SUELEN APARECIDA SUPHORONSKI

FITOGÊNICOS, ÁCIDOS ORGÂNICOS E PROBIÓTICOS NA ALIMENTAÇÃO DE TILÁPIAS DO NILO E SEUS EFEITOS NA IMUNIDADE, PRODUTIVIDADE, RESISTÊNCIA A DOENÇAS E MICROBIOTA INTESTINAL

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

Orientador: Prof. Dr. Ulisses de Pádua Pereira

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Londrina, 13 de dezembro de 2019.

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“Se eu vi mais longe, foi por estar sobre ombros de gigantes”.

Isaac Newton

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RESUMO

O Brasil é o quarto país maior produtor de tilápias, porém com a intensificação da produção, a exigência de manejos e monitoramento aumenta, e se estes não forem realizados adequadamente podem ocorrer o aparecimentos de doenças. As bacterioses, como estreptococcose e francicelosse, estão entre as principiais causas de perdas dos produtores. A principal forma utilizada atualmente para prevenir e tratar essas doenças é a antibioticoterapia. Contudo, sabe-se que o uso indiscriminado de antimicrobianos pode causar seleção de micro-organismos resistentes e comprometer, inclusive a saúde humana. A utilização de vacinas nem sempre é viável ou ainda não existem vacinas para certos patógenos causadores de doenças para os peixes. Por isso métodos alternativos estão sendo desenvolvidos e utilizados na piscicultura, como a utilização de probióticos, ácidos orgânicos e fitogênicos na alimentação de animais, auxiliando na melhora do sistema imunológico. O objetivo desse trabalho, foi avaliar a utilização de aditivos alimentares quanto a performace, parâmentros hematológicos, imunológicos, resistência a doenças, histologia e microbiota intestinal de tilápias do Nilo, para isso foram realizados dois experimentos. No primeiro experimento, foi avaliado os efeitos da suplementação com A-Live (fitogênico), individualmente e/ou em combinação com Aquaform (diformato de potássio) no desempenho do crescimento de tilapias do Nilo (*Oreochromis niloticus*), parâmetros imunes inatos, microbiota intestinal e resistência à *Francisella noatunensis* subsp. *orientalis*. Cada grupo continha 140 individuos e foram três tratamentos com diferentes concentrações dos produtos. Após o desafio verificou-se que a mortalidade foi significativamente menor nos grupos tratados, além desses grupos apresentarem melhor desempenho de crescimento e também nos parâmetros de imunidade. Mostrando assim que ocorre um efeito benéfico com o uso desses produtos em tilápias do Nilo. No segundo experimento, foi avaliado a administração na ração e/ou água do probiótico *Enterococcus faecium*, isolado de tilápias do Nilo. Com a bactéria probiótica foram realizados testes de inibição e também o sequenciamento do seu genoma completo antes dos testes *in vivo*. Tilápias do Nilo (45 por grupo com duas repetições) receberam durante 38 dias o probiótico. Após esse período encontramos diferença significativa no ganho de peso médio final no grupo G1, e nos trombócitos dos grupos que receberam probiótico na ração (G1 e G3). *Cetobacterium* foi o gênero mais abundante da microbiota intestinal em todos os grupos, incluindo CN. Os grupos foram reordenados e desafiados com *F. noatunensis* subsp. *orientalis* e *Streptococcus agalactiae*. Após o desafio verificou-se diferença estatística na mortalidade de peixes infectados com *S. agalactiae* do grupo CN em comparação com os grupos tratados. *Enterococcus faeicum* mostrou-se eficaz para melhorar a imunidade dos peixes. Dessa forma estudos utilizando produtos alternativos ao uso de antimicrobianos como ácidos orgânicos, fitogênicos e probióticos autoctones, são de suma importância para prevenir o aparecimento de bactérias resistentes, já que estes estimulam o sistema imune, melhoram a performace e ainda não interferem na micro-comunidade aquática local.

Palavras-chave: *Francisella noatunensis* subsp. *orientalis*, componentes fitogênicos, probióticos, *Streptococcus agalactiae*, tilapicultura.

SUPHORONSKI, Suelen Aparecida. Phytogenics, organic acids and probiotics in the feeding of Nile tilapia and their effects on immunity, productivity, resistance to diseases and intestinal microbiota. 2019. 93p. Dissertation (Master in Animal Science) - State University of Londrina, Londrina, 2019.

ABSTRACT

Brazil is the fourth largest tilapia producing country, however with the intensification of production, the requirement for management and monitoring increases, and if these are not carried out properly, diseases may occur. Bacterioses, such as streptococcosis and francicellosis, are among the main causes of loss for producers. The main way currently used to prevent and treat these diseases is antibiotic therapy. However, it is known that the indiscriminate use of antimicrobials can cause selection of resistant microorganisms and compromise, including human health. The use of vaccines is not always feasible or there are still no vaccines for certain disease-causing pathogens for fish. Therefore, alternative methods are being developed and used in fish farming, such as the use of probiotics, organic and phytogenic acids in animal feed, helping to improve the immune system. The objective of this work was to evaluate the use of food additives in terms of performance, hematological, immunological parameters, resistance to diseases, histology and intestinal microbiota of Nile tilapia. For this, two experiments were carried out. In the first experiment, the effects of supplementation with A-Live (phytogenic), individually and / or in combination with Aquaform (potassium diformate) on the growth performance of Nile tilapia (Oreochromis niloticus), innate immune parameters, intestinal microbiota were evaluated and resistance to Francisella noatunensis subsp. orientalis. Each group contained 140 individuals and there were three treatments with different concentrations of the products. After the challenge, it was found that mortality was significantly lower in the treated groups, in addition to those groups presenting better growth performance and also in the parameters of immunity. Thus showing that there is a beneficial effect with the use of these products in Nile tilapia. In the second experiment, the administration in the feed and / or water of the probiotic Enterococcus faecium, isolated from Nile tilapia, was evaluated. Inhibition tests were carried out with the probiotic bacteria and also the sequencing of its complete genome before in vivo tests. Nile tilapia (45 per group with two replications) received the probiotic for 38 days. After this period, we found a significant difference in the final average weight gain in the G1 group, and in the thrombocytes of the groups that received probiotic in the diet (G1 and G3). Cetobacterium was the most abundant genus of the intestinal microbiota in all groups, including CN. The groups were reordered and challenged with F. noatunensis subsp. orientalis and Streptococcus agalactiae. After the challenge, there was a statistical difference in the mortality of fish infected with S. agalactiae in the CN group compared to the treated groups. Enterococcus faeicum was shown to be effective in improving fish immunity. Thus studies using alternative products to the use of antimicrobials such as organic acids, phytogenics and autochthonous probiotics, are extremely important to prevent the appearance of resistant bacteria, since they stimulate the immune system, improve performance and do not yet interfere in the micro-community. local aquatic.

Key words: *Francisella noatunensis* subsp. *orientalis*, phytogenic components, probiotics, *Streptococcus agalactiae*, tilapiculture.

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REVISÃO DE LITERATURA

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ARTIGO 2

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LISTA DE ABREVIATURAS E SIGLAS

BLAST:	Basic Local Alignment Search Tool
CAPES:	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA/UEL:	Comissão de Ética no Uso de Animais/ Universidade Estadual de Londrina
CFU:	Unidades Formadoras de Colônia
CNPq:	Conselho Nacional de Desenvolvimento Científico e Tecnológico
FAO:	Organização das Nações Unidas para Agricultura e Alimentação
LABBEP:	Laboratório de Bacteriologia em Peixes
MRS:	Man, Rogosa e Sharpe Lactobacillus Kasvi®
NCBI:	National Center for Biotechnology Information
PEIXE BR:	Associação Brasileira da Piscicultura
RAST:	Rapid Annotation using Subsystem Technology

SUMÁRIO

1 INTRODUÇÃO

O Brasil ocupa lugar de destaque no cenário da aquicultura mundial, sendo o quarto maior produtor de tilápias do mundo (PEIXEBR, 2019). Porém com o crescimento acelerado da aquicultura surge novos desafios, como a biosseguridade na produção (MIAN et al., 2009). Atualmente, a principal medida terapêutica utilizada no caso de surtos de bacterioses em peixes é a antibioticoterapia, porém o uso indiscriminado de antimicrobianos para controle de doenças aumenta a pressão seletiva exercida sobre as comunidades microbianas e estimula o surgimento natural de resistência bacteriana. Além disso, pode causar sérios riscos ao meio ambiente e segurança alimentar da população humana (BIDHAN, 2014). Para evitar o uso indiscriminado de antimicrobianos, outros métodos profiláticos alternativos estão sendo utlizados, tais como probióticos, prebioticos, compostos fitogênicos e ácidos orgânicos. Estes métodos demonstraram ser ferramentas promissoras que podem estimular o sistema imune auxiliando no combate às doenças (MOURIÑO et al., 2012).

Já existam vários trabalhos com a utilização de probióticos em peixes, porém esses produtos normalmente são importados de outros lugares e/ou de espécies diferentes, o que pode interferir na microbiota intestinal dos peixes que a recebem e também no ambiente em que estes animais vivem. Da mesma forma que produtos como, compostos fitogênicos e ácidos orgânicos normalmente são avaliados separadamente e não se têm muitos dados do seu efeito em peixes quando utilizados em associação.

Por isso um probiótico autóctone, *Enterococcus faecium* cepa LAC7.2, foi isolado, identificado e produzido para ser utilizado na alimentação de tilápias do Nilo. Ainda, os produtos comercias da Addcon- Salus (Brasil) e MiXscience (França), sendo: diformato de potássio, um sal orgânico com nome de Aquaform® e o composto fitogênico, derivados de extratos vegetais da Família Alliaceae (A-live ®) foram testados na dieta de tilápias do Nilo.

Portanto o objetivo desse trabalho foi verificar os parâmetros hematológicos, imunológicos, resistência a doenças e histologia e microbiota intestinal em tilápias do Nilo (*Orechromis niloticus*) após a utilização do *Enterococcus faecium* cepa LAC7.2 e dos produtos A-live® e Aquaform®, separados ou em associação.

2 REVISAO DA LITERATURA

2.1 TILAPICULTURA

Segundo dados da Organização das Nações Unidas para Agricultura e Alimentação a produção global aquícola (incluindo plantas aquáticas) foi de 110,2 milhões de toneladas. Mais uma vez o grupo mais produzido foi o de peixes, com 54,1 milhões de toneladas, seguido de algas com 30,1 milhões, moluscos com 17,1 milhões e crustáceos com 7,9 milhões de toneladas produzidas. Dentre as espécies mais produzidas estão as carpas e em quarto lugar a tilápia do Nilo (*O. niloticus*). Hoje, ainda a produção global pesqueira é maior que a produção aquícola, mas a estimativa até 2030 é que os números de invertam e que a aquicultura contribuia com 60% do pescado para consumo humano (FAO, 2018).

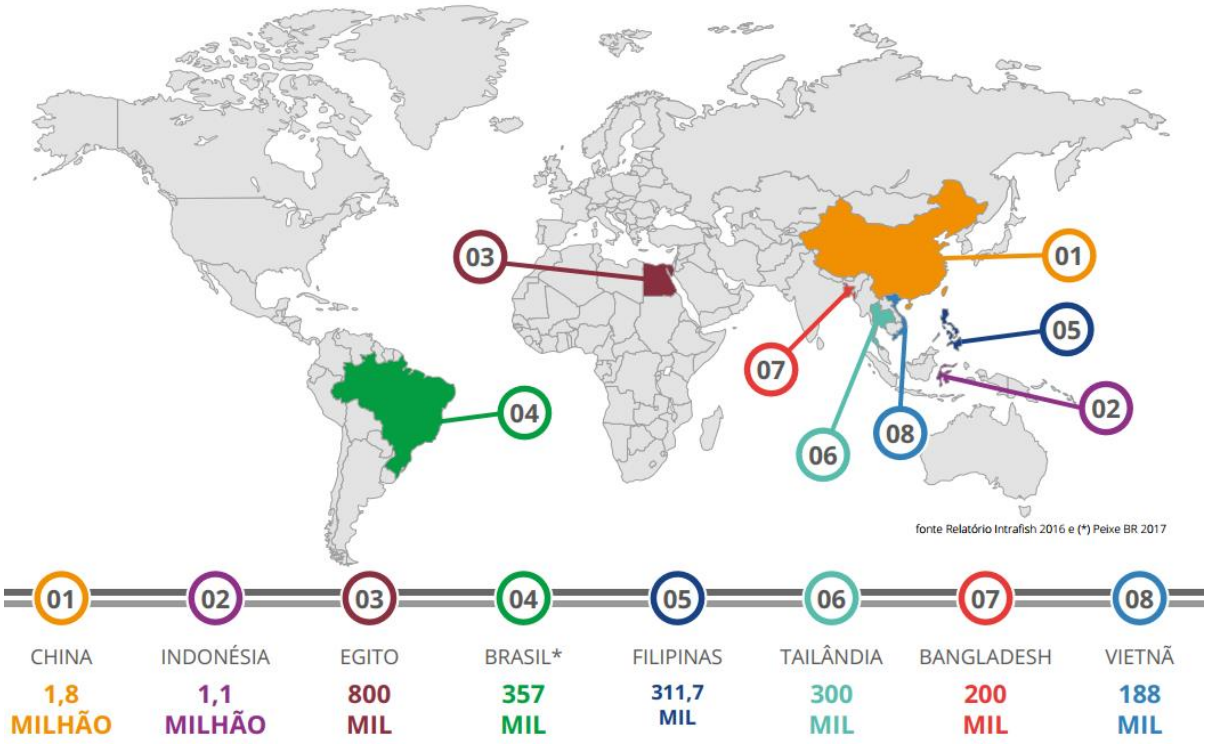
No Brasil, a produção de peixes aumentou 4,5% em 2018 e atingiu 722.560 toneladas, sendo que a espécie mais produzida foi a tilápia. A produção de tilápia cresceu 11,9%, chegando a um total de 400.280 toneladas, com essa produção o Brasil ficou em quarto como maior produtor de tilápia do mundo, ficando atrás apenas de China, Indonésia e Egito (Figura 1). O Paraná em 2018 foi o estado que mais produziu peixes (129.900 toneladas) em seguida Santa Catarina (73.200 toneladas) e Rondônia (72.800 toneladas). Em relação à tilapicultura, o Paraná também está em primeiro lugar em produção, com 123.00 toneladas, em segundo São Paulo com 69.500 toneladas e em terceiro Santa Catarina com 33.800 toneladas (PEIXEBR, 2019).

