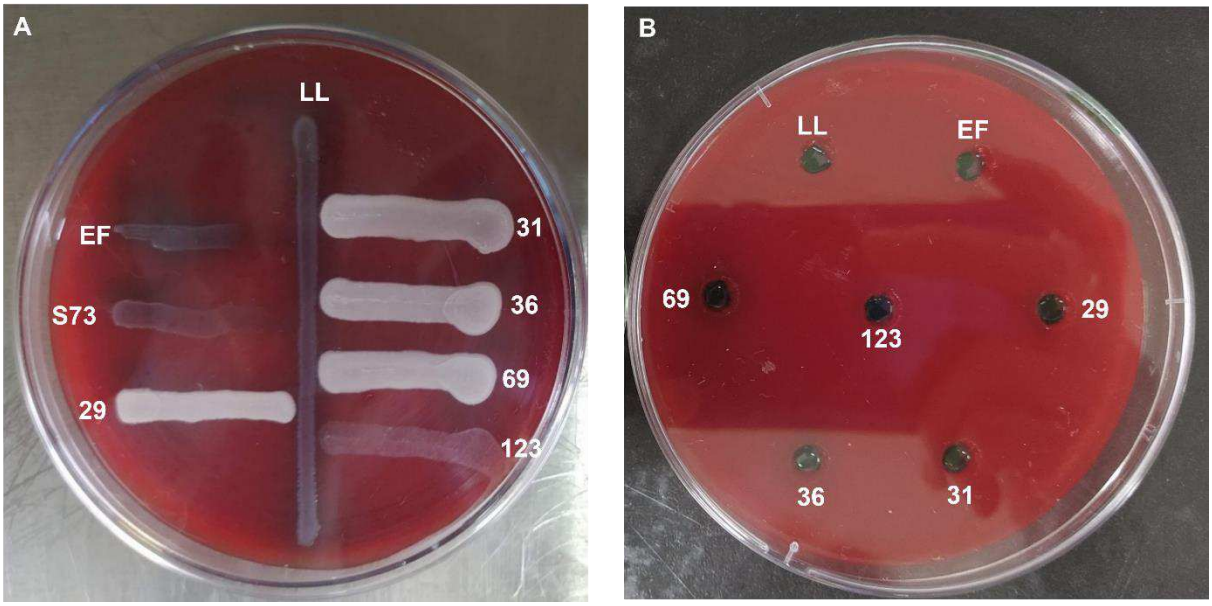
(B)



(C)



Supplementary Figure 2 – Antagonistic activity assays. (A) The cross-streak method. (LL) *L. lactis* (LAC9); (EF) *E. faecium* (LSC7.2); (S73) *S. agalactiae* serotype III; (29) *Y. lipolytica* (QU29); (31) *Y. lipolytica* (QU31); (36) *Y. lipolytica* (QU36); (69) *Y. lipolytica* (QU69); (123) *Y. lipolytica* (QU123); and (B) the agar well diffusion method.



8 CONSIDERAÇÕES FINAIS

O uso de imunoestimulantes, como probióticos, tem-se tornado frequente e visa difundir uma aquicultura sustentável, promovendo segurança alimentar e diminuição no uso de antimicrobianos.

Neste estudo, foi possível observar os efeitos benéficos promovidos por uma combinação de ácidos orgânicos e uma associação de probióticos em tilápias do Nilo. Evidenciaram-se incrementos no desempenho zootécnico, melhora em parâmetros imunes, assim como melhor taxa de sobrevivência contra *Francisella orientalis* e *Streptococcus agalactiae*.

Com os resultados positivos deste estudo, outras formas de administração podem ser estudadas, como a administração via água, ou diferentes dosagens para se obter resultados mais promissores, bem como explorar períodos mais curtos de administração, o que poderia ser benéfico para o produtor. Tais estudos também devem ser avaliados com espécies nativas, como tambaqui e pacu, os quais apresentam grande viabilidade econômica, porém com uma exploração ainda tímida.

Alguns testes adicionais podem ser empregados para melhor caracterização das cepas probióticas, como testes para avaliar a secreção de moléculas funcionais, compostos antioxidantes e caracterização e/ou quantificação de enzimas produzidas. As novas tecnologias ômicas, como transcriptômica, proteômica e metabolômica, também podem auxiliar ou acelerar no desenvolvimento de novos produtos probióticos.

Finalmente, estudos mais amplos de combinações entre cepas de probióticos contra uma maior gama de patógenos, como vírus e fungos, são necessários.

ANEXOS

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

19/10/2021 870210096313 09:22

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2021 020920 8

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE ESTADUAL DE LONDRINA

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 78640489000153

Nacionalidade: Brasileira

Qualificação Jurídica: Instituição de Ensino e Pesquisa

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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 19/10/2021 às 09:22, Petição 870210096313

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): PROBIÓTICO COM ATIVIDADE ANTIMICROBIANA E IMUNOMODULADORA PARA PREVENÇÃO E CONTROLE DE BACTERIOSES EM TILÁPIAS E POTENCIALIZADOR VACINAL CONTRA ESTREPTOCOCOSE

Resumo: A presente invenção refere-se a um probiótico a base de Lactococcus lactis cepa LAC9 isolada de pirarucu saudável e os métodos e processos da produção deste probiótico e de sua administração na alimentação de peixes, a fim de obter ganhos zootécnicos e imunológicos, incrementando o ganho de peso e crescimento e limitando, assim, o impacto das bacterioses sobre esses animais, em especial para aqueles criados em cultivo intenso. Adicionalmente, a o fornecimento deste probiótico aumentou de forma significativa a eficácia vacinal em tilápias desafiadas com Streptococcus agalactiae. Este probiótico e sua aplicação na piscicultura brasileira, é eficaz e trouxe incrementos zootécnicos, de alguns parâmetros imunológicos e reduziu a mortalidade causada por estreptococose. A utilização de um probiótico oriundo de peixes brasileiros é um método seguro para peixes de produção, por se adaptar melhor ao trato digestório e oferecer menos riscos de reações adversas, assim como gerando menor impacto para o ambiente.