A tilápia do Nilo é um peixe onívoro, rústico, que se adapta facilmente ao confinamento em sistemas intensivos de criação, tolerando baixos níveis de oxigênio e elevadas concentrações de amônia, motivos pelos quais tornaram também possível seu cultivo comercial em águas salobras ou salgadas (KUBIZA, 2005). Porém, mesmo com toda essa rusticidade, quando a sua criação é realizada em sistema intensivo com alta densidade populacional e não são tomadas as medidas necessárias de biosseguridade, há um alto risco de ocorrer doenças infecciosas e parasitárias (MARCUSSO, 2017).

Os principais agentes etiológicos associados aos casos de mortalidade em psiciculturas são protozoários, vírus, fungos e bactérias, porém as bactérias destacam-se e podem causar surtos que chegam a dizimar até 50% dos animais (ASSEFA E ABUNNA, 2018)

Os principais patógenos bacterianos associados à doença da tilápia do Nilo são *Streptococcus agalactiae, S. iniae, S. dysgalactiae, Flavobacterium columnare e Francisella noatunensis* subsp. *orientalis* (FIGUEIREDO et al., 2012).

Figura 1 Maiores Produtores Mundiais de Tilápia



Fonte: PeixeBR (2019).

2.2 ESPREPTOCOCOSE

As bactérias causadoras de estreptococose em peixes são cocos Gram positivas, oxidase negativa e incluem as seguintes espécies: *Streptococcus iniae, S. agalactiae, S. parauberis, S. difficile, S. shiloi e S. dysgalactiae* (PRADEEP et al., 2016).

Atualmente, infecções causadas por *Streptococcus* spp., especialmente *S. agalactiae* e *S. iniae*, são as mais comuns e causam significativas perdas econômicas para a tilapicultura. Sua prevalência e severidade dependem de múltiplos fatores ambientais, incluindo temperaturas de água morna (~28ºC), aumento dos níveis de amônia, baixos níveis de oxigênio dissolvido, causados por manejo e alta densidade de estocagem (LIU et al., 2016). A estreptococose causa septicemia, exoftalmia, meningoencefalite nos peixes, causando surtos agudos com alta mortalidade ou de forma crônica, principalmente no verão (EVANS et al., 2008).

S. agalactiae pode infectar outros animais, incluindo o homem, e com base na composição do polissacarídeo capsular, as espécies podem ser subdivididas em dez sorotipos (Ia, Ib e II a IX) (CHIDEROLI et al., 2017; EVANS et. al., 2008). No Brasil apenas os tipos Ia e Ib foram relatados, porém em 2016 houve um surto com alta mortalidade na região nordeste e após isolamento foi comprovado através da biologia molecular que se tratava do sorotipo III. Ainda, nesse estudo verificou que o sorotipo III é multiresistente e bom formador de biofilme, aumentando assim as chances de contaminação (CHIDEROLI et al., 2017). Em estudo sobre a caracterização e virulência de *S. agalacteae* de diferentes espécies, bovina, humana e de peixes, foi possível verificar que algumas cepas bovina e humana são capazes de infectar peixes e causar meningoencefalite, demonstrando que pode ocorrer infecção em diferentes espécies de hospedeiros pela mesma cepa (PEREIRA et al., 2010).

Para a estreptococose existem alguns tipos de vacinas como inativada, viva atenuada, recombinante, porém a que se destaca com a maior eficácia é a vacina inativada e a forma de aplicação da vacina a intraperitoneal (LIU et al., 2016). Porém o manejo para a vacinação é difícil visto que é realizado individualmente. Outro desafio é eficácia da vacina, já que essa pode variar devido à existência de diferentes sorotipos e os perfis genéticos das cepas circulantes (CHEN et al., 2012).

2.3 FRANCICELOSE

Francisella spp. pertence à família Francisellaceae e são bactérias Gram-negativas, estritamente aeróbicas, não esporuladas, cocobacilos não-móveis,

capsulados e intracelular facultativo (SCHRALLHAMMER et al., 2011). O gênero *Francisella* spp. pode ser classificado em: *F. tularensis*, que engloba as subespécies *tularensis, mediasiatica, novicida* e *holarctica; F. halioticida*; *F. hispaniensis*; e *F. noatunensis,* que engloba as subespecies *noatunensis* e *orientalis*; *F. persica* e *F. philomiragia*. Essa bactéria pode causar doença a diversos hospedeiros vertebrados.

A franciselose nos peixes pode ser causada pelas espécies *F. noatunensis* subsp *orientalis* e *F. noatunensis* subsp *noatunensis*. Os principais sinais clínicos apresentados pelos peixes acometidos com essa doença são: letargia, melanose, exoftalmia e natação erradica (SOTO et al., 2009). É possível observar através de exame macroscópico de peixes autopsiados a presença de nódulos brancos, principalmente em rim e baço (VOTEJTECH et al., 2009). Além da alta densidade populacional, o estresse térmico é um dos fatores que predispõem à franciselose, pois a doença se manifesta principalmente em estações frias com temperaturas de aproximadamente 21ºC (SOTO et al., 2009).

Várias espécies de peixes podem ser afetadas por essa bactéria como: *Oreochromis* sp (tilápia), *Gadus morhua* (bacalhau do Atlântico), *Salmo salar* (Salmão do Atlântico), *Morone chrysops* (baixo branco), *M. saxatilis* (robalo riscado) e *Parapristipoma trilinineatum* (grunhido de três linhas) (COLQUHOUN; DUODU, 2011). Relacionado com estudos de sensibilidade, em uma cepa de *Francisella* spp. isolada de peixe foi verificado resistência em seis dos dez antimicrobianos testados, sendo eles: trimetoprim, sulfametazol, penicilina, ampicilina, cefalosporinas de segunda geração e eritromicina (OTTEM et al., 2007).

A produção de vacinas para *Francisella* spp. e outros patógenos intracelulares é um desafio visto que a bactéria possuem mecanismos de evasão do sistema imune, fazendo com que a vacina apresenta baixa eficácia (PECHOUS; MCCARTHY; ZAHRT; 2009; SOTO et. al., 2009), por isso ainda não há vacina comercial disponível no Brasil ou no mundo para essa doença.

2.4 PROBIÓTICOS

Dentre as práticas sanitárias alternativas ao uso de antibióticos, destaca-se a utilização de aditivos alimentares, como os probióticos que auxiliam no aumento da capacidade imunológica dos peixes.

O termo probiótico é derivada do latim e grego e tem como significado “para a vida”, sendo definido como micro-organismos vivos que oferecem benefícios à saúde do hospedeiro, quando consumidos em quantidades adequadas, proporcionando ação protetora contra patógenos e benefícios nutricionais (HOSSAIN; SADEKUZZAMAN; HA, 2017). A maioria dos probióticos são bactérias ácido lácticas mas podem ser também leveduras, sendo os principais gêneros: *Lactobacillus bacteriano, Bifidobacterium, Lactococcus, Carnobacterium, Enterococcus, Streptococcus, Pediococcus, Propionibacterium, Leuconosto, Bacillus* e Leveduras *Saccharomyces* (AMARA; SHIBL, 2015).

Evidências provam que a microbiota complexa presente no trato gastrintestinal dos animais é eficaz no fornecimento e resistência à doenças. Porém, a composição dessa flora pode ser alterada por influências alimentares e ambientais, tornando o animal suscetível a certas doenças e também reduzindo sua eficiência na utilização de alimentos. Por isso muitos estudos estão sendo realizados com probióticos, visando reestabelecer a condição natural da flora gastrintestinal (AMARA; SHIBL, 2015).

Algumas características são critérios de seleção para um probiótico, como: ser atóxico, não patogênico, capaz de sobreviver e metabolizar no ambiente intestinal, resistindo ao baixo pH do estômago, ácidos orgânicos, bile e enzimas presentes no intestino e ser estável sob armazenamento e condições de campo (HOSSAIN; SADEKUZZAMAN; HA, 2017). Por isso ensaios *in vitro* devem ser realizados para permitir uma triagem preliminar do potencial probiótico e seus atributos, como resistência a condições gastrointestinais, capacidade de desconjugação de sais biliares e propriedades de adesão para o intestino (REPALLY et al., 2018).

As mudanças no ambiente intestinal, devido as probióticos, incluem a produção de compostos responsáveis pela atividade antimicrobiana, competição com

potenciais patógenos por sítios de ligação ou estimulação da função da mucosa intestinal, aumento das respostas imunes como as citocinas reguladoras, inibição da virulência, da expressão gênica ou proteica em patógenos gastrintestinais e melhora da estrutura e digestão do intestino (KIRON, 2012).

Estudos recentes mostraram o efeito benéfico (aumentando a imunidade e reduzindo a mortalidade frente a patógenos) de micro-organismos probióticos em tilápias do Nilo (STANDEN et al., 2016), surubim (PEREIRA et al., 2016), linguado (PARK et al., 2016) e juvenis de truta arco-íris (SAFARI et al., 2016). Estudos realizados com tilápia do Nilo e juvenis de esturjão (TA'ATI et al., 2011) relatam que os animais que receberam probióticos juntamente com a ração obtiveram maiores ganhos de peso em comparação com os grupos não tratados.

A utilização de *Bacillus amyloliquefaciens* na dieta de *Catla catla* na dosagem de 10^9 UFC/grama demonstrou significativo aumento do teor de proteína, melhora em parâmetros imunológicos e resistência a doenças (DAS et al., 2013). Ainda, estudos comparando dois tipos de probióticos, *Bacillus subtilis* WB60 e *Lactobacillus plantarum* KCTC3928, na alimentação de *Anguilla japonica*, demonstrou resultados melhores na imunidade e performace, incluindo parâmetros de histologia do intestino, na dosagem de 10^8 UFC/grama com o *Bacillus subtilis* (LEE et al., 2017).

Atualmente no mercado encontramos diferentes fórmulas comerciais de probióticos testadas e utilizadas em peixes. Porém observa-se que probióticos isolados de outros animais ou em espécies de peixes que não sejam o alvo, podem apresentar resultados controversos, sendo necessário o isolamento e desenvolvimento de probióticos espécie-específicos, ou seja, probióticos autóctones (MOURIÑO, 2010). Além disso, muitos dos probioticos foram isolados em outros países, o que pode influenciar negativamente a microflora aquático das bacias hidrográficas do Brasil, fazendo necessário o estudo de probiótico autóctones e que sejam utilizados na mesma região que foram isolados. Por isso o desenvolvimento de probióticos isolados no Brasil é de suma importância, assim esse trabalho abordará sobre o isolamento, estudos *in vitro* e *in vivo* de *Enterococcus faecium* strain LAC 7.2 e ainda a utilização da bioinformática para estudo genômico comparativo, descoberta *in sílica* de metabólitos secundários e de fatores de virulência.

2.5 COMPOSTOS FITOGÊNICOS E ÁCIDOS ÓRGANICOS

Os compostos fitogênicos são derivados de plantas e que adicionados a ração podem melhorar o crescimento e o desempenho sanitário dos peixes. Essas substâncias bioativas possuem propriedades diferentes como antioxidante, antimicrobiano, anticarcinogênico, analgésico, inseticidas, antiparasitário e anticoccidial, além disso ajudam a melhorar a palatabilidade dos alimentos, aumentando assim a performance dos peixes. Esses componentes podem ser originários de diferentes partes das plantas como folhas, raízes, tubérculos e frutas e serem utilizados nas formas de: óleo, pó ou extrato. (ALEMAYEHU et al., 2018; BHARATHI et al., 2019)

Estudos realizados por exemplo, com alho (*Allium sativum*) demonstraram que independente da forma (natural, óleo ou pó) que os peixes do experimento (*O. niloticus*) receberam o composto fitogênico, eles tiveram aumento significativo no crescimento, na atividade antioxidante e menor mortalidade (METWALLY, 2009). Outro trabalho utilizando tilápias do Nilo suplementadas com óleo essencial e que foram desafiadas com *Aeromonas hydrophila*, apresentaram melhor desempenho de crescimento e maior sobrevida comparando com o grupo controle.

Um exemplo de compsto fitogênico é o produto A-live® desenvolvido pela empresa Addcon- Salus (Brasil) e MiXscience (França) que é uma mistura de extratos de plantas da família Alliaceae e argilas minerais formulados para ter alta dispersão na água, facilmente aplicável na aquicultura e que pode ser utilizado em peixes e camarões.

Os ácidos orgânicos são compostos carboxílicos, considerados ácidos fracos pois se dissociam parcialmente na água para formar um íon hidrogênio e um íon carboxilato. Os ácidos orgânicos (e seus sais), como ácido fórmico, cítrico, acético, propiônico e butírico, entre outros, já são utilizados como acidificantes com sucesso na indústria de ração animal, incluindo a aquicultura, para melhorar o desempenho e a saúde intestinal (CASTILLO et al., 2014).

Estes agem na diminuição do pH do estômago e intestino, reduz o tempo de esvaziamento do trato digestivo, aumenta a atividade de pepsina e a retenção de

nitrogênio, melhorando a digestibilidade, absorção e transporte de mineirais (REDA et al., 2016) e ainda podem penetrar na parede de certas bactérias, inibindo assim o crescimento de bactérias patogênicas. Suas ações dependem de vários fatores, como espécies de peixes, tamanho, idade, tipos e níveis de ácidos orgânicos, controle de ração e qualidade da água (ALEMAYEHU et al., 2018; BHARATHI et al., 2019).

Observou-se que em tilápias do Nilo que receberam diformato de potássio em diferentes concentrações: 0,2% e 0,3% exibiram melhorias significativas na performance e a mortalidade cumulativa por *Aeromonas hydrophyla* foi menor que do grupo controle. Além disso o grupo que recebeu a 0,3% também teve estímulo da flora intestinal benéfica e de sistema imune (ELALA; RAGAA, 2015).

A empresa Addcon- Salus (Brasil) e MiXscience (França) também produz um ácido orgânico, Aquaform®, que é o diformato de potássio utilizado em peixes e camarões. Por isso em um outro experimento, estes produtos, A-live® e Aquaform® foram testados isolados ou em associação para verificar sua eficácia em tilápias do Nilo.

2.6 GENOMA

O estudo do genoma na área da bacteriologia iniciou-se em 1995 através do primeiro sequenciamento completo do genoma da *bactéria Haemophilus influenzae* por meio da técnica de Sanger (FLEISCHMANN et al., 1995).

Porém o alto custo era um dos entraves para o desenvolvimento de projetos de sequenciamento. Em 2005, com o advento da primeira tecnologia de sequenciamento *high throughput* ou *NGS* (sequenciamento de próxima geração) os custos diminuíram, o que facilitou o sequenciamento para a obtenção das *reads* (leituras) dos genomas (LOMAN et al., 2012). E dentre as tecnologias de NGS, podemos destacar as plataformas de segunda geração: 454 GS FLX system (Roche), Illumina GA IIx (Illumina), HeliScope (Helicos) e SOLiD 5500 XL system (ABI) (KAUR; MALIK, 2013).

A genômica comparativa estuda as similaridades e diferenças entre os organismos através da comparação do seu material genético. Com isso é possível a identificação de regiões codificantes no genoma, que por sua vez, permite a

caracterização da provável função dessas regiões codificantes, plasticidade genômica e estabelecimento de relações filogenéticas entre espécies e cepas (SIVASHANKARI; SHANMUGHAVEL, 2007; OGIER et al., 2010). Ainda, com o estudo comparativo do genoma podemos definir a diversidade genômica entre espécies correlatas e intra-espécies.

Atualmente há uma grande diversidade de genomas bacterianos disponíveis em banco de dados, tanto de micro-organismos que causam doenças quanto de potenciais probiótico, o que permite estudos aprofundados em diversas áreas como a filogenômica. Os estudos de filogenética permitem avaliar diferenças a nível de nucleotídeos, utilizando genomas completos (DELSUC et. al., 2005).

A construção de árvores filogenômicas e inferência de divergências evolucionárias podem ser realizadas utilizando o software Gegenees. O Gegenees identifica genes comumente compartilhados entre os genomas e cria uma matriz de distância baseada na porcentagem de similaridade entre os conteúdos variáveis dos genomas em questão (ÅGREN et al., 2012).

Outros programas podem ser utilizados para a detecção de metabólitos secundários, como o Anti-SMACH, fornecendo diversas informações relevantes sobre a atividade bacteriana. No caso de probiótico, essas informações sobre metabóltios pode ser de suma importância para o conhecimento do potencial inibitório e imunológico da bactéria (BERTRAND et. al., 2014).

Assim, novas ferramentas tecnológicas podem ser utilizadas para o conhecimento de bactérias e outros micro-organismos que podem ser de suma importância para a saúde humana e animal.

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3 HIPÓTESE

Os produtos A-live® e Aquaform® fornecidos para tilápias do Nilo melhoram o seu desempenho zootécnico e/ou sua resposta imunitária contra francicelose.

O uso da bioinformática possibilta o conhecimento sobre a cepa com potencial probiótico de *Enterococcus faecium* LAC 7.2 e este, fornecido na ração estimula resposta imune contra doenças bacterianas.

4 OBJETIVOS

4.1 Objetivo Geral

-Avaliar a ação A-live® e/ou Aquaform® e *Enterococcus faecium* LAC 7.2, em diferentes experimentos, com relação ao crescimento, imunidade, morfologia, microbiota gastrintestinal e na proteção contra estreptococose e franciselose em tilápias do Nilo.

4.2 Objetivos Específicos

- Avaliar o efeito do A-live® e Aquaform® e do *E. faecium* LAC 7.2 fornecidos na ração nos: índices zootécnicos, tais como ganho de peso, conversão alimentar, dentre outros; nos parâmetros hematológicos e imunidade; na morfologia e microbiota intestinal e na sobrevivência de tilápias do Nilo desafiadas com *Streptococcus agalactiae* e/ou *Francisella noatunensis* subsp. *orientalis*.
- Sequenciar o genoma de *E. faecium* LAC 7.2, fazer a montagem, anotações e depositar no GenBank (NCBI).
-Fazer testes *in vitro* para verificação do potencial probiótico do *E. faecium* LAC 7.2.

5 ARTIGO PUBLICADO NO PERIÓDICO SCIENTIFIC REPORTS: “Effects of a phytogenic, alone and associated with potassium diformate, on tilapia growth, immunity, gut mcrobiome and resistance against francisellosis”

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Effects of a phytogenic, alone and associated with potassium diformate, on tilapia growth, immunity, gut microbiome and resistance against francisellosis

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This work evaluated the effects of dietary supplementation of A-Live (phytogenic) either individually or in combination with Aquaform (potassium diformate, acidifier) on juvenile Nile tilapia (*Oreochromis niloticus*) growth performance, innate immune parameters, gut microbiome, and resistance against *Francisella noatunensis* subsp. *orientalis* challenge. Each experimental group contained 140 fishes (34.3 ± 0.33) in two 150L tanks. The experimental design consisted of five groups: a negative control; treated groups (G1, G2, G3) supplemented with different concentrations of A-Live and Aquaform in the feed; and a positive control (PC) for pathogen infection. Groups G1, G2, G3, and PC were challenged with *Francisella* spp. after 15 days. After infection, the mortality was significantly lower in groups G1, G2, and G3 ($p < 0.01$). Furthermore, these groups showed significant increase ($p < 0.05$) in daily weight gain, feed conversion rate, and specific growth rate. The PC group presented increase ($p < 0.05$) in the leukocytes and neutrophils number. Innate immunity parameters showed no difference between treatments after infection. Microbiome analysis revealed an increased number of bacteria belonging to the Vibrionaceae family after pathogen infection suggesting a secondary pathogen function of these bacteria. These results validate the beneficial effects of these products in tilapia farming.

World aquaculture is the sector of animal production that has been showing the highest rate of growth in recent decades[1–3]. Despite the high potential to increase production of aquatic organisms, there are still many barriers to the development of this sector in many countries[4–6]. This rapid growth has led producers to use intensive and super-intensive production systems that have positively increased productivity but have also increased the disease susceptibility of fish[7]. Some bottlenecks limit production in this sector, such as the availability of equipment and adequate rations, intensive production systems, environmental licenses, imports, low disclosure[8] and outbreaks of infectious diseases, which are highlighted as one of the main problems of aquaculture, especially in fish farming[9,10]. Protozoa, viruses, fungi, and bacteria are the main etiological agents associated with mortality in tilapia farms. The bacterial disease outbreaks may decimate up to 90% of the affected batches[11–13].

The main bacterial pathogens associated with Nile tilapia disease are *Streptococcus agalactiae*, *S. iniae*, *S. dysgalactiae*, *Flavobacterium columnare and Francisella noatunensis* subsp. *orientalis*[13–16]. In recent years, outbreaks caused by *Francisella* spp. have gained notoriety as the major pathogen of tropical fish cultivated worldwide[17,18]. Francisellosis is an emerging disease that has spread to several countries in the last two decades[17,18] due to the

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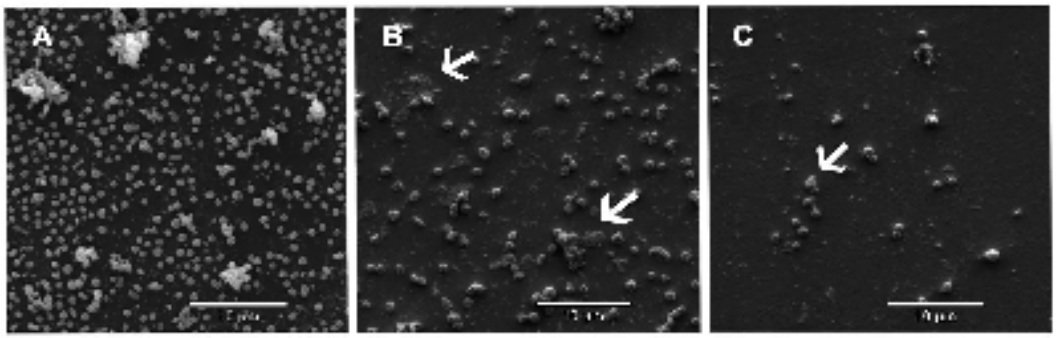

Figure 1. Visualization of number of cells of *Francisella* spp. F1 strain by electron microscopy after 30 min of exposure with both products applied either at 0.1% or 1%, (**A**) (*Francisella* spp. without the products); (**B**) (*Francisella* spp. with A-Live 0.1% and Aquaform 0.1%); (**C**) (*Francisella* spp. with A-Live 1% and Aquaform 1%). Cellular damages are shown using white arrows (**B**,**C**).

absence of commercially available effective vaccines and difficulties in antibiotic treatment because the disease is caused by a facultative intracellular pathogen[19,20].

Currently, the main therapeutic measure for francisellosis outbreaks is antibiotic therapy. However, its indiscriminate use may lead to the development of antibiotic resistance and poses potential risk to the environment, public health and food safety. In many cases, antibiotics (administered on feed) may have a limited efficacy when fish present hyporexia/anorexia, which is one of the first clinical signs of disease[19] or due to the late onset of treatment after the loss of affected fish batches[21,22]. Therefore, the search for less harmful and more environmentally friendly treatments is of major importance.

Due to the intrinsic properties of dietary supplements such as phytogenic compounds combined with or without organic acids appear to be one of the most promising alternatives to prevent microbial infections[23–25]. Indeed, phytogenic compounds are defined as plant-derived natural bioactive compounds which show positive effects on animal growth and health, and they often encompass essential oils (EOs), botanicals and herbal extracts[24]. Recent studies have reported that phytogenic compounds are capable of promoting improved performance, stimulating the innate immune response, and decreasing mortality against bacterial challenges in fish. Common carps (*Cyprinus carpio*) supplemented with EOs of *Litsea cubeba* presented better growth performance and when challenged with *Aeromonas hydrophila* they had a higher relative survival than the control group[26]. Phytogenic compounds derived from *Ocimum gratissimum* and *O. americanum* have been shown to be more efficient in promoting increased activity of the complement system in catfish (*Rhamdia quelen*)[27]. Nevertheless, studies evaluating the effect of phytogenic compounds on growth, immunity, and resistance to diseases in Nile tilapia are rare.

Furthermore, organic acids (and their salts), such as formic, citric, acetic, propionic, and butyric acid, among others, are already used as acidifiers with success in the animal feed industry, including aquaculture[28–30]. The main function of these organic acids is the reduction of the pH of the digestive tract, kill pathogenic bacteria, reduce the digestion tract emptying time, increase pepsin activity and nitrogen retention, and improve the digestibility, absorption and transport of minerals[25]. However, the efficacy of these food supplements in the prevention and/or control of different bacterial diseases in tilapia (especially francisellosis) and their effect on the intestinal bacterial population has been poorly studied.

The combination of a phytogenic compound with antimicrobial activity and an organic acid seems to be an efficient and reliable alternative to antibiotic administration. Thus, the aim of this study was to measure the *in vitro* activity of phytogenic compound A-Live (MiXscience, France) individually or in combination with an organic acid Aquaform (Salus, Santo Antônio da Posse, SP, Brazil) against *Francisella noatunensis* subsp. *orientalis* and also evaluate the *in vivo* potential of the products treatment on growth performance, innate immunity, gut microbiome and francisellosis resistance in juvenile Nile tilapia (*Oreochromis niloticus*).

Results

In vitro antibacterial activity of products against *Francisella noatunensis* subsp. orientalis.

Aquaform had inhibitory effects against *Francisella* spp. at concentrations ranging from 0.5 to 10%, showing halos of 13 to 20 mm. For A-live, inhibition values extended from 0 to 30 mm. The products, when used isolated, did not show inhibitory effects against the bacteria at a concentration of 0.1%. However, when used in combination, the products showed an inhibitory halo of 15 or 18 mm at a low concentration (0.1%) in 2 consecutive replicates.

The results of electron microscopy showed a decline in the cell number of *Francisella* spp. after 30 min in the presence of either Aquaform or A-Live individually. However, a smaller number of bacterial cells were visualized in the presence of A-Live than in Aquaform, suggesting that A-Live was capable of causing rapid damage to *Francisella* spp. (Supplementary Fig. 1). Additionally, the combination of Aquaform and A-Live at a concentration of 0.1% (of each product) showed a reduction in the number of *Francisella* spp. cells and a large amount of destroyed cell fragments. The combination of products at 1% revealed a drastic reduction in cell number when compared to the cell number reduction in other concentrations of mixture of products (Fig. 1) or when treated individually (Supplementary Fig. 1).

Analyzed period	Groups	Initial weight (g)	Final weight (g)	Daily weight gain (g)	Food consumption/ fish/day (g)	Feed conversion rate (FCR)	Specific growth rate (SGR)
7 days of acclimation and 15 days of experimental treatment	NC	35	50.6	0.74[a]	1.63	2.19[a]	1.75[a]
	PC	36	51.1	0.72[a]	1.66	2.31[a]	1.66[a]
	G1	35	49.8	0.70[a]	1.56	2.21[a]	1.67[a]
	G2	35	56.9	1.04[a]	1.93	1.85[a]	2.31[a]
	G3	33	52.7	0.94[a]	1.71	1.82[a]	2.22[a]
14 days post *Francisella* spp. Challenge	NC	50.6	60.7	0.72[a]	1.01	1.40[a]	1.29[a]
	PC	51.1	57.4	0.45[b]	0.9	2.00[b]	0.83[b]
	G1	49.8	56	0.44[b]	0.77	1.74[b]	0.83[b]
	G2	56.9	66.3	0.67[a]	0.9	1.34[a]	1.09[a]
	G3	52.7	61.25	0.61[a]	0.8	1.31[a]	1.07[a]

Table 1. Growth performance of fish from different treatments before and after disease challenge with *F. noatunensis* subsp. *orientalis* strain F1. *Different letters (a and b) indicate significant difference between the treatments ($p < 0.05$). NC, negative control; PC, positive control; G1, 0.2% A-Live treatment; G2, 0.2% A-Live and 0.2% Aquaform treatment; G3, 0.5% A-Live and 0.2% Aquaform.

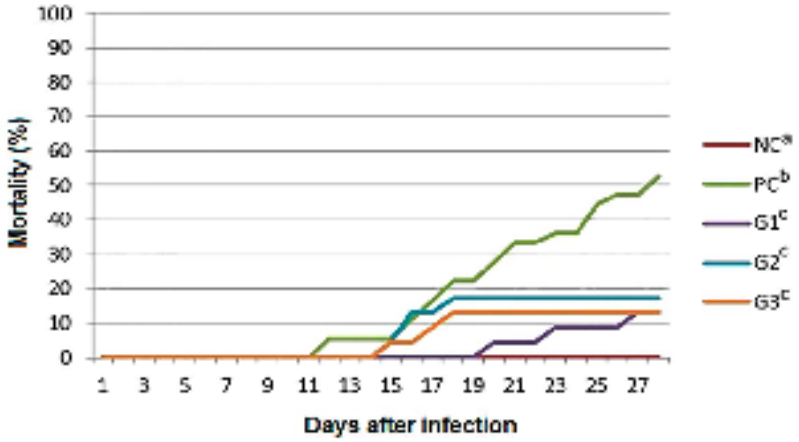

Figure 2. Cumulative mortality observed in the different groups after disease challenge by immersion with *Francisella noatunensis* subsp. *orientalis*. NC negative control: No challenge with bacteria and no A-Live or Aquaform (2 tanks); PC positive control: Challenge with *Francisella* spp. and no A-Live or Aquaform; G1- fish that received A-Live at 0.2% in the feed for 15 days prior to experimental infection with *Francisella* spp. (2 tanks); G2- fish that received the product A-Live at 0.2% and Aquaform at 0.2% in the feed for 15 days prior to experimental infection with *Francisella* spp.; G3- fish that received the product A-Live at 0.5% and Aquaform at 0.2% in the feed for 15 days prior to experimental infection with *Francisella* spp. (2 tanks).

Growth performance and disease challenges. Prior to pathogen challenge, groups G2 and G3 showed a superior feed conversion ratio (FCR) and specific growth rate (SGR) than that in two groups that did not receive the products in the feed (NC and PC) and G1 group. The G2 group presented better daily weight gain and SGR than that in other groups. Group G1 showed similar results when compared with the PC and NC groups. Even after disease challenge, the G2 and G3 groups presented a better daily weight gain, FCR, and SGR than that of the PC and G1 groups but did not differ from that of the NC group (Table 1).

The cumulative mortality of fishes in the group PC, G1, G2 and G3, which were pathogen challenged through feed, was 80, 65, 70, and 77%, respectively (Supplementary Fig. 2). Daily feed consumption was higher in groups G2 and G3 than in group G1 during the challenge period. Only G1 group showed a significant difference in cumulative mortality when compared to PC group ($p < 0.05$). In the pathogen challenge by immersion, cumulative mortality observed in PC, G1, G2 and G3 groups was 52, 13, 17 and 13%, respectively (Fig. 2). There was a significant difference in the mortality of G1, G2 and G3 groups compared with the mortality of the PC group ($p < 0.0001$).

Not all the surviving fish in the treated groups showed francisellosis lesions on the last day of the immersion experiment. In the PC and G1 groups, all surviving fish had granulomatous lesions in the cranial kidney and spleen. However, in the G2 and G3 groups, 15% and 35% of animals, respectively, did not exhibit francisellosis lesions in these organs. Additionally, these samples were negative in the CHAH culture. No lesions were observed in the kidney and spleen of the NC group. No mortality was observed in the oral or immersion negative control groups, indicating that the control was reliable.

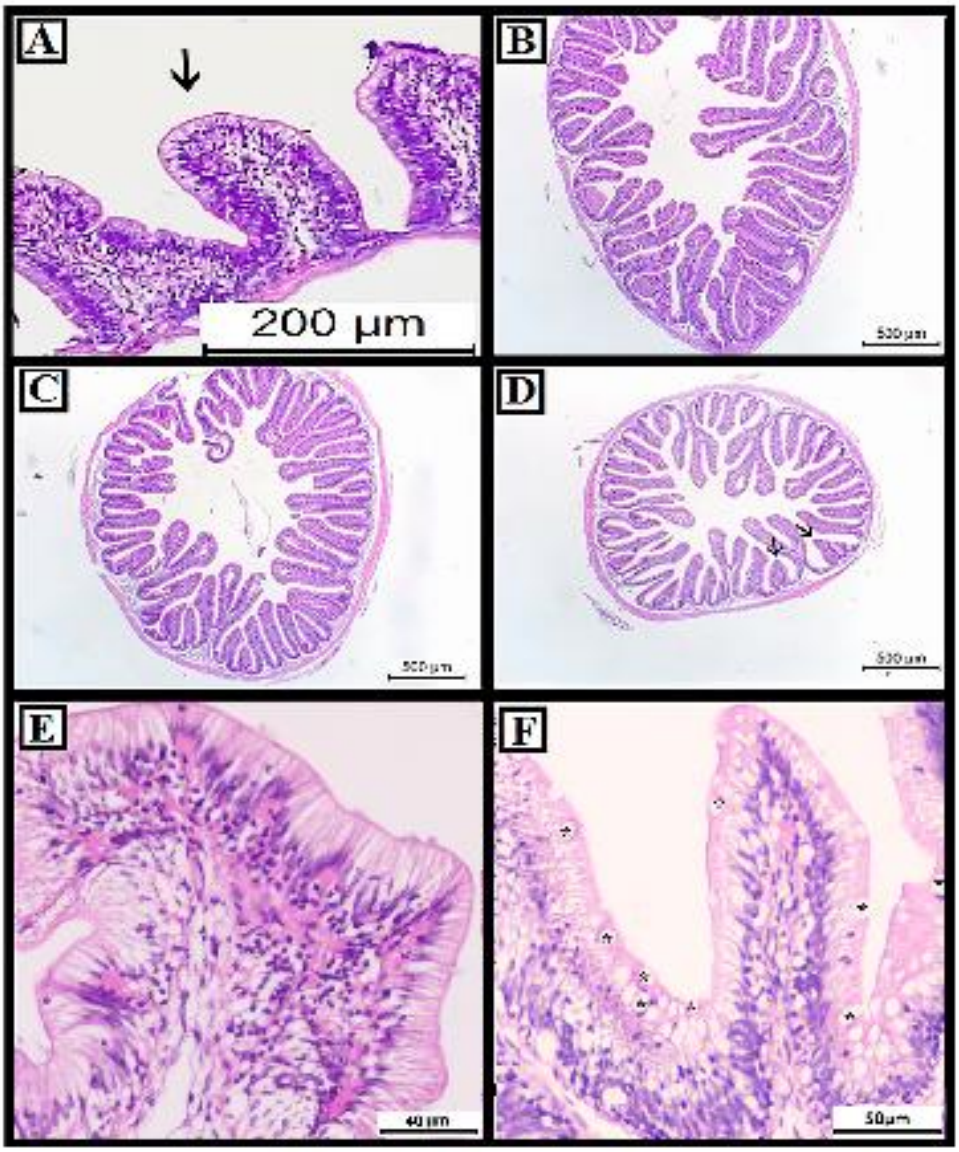

Figure 3. Histomorphology of the gut with different treatments after 15 days of experimental infection. Shrinkage of intestinal villi are shown using black arrow. Goblet cells are denoted by "*". (**A**) G1 group (A-Live at 0.2%); (**B**) G2 group (A-Live at 0.2% and Aquaform at 0.2%); (**C**,**F**): G3 group (A-Live at 0.5% and Aquaform at 0.2%); (**D**,**E**) PC group.

Intestine histology and morphology. After 15 days of treatment with the products, a higher quantity of digesta was observed in groups G2 and G3, suggesting that it might be owing to the higher feed intake observed in these groups (Supplementary Fig. 3). Autolysis was observed in the apical region of the intestinal villi in G2 and G3 groups, probably because of the presence of lumen ingestion since these groups had a higher feed consumption (Supplementary Fig. 3). In group G3, hyperplasia of goblet cells was observed (Fig. 3F). After infection, aggregates of lymphoid cells were observed in the PC group (Fig. 3E) In G1, shrinkage of some intestinal villi was visualized (Fig. 3A). No evident variations in the villi such as diffused shrinkage was found in groups G2 and G3 villi (Fig. 3B,C).

Blood and innate immune parameters. The blood parameters are shown in Table 2. Some of the indicated characteristics are the significant increase ($p < 0.05$) in leukocytes and neutrophils after infection in the PC group compared to those in the G1 group. Before infection, there was a significant decrease ($p < 0.05$) in the number of erythrocytes (in G2 and G3), thrombocytes (in G1), hemoglobin (in G2), and monocytes (in G1, G2, and G3) compared to those in the NC group. Hematimetric indices and glucose showed no difference ($p > 0.05$) among the groups before or after infection.

Analyzed period	Pre-challenge				Post-challenge			
Groups	NC	G1	G2	G3	PC	G1	G2	G3
Hematocrit (%)	39.6 ± 3.58	29.0 ± 0.79	31.2 ± 1.82	27.2 ± 2.36	31.4 ± 1.24	34.8 ± 0.14	31.2 ± 0.13	27.8 ± 0.18
Hemoglobin (g/dL)	6.34 ± 0.17^{a}	5.73 ± 0.08^{ab}	5.25 ± 0.13^{b}	5.68 ± 0.08^{ab}	4.98 ± 0.18	5.92 ± 0.59	4.66 ± 0.56	4.7 ± 0.77
Erythrocytes (10^{6}/µL)	1.96 ± 0.07^{a}	1.68 ± 0.02^{ab}	1.23 ± 0.04^{b}	1.35 ± 0.06^{b}	1.86 ± 0.08	1.91 ± 0.07	1.43 ± 0.02	1.43 ± 0.04
Thrombocytes (10^{6}/µL)	32.20 ± 1.13^{ab}	27.90 ± 1.69^{b}	Ag.	38.95 ± 0.63^{a}	28.15 ± 1.46	30.25 ± 0.96	27.61 ± 1.33	27.86 ± 0.93
Leukocytes (10^{3}/µL)	53.79 ± 2.04	55,12 ± 1.58	51.75 ± 0.91	39.34 ± 2.83	64.12 ± 0.32^{a}	51.56 ± 1.21^{b}	55.10 ± 1.87^{ab}	53.16 ± 1.01^{b}
Lymphocytes (10^{3}/µL)	28.89 ± 1.03	30.54 ± 0.67	30.05 ± 0.82	24.22 ± 1.65	31.10 ± 0.38	30.50 ± 0.55	30.18 ± 1.21	29.32 ± 0.89
Neutrophils (10^{6}/µL)	21.49 ± 0.98	23.30 ± 0.85	21.49 ± 0.74	14.62 ± 1.40	29.70 ± 0.97^{a}	20.47 ± 1.20^{b}	24.04 ± 1.15^{ab}	22.63 ± 0.28^{ab}
Monocytes (10^{3}/µL)	3.04 ± 0.25^{a}	0.84 ± 0.27^{ab}	0.19 ± 0.08^{b}	0.35 ± 0.13^{b}	0.81 ± 0.23	0.55 ± 0.16	0.72 ± 0.20	1.20 ± 0.21
Eosinophils (10^{3}/µL)	0.36 ± 0.13	0.19 ± 0.09	0 ± 0	0,10 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Basophils (10^{3}/µL)	0 ± 0	0.23 ± 0.12	0 ± 0	0.03 ± 0.01	0 ± 0	0.04 ± 0.02	0.12 ± 0.03	0 ± 0
MCV (fL)	203.39 ± 16.01	173.13 ± 4.23	272.13 ± 26.32	221,87 ± 27.42	172.48 ± 8.97	183.91 ± 5.67	222.61 ± 11.14	201.47 ± 10.43
MCHC (g/dL)	19.28 ± 2.50	19.91 ± 0.45	17.70 ± 1.10	23.63 ± 2.08	15.97 ± 0.28	15.64 ± 0.39	15.69 ± 0.80	16.86 ± 0.38
Glucose (mg/dL)	30.15 ± 1.28	31.57 ± 2.66	31.65 ± 2.76	43.29 ± 3.20	122.58 ± 13.26	91.50 ± 6.78	80.16 ± 7.25	76.31 ± 9.90

Table 2. Blood general parameters (mean ± standard error) of experimental groups of fish supplemented with additives before and after disease challenge with *F. noatunensis* subsp. *orientalis* strain F1. *Different letters (a and b) indicate significant difference between the treatments ($p < 0.05$); * Ag = Aggregated. NC, negative control; PC, positive control; G1, 0.2% A-Live treatment; G2, 0.2% A-Live and 0.2% Aquaform treatment; G3, 0.5% A-Live and 0.2% Aquaform.

Analyzed period	Pre-challenge				Post-challenge			
Groups	NC	G1	G2	G3	PC	G1	G2	G3
Lysozyme (µg/mL)	8.77 ± 0.25^{b}	9.24 ± 0.25^{ab}	9.43 ± 0.28^{ab}	11.02 ± 0.19^{a}	16.76 ± 0.42	17.18 ± 0.36	17.82 ± 0.43	16.73 ± 0.55
Complement activity mean (µL for lysis of 50% of erythrocyte)	2.11 ± 0.23^{a}	0.82 ± 0.11^{b}	0.98 ± 0.09^{ab}	1.15 ± 0.08^{ab}	1.07 ± 0.05	1.37 ± 0.09	1.09 ± 0.10	1.06 ± 0.13
Antibacterial activity of serum (Log2 mean of dilution + 1)	0 ± 0	0 ± 0	0.32 ± 0.15	0.63 ± 0.19	0 ± 0	1.27 ± 0.29	0.63 ± 0.19	0.95 ± 0.31

Table 3. Data of serum lysozyme activity, complement activity and antibacterial activity (mean ± standard error) of fish from different groups of treatment before and after disease challenge with *F. noatunensis* subsp. *orientalis* strain F1. *Different letters (a and b) indicate significant difference between the treatments ($p < 0.05$). NC, negative control; PC, positive control; G1, 0.2% A-Live treatment; G2, 0.2% A-Live and 0.2% Aquaform treatment; G3, 0.5% A-Live and 0.2% Aquaform.

Lysozyme activity was higher in G3 group before infection. However, after infection all groups that had contact with *Francisella* spp. showed an increase in this parameter but the difference among them was not significant ($p > 0.05$) (Table 3). Before infection, serum antibacterial activity was only present in G2 and G3, demonstrating that the combination of products stimulated this immunological parameter (Table 3).

Microbiome diversity and abundance of the bacterial population. A total of 1,188,301 sequences were obtained for all groups, and 72 OTUs (operational taxonomy units) were identified. The number of sequences per group ranged from 88 to 182 thousand reads. A rarefaction curve showed that sequencing was sufficient to sequence most of the bacterial species present in the fish gut as there was little addition of new species after 10,000 sequences (Fig. 4), suggesting that the read count of the trial was representative of the intestinal bacteriome in all groups. Additionally, Supplementary Table 1 displays the total number of sequences obtained in each group.

Moreover, Mothur software was used to calculate the Shannon index, which compares the diversity of species in each group (Fig. 5). However, no significant differences were observed. The boxplot shows that NC and PC groups presented the lowest level of species diversity, while the treated groups had higher level of diversity. Thus, this information suggests that before and after infection by *Francisella* spp., the treated groups had higher diversity in the gut bacterial microbiome than that in the NC and PC groups. Groups G1 and G2 presented more bacterial diversity before infection in comparison to the bacterial diversity of groups G1 and G2 after infection by *Francisella* spp. Among all the groups, G1 was the most diverse; however, it showed a decreased diversity after 15 days of infection. Interestingly, after infection, the group (G3) that received a higher concentration of both products maintained a higher diversity at this time of the trial.

The abundance of bacterial species calculated by Mothur software is shown in Fig. 6 and Table 4. The abundance plot displays the most abundant bacteria in each sample: *Cetobacterium*, *Bacteroidales*, *Vibronaceae*, *Porphyromonadaceae*, *Romboutsia* and *Plesiomonas*. Before infection, in the NC group, the genus *Cetobacterium* was the most abundant, and the other families and genera (*Bacteroidales*, *Porphyromonadaceae*, *Romboutsia* and *Plesiomonas*) appeared in smaller numbers. On the other hand, in the treated groups, there was a decrease in the

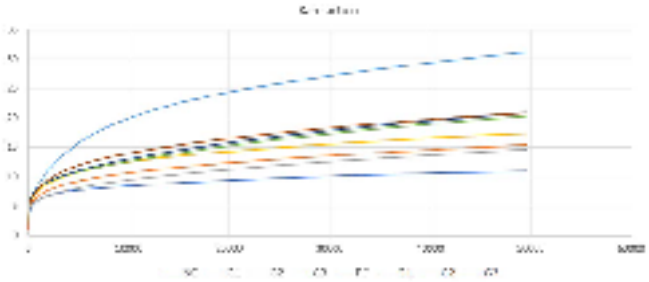

Figure 4. Rarefaction curve showing increasing species along the number of reads in different trial groups.

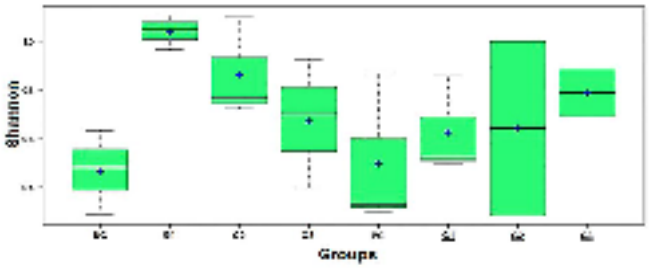

Figure 5. Shannon index in different trial groups after 15 days of treatment and after experimental infection with *Francisella* spp. (underlined).

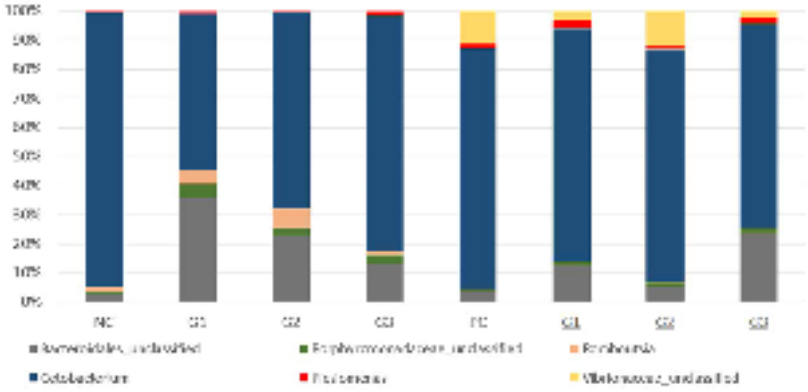

Figure 6. Abundance of noninfected and infected groups (underlined) with information on the percentage of sequences in each group. *Cetobacterium* (dark blue), Bacteroidales_unclassified (gray), Vibronaceae_unclassified (yellow), Porphyromonadaceae_unclassified (green), *Romboutsia* (pink) and *Plesiomonas* (red).

genus *Cetobacterium* in the gut microbiota. After infection, genus *Cetobacterium* remained in greater proportions in the groups, also favoring the increase in the genus of the family *Vibrionacea* (mainly in the PC and G2 groups).

STAMP analysis revealed significant difference in the proportion in the abundance results (Fig. 7). Thus, even small counts of sequence that are not seen in abundance plots are considered in STAMP analysis. Comparison of NC with G1 (Fig. 7a) showed significance in abundance of *Porphyromonadaceae*_unclassified, *Cetobacterium*, and *Bacteroidales*_unclassified. The *Porphyromonadaceae*_unclassified and *Bacteroidales*_unclassified considerably increased in the G1 group. On the other hand, *Cetobacterium* showed a decrease in abundance in the same group. *Phreatobacter*, *Rhizobiales*_unclassified and *Neisseriaceae*_unclassified were significant in the plot. Figure 7b displayed similar results, with discreet variations between the compared groups. The comparison of NC with G3 was not significant for any level of taxonomy. In Fig. 7c, the *Rhizobiales*_unclassified taxon displayed a large decrease in the PC when compared with the NC; however, there was a large standard deviation. The bacterial abundance in the infected groups (G1, G2 and G3) did not present any significant difference when compared to the bacterial abundance of the PC group. A comparison of the groups over time (before and after infection) showed different taxon among them (Fig. 7d–f). A higher number of *Cetobacterium* and *Plesiomonas* was observed in G1 (Fig. 7d), but the *Romboutsia* number decreased after infection (G1). Figure 7e displays an

Analyzed period	Pre-challenge numbers of sequences (%)				Post-challenge numbers of sequences (%)			
Taxon	NC	G1	G2	G3	PC	G1	G2	G3
Cetobacterium	46733 (94.2)	97834 (53.5)	112968 (67.3)	223726 (81.1)	105808 (82.5)	112245 (79.9)	99783 (79.9)	61951 (70)
Bacteroidales_unclassified	1486 (3.0)	65636 (36)	38781 (23.1)	36063 (13.1)	4969 (3.9)	17860 (12.7)	6612 (5.3)	21196 (24)
Vibrionaceae	0(0)	1(0)	1(0)	0(0)	14373 (11.2)	3879 (2.8)	14726 (11.8)	2077 (2.3)
Porphyromonadaceae_unclassified	207 (0.4)	8756 (4.8)	4218 (2.5)	8131 (3)	833 (0.6)	1712 (1.2)	1654 (1.3)	1445 (1.6)
Romboutsia	876 (1.8)	8419 (4.6)	10937 (6.5)	3477 (1.3)	22 (0.01)	239 (0.2)	460 (0.4)	55 (0.06)
Plesiomonas	187 (0.4)	1561 (0.8)	833 (0.5)	3221 (1.2)	1645 (1.3)	3732 (2.7)	1260 (1)	1499 (1.7)
Total of reads	49560	182643	167955	275745	128325	140541	124925	88543

Table 4. Count of sequences of the most abundant species in experimental groups. NC, negative control; PC, positive control; G1, 0.2% A-Live treatment; G2, 0.2% A-Live and 0.2% Aquaform treatment; G3, 0.5% A-Live and 0.2% Aquaform.

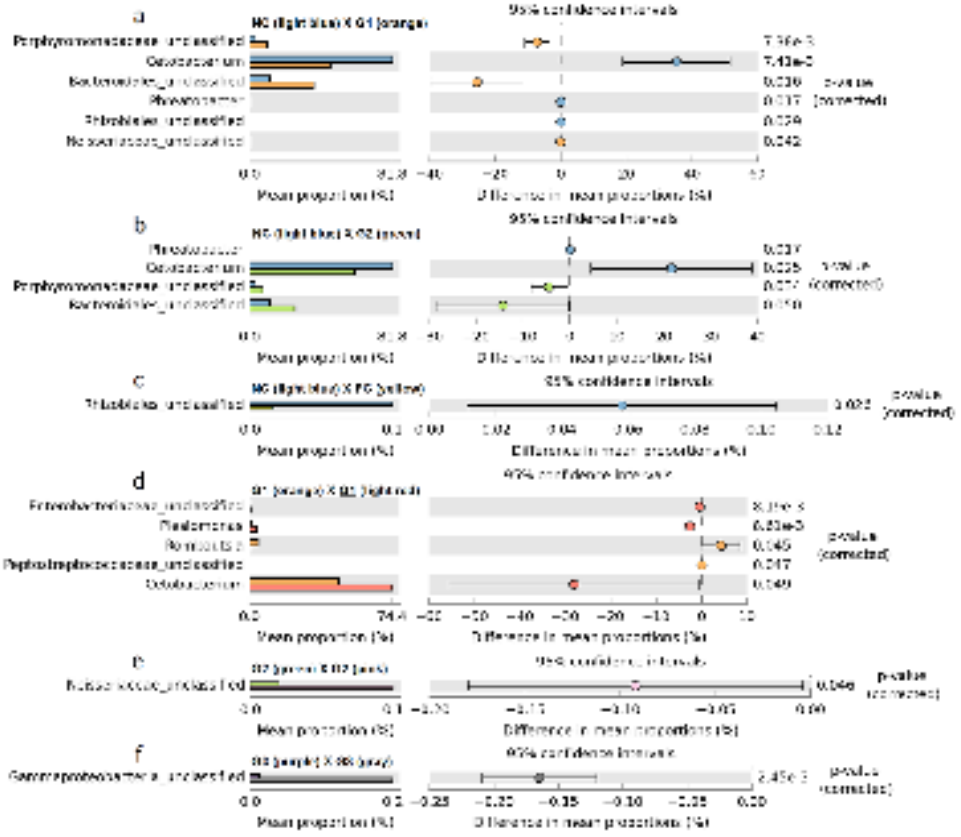

Figure 7. Comparisons of bacterial abundance among groups. The left side presents the abundance proportion of each sample. The right side shows the difference in abundance within 95% confidence intervals with the p-value of the significance test. Only significant results are displayed ($p < 0.05$).

increase in *Neisseriaceae*_unclassified sequences in G2; however, this result presented a large standard deviation. Finally, Fig. 7f shows a large increase in the G3 group of *Gammaproteobacteria*_unclassified.

Discussion

The products demonstrated very promising *in vitro* as well as *in vivo* results, with the combination of the products providing better benefits. *In vitro* data showed an inhibitory halo (for *Francisella* spp.) of 15 or 18 mm when using Aquaform and A-Live in combination, each at a low concentration (0.1%). Electron microscopy showed that the combination of 1% products resulted in a drastic reduction in the bacterial cell counts when compared with the other concentrations. Similar results were found in experiments using *Aeromonas* spp. and that there were inhibitory halos from a mixture of formic acid, propionic acid and calcium propionate[25].

In vivo results showed better FCR and SGR in groups G2 and G3, which received the combination of the two products at A-Live concentrations of 0.2% and Aquaform of 0.2% and at A-Live of 0.5% and Aquaform of 0.2%, respectively. Mortality after infection was smaller in all groups receiving the products than in the positive control

group. Additionally, mortality rate was significantly lower in the fish challenged with *Francisella* spp. by immersion than in the positive control group. These results reinforce that future studies with nutritional additives should use immersion challenge method, which resembles the natural infection, to obtain a more reliable data.

The autopsy analysis of the G2 and G3 groups showed that 15% and 35% of the animals, respectively, did not present characteristic lesions of francisellosis. These results along with immunity parameters and mortality data suggest that these high efficacy products can be an alternative strategy for prevention of francisellosis outbreaks in tilapia. In addition, the interference of products in pathogen homeostasis should also be explored in future studies through global gene expression and proteomic approaches. A previous study on tilapias showed an increase in weight gain, specific growth rate and apparent protein digestibility[31]. Additionally, the authors found a decrease in mortality rate (*Aeromonas hydrophila* challenge) and increase in serum lysozyme and phagocytic activity of the group treated with concentration of 0.3% potassium diformate. They also reported a decrease in intestinal pH in the treated groups, which resulted in increased beneficial bacteria count in the intestinal gut. Similarly, the innate immunity in current study, demonstrated a higher antibacterial and complement activity besides lower mortality in treated groups. Although intestinal pH is not assessed, the beneficial effects in fish in the present study may be associated with the antimicrobial effect of Aquaform (potassium diformate) in the intestinal tract by increasing intestinal health and nutrient absorption. In addition, it also suggested a synergistic effect with the A-live, which may have provided a positive effect to the intestinal and overall health of the animals even though no noticeable differences were observed in microbiome data. This hypothesis is supported in the present study in groups G2 and G3 data, both before and after the challenge, and in the *in vitro* results.

In the histological analysis of the intestine, it was observed that, post infection, the PC group presented aggregates of lymphoid cells and obtained low performance indices (growth and survival). On the other hand, the G2 and G3 groups that presented better performance indices and had higher villi based on intestinal histology. Also, in G3 group hyperplasia of goblet cells was observed. These results corroborate those found in other studies, where supplementation of organic acids in the diet improves mucus[32] and nutrient digestibility in tilapia[33].

Additionally, the use of products improved some blood and immunological parameters that may be related to the disease resistance in fish. The results of blood parameter analysis (mainly the increase in neutrophils in the PC group) indicated an acute inflammatory response to infection by *Francisella* spp., with the migration of neutrophils in large quantities to the site of infection resulting in the characteristic lesions of francisellosis in fish[34]. Alternately, in the treated groups, moderate increase in this type of immune cell may be due to an immunomodulation resulting from ingestion of the products, as shown in previous studies[25,35]. This immunomodulation hypothesis is reinforced by the results found in the G2 and G3 groups, with some fish presenting minor or even an absence of granulomatous lesions in the spleen. These two groups also showed the best results in PCR after infection, which may be related to reduction in the damage caused by the pathogen and an enhanced recovery of digestive functions. In addition, the product A-Live at the highest dietary concentration might have stimulated the innate immune system in different ways, which would explain the increase in the number of thrombocytes in the G3 group. The decrease in erythrocyte and hemoglobin values might be associated with physiological compensation, since they were within normal range for the species. Cytokine-related studies are needed to better understand the evolution of disease, pathogenicity and its relationship with the immune system in normal and treated fish. In addition, the active molecules harbored in A-Live (derivate from plant extracts) also should be explored. Research on the effect of purified bioactive molecules will aid in understanding the effect of these molecules, which may result in a more effective product for use in prevention of fish diseases. Glucose increased significantly in all groups after infection, which was expected since there is an increase in the hyperglycemic hormone cortisol during infections, which results in an increase in gluconeogenesis[36].

Innate immunity parameters, in particular phagocytic, lysozyme and complement activities, have been used as indicators of the effects of inherent or external factors on the immune system and the disease resistance of fish. Among several factors that stand out are dietary and food additives[37,38], such as immunostimulants and probiotics[39,40], as well as the effects of diseases and vaccination[41,42].

The level of serum lysozyme is an important index of innate immunity in fish. Lysozyme of fish having lytic activity against bacteria is well understood. Moreover, it has opsonic properties and helps to activate the complement system and phagocytes. Lysozyme activity is activated when the outer cell wall of Gram-negative bacteria bursts due to the action of complement and other enzymes that expose the inner peptidoglycan layer. The lysozyme then becomes effective and consequently increases its level and activity[43,44]. We observed in this study that, even before infection, the groups that were fed the products had higher lysozyme activity in comparison to the NC group, although this difference was not significant. When comparing the G3 group with the NC, we observed a significant increase in lysozyme activity. Post infection, all the groups had increased lysozyme activity. Thus, these results demonstrated that the products added to the diet displayed immunostimulatory role[25], which suggest that dietary acidifiers have beneficial effect in aquaculture production assisting disease resistance.

Before infection, serum antibacterial activity was only present in G2 and G3 groups, indicating that the products stimulated this immune parameter. However, after infection all the treated groups (G1, G2 and G3) showed antibacterial activity. This result may be explained by the absorption and/or serum presence of active compounds in the diet or by the indirect stimulation of antibacterial serum components by the products in the feed which is quickly stimulated after a trigger (in this case, the infection by pathogen).

Before infection, we observed that all groups receiving the products showed better results of serum complement activity than that of the NC group. A significant difference in serum complement activity was observed in G1 group when compared to the serum complement activity of NC group. After infection, there was no significant difference between the groups, but G3 provided the best result. Similar results were found in other studies, where an improvement of the immune system from dietary supplementation has been reported[25,31].

Studies have been conducted in vertebrates concerning host and intestinal microbiota interactions and demonstrated an integral role in ontogenetic development, especially when related to immune system modulation[45–47].

In addition to competition for readily available carbohydrates in the diet, intestinal microbes are able to extract energy from dietary polysaccharides that are indigestible by the host[48].

In general, microbiome analysis showed a decrease in *Cetobacterium* and an increase in *Bacteriodales* in the treated groups before infection (Fig. 4). After infection, more discrete differences were observed in the microbiome between the groups (Table 4 and Supplementary Fig. 4). Further studies investigating the microbial composition of the fish gut are necessary to provide a comprehensive understanding of the influence of diet on the health status of fish. For example, the order *Bacteroidetes* was found in abundance in this study, as well as in marine herbivorous fish[49] and other non-fish gut flora[50].

The dominant phyla in the gut microbiome of Nile tilapia are generally *Proteobacteria* and *Fusobacteria*, although the relative abundances of these phyla vary between studies[51–54]. Microbiota analysis of tilapia gut in this study revealed *Cetobacterium* as the most prevalent genus in all intestinal samples. *Cetobacterium* has been recognized as a common member of the microbiome of grass, Asian bighead, common and Crucian carps[50,55–58], and it may be considered a core genus among carps. This bacterium is a micro-aerotolerant anaerobe that is capable of producing vitamin B-12 and antimicrobial metabolites[59]. Thus, this interaction between pathogen and host suggests that tilapia may take advantage of the physiological benefits of this microorganism.

Our results are in agreement with a previous report[60], which suggested that *C. somerae* is a commensal microbe with a highly abundant nucleus inside the intestine of catfish. Moreover, in the same study, the authors found the species *Plesiomonas shigelloides*, another bacterium belonging to the family *Enterobacteriacea* and commonly found in freshwater fish and aquatic environments. Additionally, *Plesiomonas shigelloides* is commonly found in the intestine of fish from tropical and subtropical countries such as Japan and Thailand, several countries in Africa, Australia and Brazil[61–64]. In this study, *Plesiomonas* spp. was present in all groups, even in small amounts. Considering that after infection only G1 group showed an increase in *Plesiomonas* spp. in the microbiota, and all remaining fish in this group presented spleen lesions from francisellosis, it was hypothesized that *P. shigeloides* played a role as a secondary/opportunistic pathogen in the disease evolution when group G1 showed worse result in feed conversion rate (FCR) than that of G2 and G3 groups.

Another genus that clearly appeared before infection was *Romboutsia*. This genus was previously known as *Clostridium*, and little information is available about this species[65]. Recently, a new species isolated from the small intestine of rats was described as *Romboutsia ilealis* CRIB^T. Several studies have explored the link between the diet and gut microbiota due to the potential dietary properties presented by some species. The data shown here suggested that the products stimulated the increase in number of the genus *Romboutsia* before infection (groups G1 and G2 in Fig. 6). Additionally, an increase in *Bacteriodales* and an unclassified genus of the *Porphyromonadaceae* family was observed, suggesting a connection with a microbiota modulation observed in the treatment groups (as demonstrated in Fig. 7a,b).

The increase in the unclassified genus of the *Vibrionaceae* family in the infected groups might be related to the participation of this presumable pathogen in the symbiotic and commensal microbiota[60]. Interestingly, the group G3 after infection showed a small proportion of this bacteria family, suggesting an inhibitory effect of treatment against *Vibrionaceae* family. Fish with francisellosis are probably more susceptible to opportunistic pathogens, and an increase in this bacterium in the PC group suggests that francisellosis caused the fish to be more susceptible to secondary pathogens. It is noteworthy that minimal number of *Gammaproteobacteria* was detected after *Francisella* spp. infection, (Fig. 6). There are no reports about *Francisella* spp. pathogenesis affecting the fish gut; however, these results suggest that this pathogen may have few tropisms to this tissue.

There is global demand to reduce the use of antibiotics in animal production. Consequently, in the last decade, new alternatives such as acidifying products and plant extracts, have been widely explored for these economic activities, mainly in swine and poultry industries. More recently, the demand for these studies has increased in aquaculture, including fish farming. Accordingly, the data from this tilapia study indicated that the combination of Aquaform and A-Live presented the best results for growth, blood and immunological parameters and lower mortality in francisellosis-challenged model. Thus, the beneficial effects of these products on tilapia production were validated. Furthermore, studies on the effect of these additives on fish production are necessary to evaluate their efficacy against other diseases and/or stress conditions that may commonly occur in aquaculture.

Materials and Methods

Antibacterial activity of the products against *Francisella* spp. Addcon- Salus (Brazil) and MiXscience (France) provided the organic salt (Aquaform, potassium diformate) and vegetal-derived product (A-live, derived from plant extracts) used in this study, respectively. The tested phytogenic is a blend of plant extracts and mineral clays formulated to have high dispersion in water easily applicable in aquaculture. Antimicrobial activity of the tested phytogenic comes from sulfur organic compounds of the extracts from *Alliaceae* family (mainly garlic, onion, and leek in the present case). The *Alliaceae* family includes 13 genera and 600 species. Main representatives are onion, garlic, leek, shallots, and chives. The analysis consists of an *in vitro* bacterial growth inhibition test, which initially requires the bioactive compounds of the product to remain in aqueous solution. Thus, the solubility test of the products was performed using sterilized water, methanol (10, 20 and 30% in water) and ethanol (10, 20 and 30% in water). The best solvent (with low precipitation of both products) was 20% ethanol. The products were filtered using a 0.22 µm membrane. The products were tested at concentrations of 10, 5, 1, 0.5 and 0.1% individually and in combination; when combined, the ratio of the products was 1:1. *Francisella noatunensis* subsp. *orientalis* strain F1 was used at the 0.5 McFarland scale and plated on cystine heart agar supplemented with bovine hemoglobin (CHAH). Sterile discs were soaked separately with 10 µL of the different concentrations in duplicate. Petri dishes with a diameter of 9 cm were used, with two discs allocated for each dish. The plates were incubated at 28 °C for 72–96 h for further measurement of the inhibition halo, as previously described[25].

Electron microscopy of *Francisella* cells when exposed to products. This method aims to evaluate the possible physical damage caused by the products to the pathogen *Francisella* spp. using electron microscopy. After 72 h culture, colonies of the F1 strain were suspended in sterile saline, at the 1 McFarland scale, containing different concentration of products and incubated at 28 °C for 30 min. The samples were centrifuged for 5 min at 2000 rpm, resuspended in 100 µL of fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.0) and transferred to 24-well polystyrene microtiter plates (Fisher Scientific, Kamstrupvej, Roskilde, Denmark) with glass coverslips precoated with a thin layer of poly-L-lysine (Sigma, Saint Louis, MO, USA). After 1 h, the volume was adjusted to 500 µL using fixing solution to avoid cell adherence to the coverslips and incubated at 25 °C for 12 h. The samples were post fixed in 1% OsO_4 (Electron Microscopy Sciences, Washington, PA, USA) and dehydrated in an ethanol series (30, 50, 70, 90, and 100°GL). Samples were subjected to critical-point drying with CO_2 BALTEC CPD 030 Critical Point Dryer (Shapiro Center for Engineering and Physical Science Research, New York, USA), coated with gold in BALTEC SDC 050 Sputter Coater (Capovani Brothers Inc., New York, USA) and observed under a scanning electron microscope (FEI Quanta 200, Netherlands).

Fish. A total of 720 healthy juvenile Nile tilapia (*O. niloticus*) were obtained from a commercial hatchery from Paraná state, Brazil (mean individual initial weight was 34.3 ± 0.33 g). The fish were divided into 3 treatment groups (432 tilapia) distributed into 2 replicates (72 tilapia each) and 2 control groups (288 tilapia) distributed into 2 replicates (72 tilapia each). The animals were stocked in 150L tanks containing dechlorinated water with continuous renewal (80% of volume daily) and the temperature was maintained at approximately 25 °C, and the fish were fed three times a day until apparent satiation (resulting in approximately 6% ± 0.3% of biomass). Water parameters (pH of 6.8 to 7.2, total ammonia < 0.4 mg/L, and absence of chlorine) were measured daily and maintained throughout the experimental period. The presence of oxygen was maintained by two aerators, resulting in dissolved oxygen of 5.4 mg/L on an average. Before the start of the feeding trial, the fish underwent a period of acclimatization for 7 days, and behavior/signs of disease were observed (exophthalmia, erratic swimming, skin lesions, and others). Microbiological diagnosis was also performed before the experiment, in which 20 fish were randomly sampled and euthanized by a high-dose of benzocaine (200 mg/mL). Aseptically, fragments of brain, liver, cranial kidney, and spleen were streaked on Mueller Hinton agar (Kasvi, São José dos Pinhais, PR, Brazil) enriched with 5% defibrinated ovine blood and in CHAH. The plates were incubated at 28 °C for 5 days for confirmation of the health status of fish (no bacterial growth in the plates). All animal procedures were approved by the Ethic Committee on Animal Use of State University of Londrina (Approval number CEUA/UEL-7327.2017.39) and all experiments were performed in accordance with relevant guidelines and regulations.

Basal diet. An extruded feed (without organic acids and phytogenics) for early stage tilapia with 36% crude protein and 2.6 mm diameter was used. The nutrient composition of the diet is shown in Supplementary Table 2. For *in vivo* studies, the goal was to homogenously add the products to the feed, to enable it to reach and act locally at the digestive tract of the fish and for the absorption of bioactive molecules in the digestive tract. These steps were carefully carried out by sprinkling the products in a fractioned aqueous solution of the feed (one-third part of total volume for each time). The feeds were prepared separately using 5 kg of fed which was distributed in to a tiny layer on a plastic surface (5 meters on all four sides) on the floor. The first fraction of the products diluted in water was spread and the feed was mixed for 5 min. A similar process was followed to obtain the fraction two and three of the products.

In other words, phytogenic A-Live alone or in combination with Aquaform was incorporated in commercial feed by manual mixing for 5 min, followed by spraying the water-diluted products on the feed during agitation. Further, approximately 5% (w/v) of universal vehicle, a carboxymethylcellulose-based product (Vansil, Descalvado, SP, Brazil) was used for avoiding energetic/nutritional disbalance in the feed between the groups and to coat the surface of the feed and avoid rapid leaching of products in the water. In the feed of negative and positive control groups, only the carboxymethylcellulose product was added using the same steps. Then, the feed was spread on trays and dried for 12 h in an incubator at 35 °C before use.

Experimental design and disease challenge. After acclimation, the fish were maintained under the same conditions until the end of experiment. Products were incorporated into the feed according to the experimental groups and provided daily for 15 days before the disease challenge. One day before the disease challenge, the water temperature was decreased gradually and maintained at 21 °C (±1 °C) to promote infection by *Francisella* spp., since outbreaks of francisellosis in Brazil occur at similar temperatures[14]. The experimental design is detailed below:

- NC- negative control group: No challenge with bacteria and no A-Live or Aquaform in the feed (2 tanks);
- PC- positive control group: Challenge with *Francisella* spp. and no A-Live or Aquaform in the feed (2 tanks);
- G1- group of fish that received A-Live at 0.2% in the feed for 15 days prior to experimental infection with the pathogen *Francisella* spp. (2 tanks);
- G2- group of fish that received the product A-Live at 0.2% and Aquaform at 0.2% in the feed for 15 days prior to experimental infection with the pathogen *Francisella* spp. (2 tanks);
- G3- group of fish that received the product A-Live at 0.5% and Aquaform at 0.2% in the feed for 15 days prior to experimental infection with the pathogen *Francisella* spp. (2 tanks).

The groups named G1, G2 and G3 received the products before experimental infection daily for 15 days and continued to receive the same dosage of the products after experimental infection (then named G1, G2 and G3)

until the end of the experiment (at least 21 days after infection or when no mortality was observed for 3 days). After experimental infection, all the fish were observed daily for clinical signs and mortality. Dead fish were subjected to microbiological diagnosis by aseptically streaking cranial kidney and spleen fragments onto CHAH plates.

This experimental design was performed separately twice as different experiments and at different times. At first, the disease challenge was performed by feeding the fish (pathogen was administered at 8×10^6 colony forming units (CFU)/g of feed for 24 h). In this first trial, the growth, blood parameters and mortality rate were evaluated. The second set of experiments were performed using an immersion challenge by diluting *Francisella* spp. in water (5.4×10^5 CFU/mL of water in the tank). For *in vivo* trials using immersion challenge (that is closer to natural infection of fish), only the clinical signs, mortality, and presence of francisellosis lesions in kidney and spleen (and bacterial culture of these organs) were observed.

Blood sampling. Blood samples were collected at 15 days posttreatment and 15 days post-challenge by *Francisella* spp. (five samples per group). The fish were anaesthetized with benzocaine (0.1 g/L), and blood was collected by puncturing the caudal vessel in 3 mL syringes (21 G) containing 10% anticoagulant (ethylenediaminetetraacetic acid). The blood was used to measure the hematocrit (Hct; %) using the microhematocrit method[66], and red blood cells (RBC; 10^6/μL) were counted in a Neubauer chamber following dilution at 1:200 in Dacie solution. White blood cell (WBC; 10^3/μL) and total thrombocyte counts were calculated using an indirect method[67]. For differential counting of leukocytes, the smears were stained with May-Grünwald/Giemsa/Wright stain[66]. The hemoglobin concentration (Hgb; g/dL) was analyzed by the cyanmethemoglobin method[68] using commercial kits (Labtest, Lagoa Santa, MG, Brazil) to determine the hematimetric indices of the mean corpuscular volume (MCV; fL) and mean corpuscular hemoglobin concentration (MCHC; g/dL). Glucose (Glu; g/dL) was measured using commercial kits (Labtest).

Growth performance. Fish of all replicates were counted and weighed individually on the first and last day. The final body weight (FBW), weight gain (WG), daily weight gain (DWG) specific growth rate (SGR), and feed conversion ratio (FCR) were determined[69].

Innate immune analysis. The innate immune analysis was performed in all groups (five fish for each replicate) 15 days after treatment and 15 days after challenge with *Francisella noatunensis* subsp. *orientalis* strain F1. Blood samples were collected without anticoagulant, allowed to coagulate, and centrifuged at 1400 *g* for 10 min to obtain the serum, which was stored at −20 °C.

The serum lysozyme concentration was determined in triplicates using a method based on gram-positive *Micrococcus lysodeikticus* lysis[70]. Standard solutions (0–10 ng/μL) of chicken egg lysozyme L6876 (Sigma, Saint Louis, MO, USA) were prepared at the time of analysis from frozen aliquots for generating the standard curve. Subsequently, standard and 150 μL dilutions of the sample were placed in a small tube. A suspension of *Micrococcus lysodeikticus* M3770 (Sigma, Saint Louis, MO, USA) prepared in the same buffer was added to each tube. Absorbance were measured using a spectrophotometer 33D model (Coleman, Santo André, SP, Brazil) at 492 nm. The results were expressed using the values of the optical density variation for each sample volume versus the lysozyme volume of the standard curve. The linear regression equation of the standard lysozyme curve was used to determine serum lysozyme levels (ng/μL).

Alternative complement pathway activity (ACH50) was determined following the method using rabbit red blood cells (RaRBC) as target cells for hemolysis[71]. Briefly, serially diluted serums were mixed with rabbit erythrocyte (RaRBC) suspension and incubated at 25 °C for 1 h with occasional shaking. The extent of hemolysis was estimated by measuring the optical density of the supernatant at 490 nm (OD4,4). Serum dilutions resulting in greater than 90% or less than 15% lysis were excluded from the calculation and the serum dilution that resulted in 50% lysis of RaRBC was represented as ACH50 units/μL.

Antimicrobial activity was measured in a flat-bottom 96-well microplate. Fish serum was evaluated for its antimicrobial activity against *Escherichia coli* prepared at a concentration of 0.5 McFarland scale and diluted 100 times in Poor broth (PB). Subsequently, serial dilution of the serum in PB medium was performed at a factor of 1:2 for 12 dilutions. Saline solution diluted in PB was used as a control. Twenty microliters of the diluted bacteria were added to each well containing the serum and positive control. The microplates were incubated at 28 °C for 24 h. The serum antimicrobial activity was the reciprocal of the least dilution that showed bactericidal activity[72].

Intestine histology and morphology. Five animals from each experimental group (of the two replicates) were used in this analysis. Animals were euthanized by a benzocaine overdose of 250 mg/mL (previously diluted in 5 mL of ethanol and later diluted in water to obtain a final concentration of 250 mg/L in water). Digestive tract samples (distal to pylorus) from each experimental group were collected and then fixed in 10% buffered formalin solution and processed using routine methods. They were embedded in paraffin at 60 °C to obtain cross-sections with a thickness of 5 μm and stained with hematoxylin-eosin (H&E). The slides were mounted (Entellan, Darmstadt, Alemanha) and subjected to microscopic evaluation. The abundance of goblet cells within the villi and lymphoid cells were evaluated[73].

Statistical analysis. All statistical analyses were performed using R version 3.1.3 (R CORE TEAM, 2015). The growth performance, hematological analysis, glucose, and innate immune analysis were subjected to normality and homogeneity tests, followed by analysis of variance (ANOVA) and the Tukey's test to compare arithmetic means, adopting a significance level of 5%. The data that did not present normality or homogeneity were transformed into log10 (x + 1), and the Tukey test was used at 5% significance. For quantitative variables that did not

present a normal distribution even after transformation, the Kruskal-Wallis nonparametric test was used followed by the Dunn's test with a significance level of 5%. The cumulative mortality was analyzed using the Fisher exact test with a significance level of 5% using OpenEpi v. 3.01 (https://www.openepi.com/Menu/OE_Menu.htm).

Microbiome analysis. Nine fish from each group were used for the bacterial microbiome analysis. Each DNA sample was isolated from the stools of three fishes and pooled. After 15 days of treatment (to evaluate alterations in the bacterial microbiome due to product supplementation in feed) and 15 days after infection (to evaluate alterations in the bacterial microbiome after infection), the animals in each experimental group were euthanized by benzocaine overdosage. The stool of the entire intestinal tract was removed aseptically and maintained in sterile vials with refrigeration. The samples were immediately stored in a freezer at −80 °C until processing. For total DNA extraction, the commercial QIAamp DNA Stool Mini kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions. Then, the V4 region of the 16S ribosome subunit gene[74] was amplified with primers containing overlapping regions with Illumina platform primers. After verification of the amplicon quality, the samples were sent to Neoprospecta company for sequencing using the Illumina MiSeq (paired-end library) platform with the 250-cycle V2 kit. The bioinformatics analysis was performed using MOTHUR v.1.36.1 software[75]. Taxonomic classification was obtained using the SILVA database[76]. Briefly, the sequences were filtered by quality parameters (Phred value greater than 15, elimination of reads without their respective pair, among others) and removal of chimeras. The alpha diversity was calculated with MOTHUR software using the parameters Chao1 for richness and Shannon for diversity.

The statistical significance of alpha diversity parameters among groups was analyzed by ANOVA. Additionally, abundance plots were generated at the level of the genus, family and order using MOTHUR outputs. To verify the abundance significance of taxon between groups, STAMP (Statistical Analysis of Metagenomic Profiles) was performed using parent level 1 and profile level 6 to analyze the significance between two groups using the two-sided Welch's t-test[77].

Reads from sequencing were submitted to the FASTQ format for analysis using Mothur software v.1.39.5[78] according to the MiSeq_SOP guidelines (https://www.mothur.org/wiki/MiSeq_SOP) to assemble 16S contigs and improve the quality of the sequences.

Data Availability

The authors declare that all information about this work are promptly available by contacting the corresponding author by email upaduapereira@uel.br. Also, all the raw data (file of reads in fastq format) are available in SRA database (www.ncbi.nlm.nih.gov/sra) with accession numbers SRR8585010 to SRR8585031).

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Author Contributions

S.A. Suphoronski, R.T. Chideroli, C.T. Facimoto, R.M. Mainardi, F.P. Souza and N.M. Lopera Barrero were involved in all *in vitro* and *in vivo* analyses of the study; G.F.A. Jesus and M.L. Martins helped in innate immune analysis and proofreading the manuscript; G.W. Di Santis helped in histopathology analysis; A. de Oliveira helped in electron microscopy analysis; U.P. Pereira, G.S. Gonçalves, R. Dari, and S. Frouel helped in the experimental design, data analysis, and proofreading the manuscript; U.P. Pereira coordinated all analyses of the project.

Additional Information

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Tabela Suplementar 1

Supplementary Table 1: Count of sequences within group.

Analyzed period	**Pre-challenge**				**Post-challenge**			
Groups	**NC**	**G1**	**G2**	**G3**	**PC**	**G1**	**G2**	**G3**
Number of sequences	153597	182535	167909	181564	128368	160840	124945	88543

Tabela Suplementar 2

Supplementary Table 2 Nutrients composition (%) of the commercial fed used in the study.

Nutrients	**Guarantee levels**
Moisture (max)	120 g/kg
Crude protein (min)	360 g/kg
Ether extract (min)	70 g/kg
Fiber (max)	50 g/kg
Mineral matter (max)	140 g/kg
Phosphorus (min)	6000 mg/kg
Calcium (max)	25 g/kg
Calcium (min)	10 g/kg
Vitamin C (min)	350 mg/kg

Data provided by the manufacturer.

Figura Suplementar 1

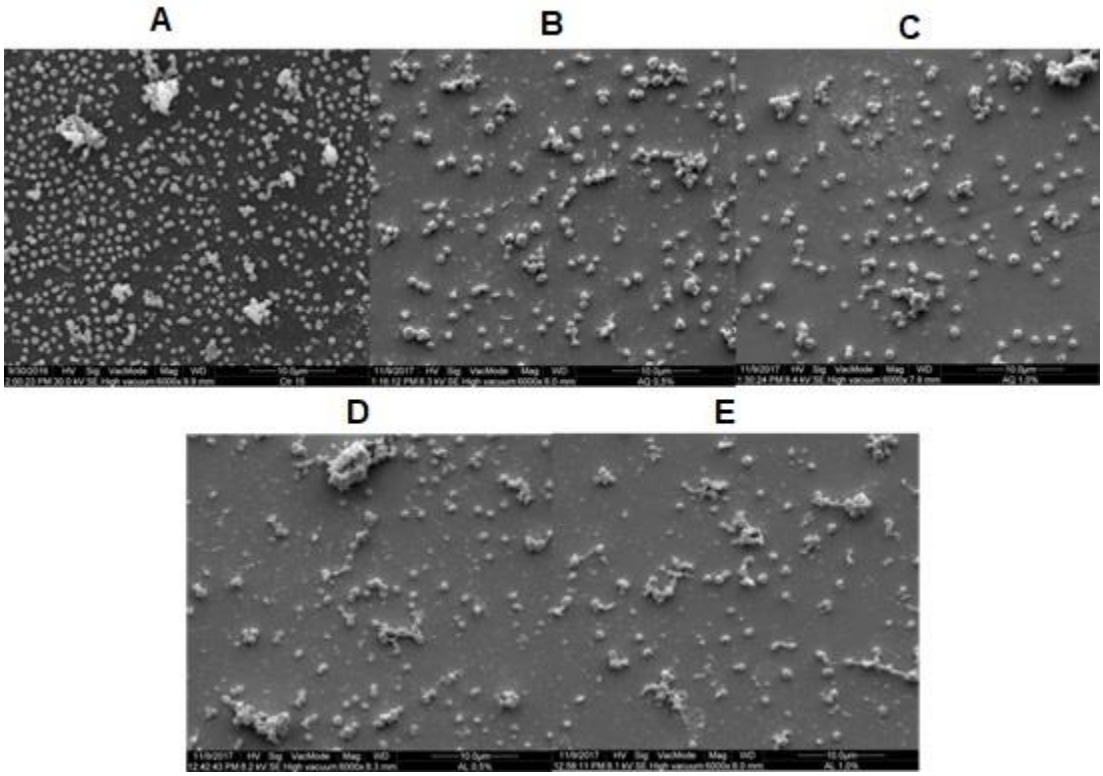


Figura Suplementar 2

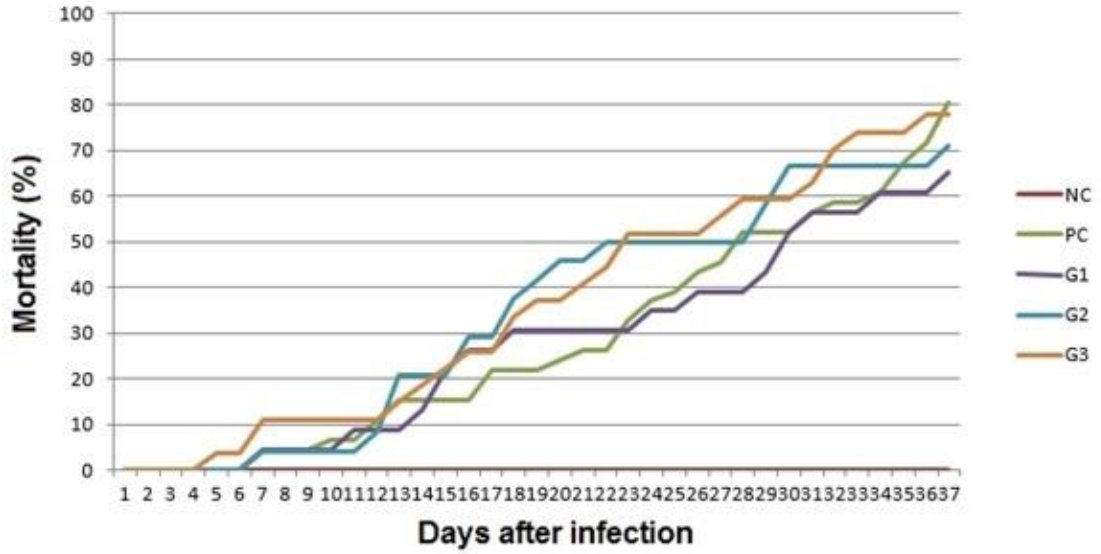


Figura Suplementar 3

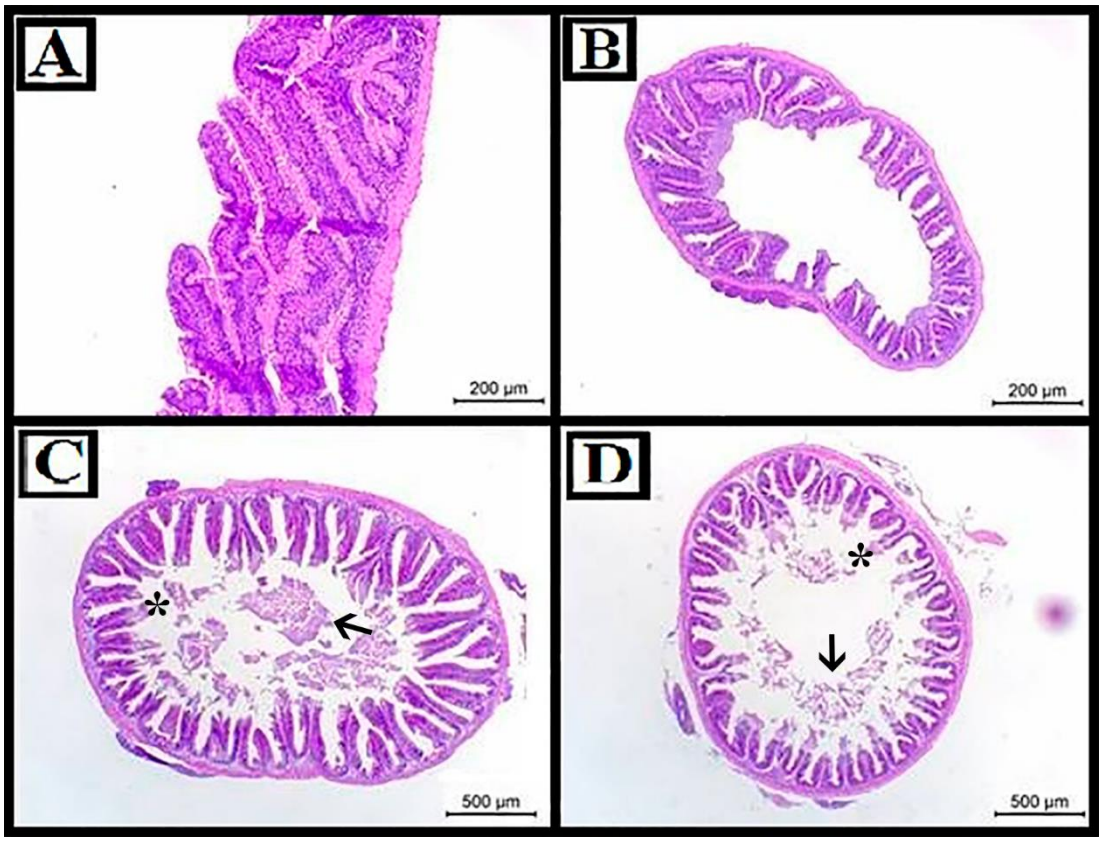
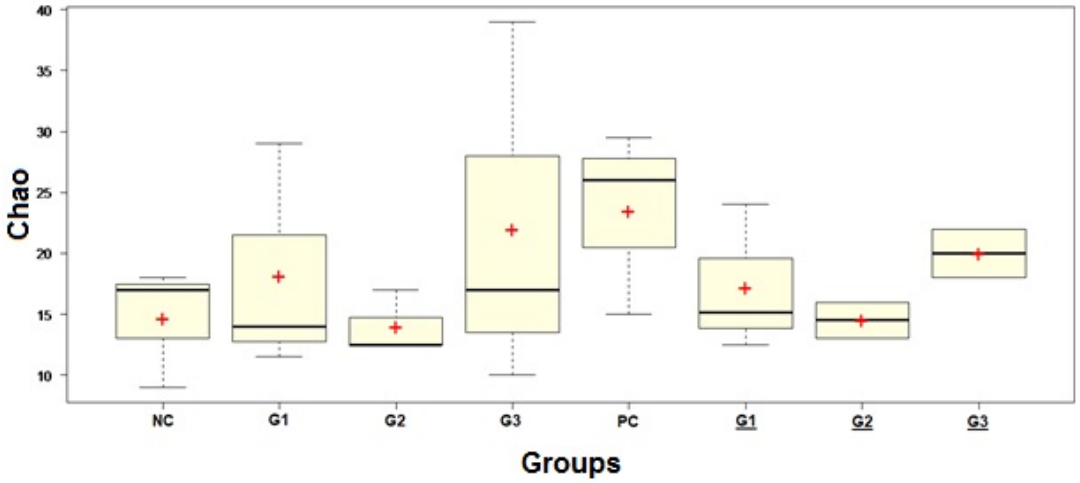


Figura Suplementar 4



6 ARTIGO NAS NORMAS DO PERIÓDICO DA AQUACULTURE RESEARCH "Effect of *Enterococcus faecium* as a water and/or feed additive on the gut microbiota, hematologics, immunological parameters and resistance against francisellosis and streptococcosis in Nile tilapia (*Oreochromis niloticus*)"

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Abstract

In the present study, we evaluated the effects of *Enterococcus faecium* administration on food and / or water on hematological, immunological, intestinal microbiota and Nile tilapia growth. Prior to the *in vivo* experiment, probiotic bacteria isolated from Nile tilapia were selected by inhibition tests. Sequencing, annotation and assembly of the complete genome of the selected bacteria and other tests were performed using bioinformatics tools. Three treatments were implemented: G1 (feeding probiotic); G2 (probiotic in water) and G3 (probiotic in food and water), and the negative control (NC). The treatment lasted 38 days and each group had 45 fish and 2 repetitions. After the groups were divided and infected with *Streptococcus agalactiae* S13 and *Francisella noatunensis* subsp. *orientalis* F1. The average final weight gain was higher in group G1. A significant increase in the number of thrombocytes was observed in the groups that received probiotic in the diet (G1 and G3). There was a statistical difference in mortality of fish infected with *S. agalactiae* S13 from NC compared to treated groups. *Cetobacterium* was the most abundant genus in the intestinal microbiota of all groups, including NC. *Enterococcus faeicum* increases the immunity of the fish that received the treatment and decreases the mortality caused by *S. agalactiae*; in addition, as an autoctone probiotic, it does not interfere with the local ecosystem, so it is a bacterium with great probiotic potential for Nile tilapia in Brazil.

Keywords: bacterioses, microbiota, probiotcs.

Introduction

Fish farming stands as one of the largest aquaculture production in the world, with Nile Tilapia (*Oreochromis niloticus*) being a species of great importance (FAO, 2018). It is estimated that the culture of this species is present and moves the economy of 135 countries in the world, and in Brazil they continue as the most produced fish (FAO, 2018; PEIXEBR, 2019).

In Brazil, it is estimated that 84 million dollars per year is lost in freshwater fish farming, including disease losses, with the bacterioses being the most important fo these (Tavares-Dias and Martins, 2017). Some bacterioses stand out for causing high mortality rates in fish, such as spretococcosis and francicellosis (Soto, Hawke, Fernandez, Morales, 2009; Chideroli et. al., 2017). Antimicrobials are used as treatment and prophylaxis for these diseases, but the indiscriminate use of antibiotics promote the selection of drug-resistant ou multi-resistant bacteria, in addition to the potential risk to the environment and public health (Merrifield, Bradley, Baker, Davies, 2010; Ruiz et al., 2019).

Therefore, the use of probiotics as an alternative strategy has been receiving increasing attention world-wide in tilapia aquaculture (Van Hai, 2015; Alemayehu, Geremew, Getahun, 2018; Bharathi et. al., 2019). Probiotics are live micro-organisms which, when administered in adequate amount, confer health benefits to the host. Several micro-organisms, including Gram-negative and Gram-positive bacteria, have been used as probiotics in fish farming such as *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Carnobacterium, Shewanella, Bacillus, Aeromonas, Vibrio, Enterobacter, Pseudomonas, Clostridium,* and *Saccharomyces* species (Nayak, 2010; Van Hai, 2015). Probiotics, either alone or in association with supplementation, can elevate phagocytic, lysozyme, complement, respiratory burst activity as well as expression of various cytokines in fish (Wang, Tian, Yao and Li, 2008; Wang et. Al., 2017; Ruiz et. Al., 2019). Also, they stimulate the gut immune system of fish with marked increase in the number of Ig(+) cells and acidophilic granulocytes (Nayak, 2010).

Studies have shown that the efficacy of probiotics is highest in the host species from which they are isolated because such strains perform better as they have already

adhered to the gut wall of the fish and are well adapted to compete with the pathogens (Ghosh, Sinha, Sahu, 2007).

It was found that Nile tilapia fingerlings supplemented with *Bacillus cereus* for 42 days in the water and feed had a significant increase in lysozyme, in addition to other immunological parameters. However, the results were better when the probiotic was added to the feed (Wang et. Al., 2017). Studies conducted with the addition of *Lactobacillus plantarum* in Nile tilapias observed that after 12 weeks the fish had higher feed efficiency, yield and final weight and there was also an increase in thrombocytes and leukocytes in these animals (Jatobá et al., 2011). Also in Nile tilapia the use of commercial probiotics after six weeks positively affected fish zootechnical performance, increased the number of goblet cells in the gut and the expression of immunity-related genes (Standen et al., 2016).

However, most studies use probiotics isolated from other species or geographic regions, which may interfer with their mode of action. When the probiotic is isolated from the host itself, it can increase the chances of colonization / adhesion, increasing its benefical effects. In this context, aiming to develop alternative methods in tilapia farming, to avoid the highly use of antibiotics, this study investigated the use of *Enteroccous faecium* as probiotic, supplemented in the diet and water of *Oreochromis niloticus* and searching for possible improvements in the health of fish and the effects on the intestinal microbiome, as well as it's growth rates and zootechnical parameters.

Materials and Methods

Probiotic bacteria selection, genome assembly and identification of metabolic regions

The *Enterococcus faecium* strain LAC7.2 selected for probiotic was isolated gastrointestinal tract healthy Nilo tilapias, arising from hatchery of city Londrina/ Paraná – Brazil in 2017. *In vitro* tests were performed to detect its probiotic potential. For this, the Nile tilapia faeces were diluted (scale 10) in 0.85% saline, plated on Man, Rogosa e Sharpe (MRS) Lactobacillus Kasvi® agar and incubated for 48 hours at 28°C Colonies were selected and characterized. Then, the selected bacteria were seeded

on MRS agar and incubated for 48 hours. Separately a solution containing Mueller Hinton agar (Kasvi, São José dos Pinhais, PR, Brazil) at 45°C with pathogenic bacteria (*Escherichia coli, Staphylococcus sp. e Streptococcus sp*.) was prepared. This solution was placed on the MRS plates (which contained the probiotic bacteria). The plates were incubated for a further 24 hours at 28°C and the inhibition halos were measured bacteria with larger halos were selected. To evaluate inhibition with *Francisella noatunensis* subsp. *orientalis* F1, it was performed on heart cystine agar (Kasvi), where the pathogenic bacteria were seeded with swab all over the plate surface, then small holes were made in the agar and the *Enterococcus faecium* LAC 7.2 filtered (0,22 μm) supernatant was deposited. The readings were taken after 48h. This test was performed in duplicate.

The genome sequencing of the *Enterococcus faecium* was performed with the MiSEQ platform (Illumina®, USA). Reads were uploaded in FASTQ format to CLC Genomics Workbench 12 (Qiagen, USA) software, for trimming and assembly steps. Genome annotation was performed in RAST - Rapid Annotation using Subsystem Technology version 2.0 (Aziz et al., 2008). The genome project was deposited in the GenBank database under the accession number CP045012.1. A summary of the project information is show in Table 1.

Phylogenetic analysis was performed using Gegenees V2.2.1 (Agren, Sundström, Håfström and Segerman, 2012) and Splitstree4 v4.15.1 with high accuracy, generating a heatmap and phylogenetic tree, respectively. The prediction of secondary metabolite clusters in the sequenced genome ere carried using antiSMASH 5.0 (Weber et. al., 2015) and RAST. The ResFinder 3.2 program was also used to detect resistance genes.

Fish

This study was conducted in (LABBEP) Fish Bacteriology Laboratory at Londrina State University, Paraná, Brazil. A total of 405 healthy juvenile Nile tilapia (*O. niloticus*) were obtained from a commercial hatchery from Paraná state, mean individual initial weight was 11,93 g. The animals were stocked in 150L tanks containing dechlorinated water with continuous renewal (80% of volume daily), the

temperature was maintained at approximately 25 °C and the fish were fed three times a day until apparent satiation. Water parameters (pH of 6.8 to 7.2, total ammonia <0.4 mg/L^{-1}, and absence of chlorine) were measured daily and main- tained throughout the experimental period. The presence of oxygen was maintained by two aerators, resulting in dissolved oxygen of 5.4 mg/L on an average. Before the start of the feeding trial, the fish underwent a period of acclimatization for 7 days, and behavior/signs of disease were observed (exophthalmia, erratic swimming, skin lesions, and others). Microbiological diagnosis was also performed before the experiment, in which 20 fish were randomly sampled and euthanized by a high-dose of benzocaine (200 mg/mL). Aseptically, fragments of brain, liver, cranial kidney, and spleen were streaked on Mueller Hinton agar enriched with 5% defibrinated ovine blood and in heart cystine agar. The plates were incubated at 28 °C for 5 days for confir- mation of the health status of fish (no bacterial growth in the plates). All animal procedures were approved by the Ethic Committee on Animal Use of State University of Londrina (Approval number CEUA/UEL-7327.2017.39) and all experiments were performed in accordance with relevant guidelines and regulations.

Experimental Design and Basal Diet

Were determined: the medium final body weight (g) and weight gain (g) (Ridha, 2006). The fish were divided in three treatment groups with two repetitions (45 fish in each) and two more control groups. This experimental design was performed during the probiotic administration period, 38 days total, the division of the groups is shown in Table 2.

Then this period, material was collected for further analysis, which will be described below. Behind fishes were infected with *Streptococcus agalactiae* S13 and *Francisella noatunensis* subsp. *orientalis* F1. One day before the disease challenge, the water temperature was decreased gradually and maintained at 21 ºC (±1 ºC) to promote infection by *Francisella* spp., since outbreaks of francisellosis in Brazil occur at similar temperatures (Ortega et. a., 2016), the infection occurred by immersion (7.1× 10^5 CFU/mL of water in the tank). The water temperature was increase gradually and maintained at 28 ºC (±1 ºC) to promote infection by *Streptococcus* sp. (Chen et.

al., 2012), the infection occurred intraperitoneally with 0.1mL/fish (8.8. × 10^5 CFU/mL). After the new organization of groups (Table 2). Fish were evaluated for 30 days for clinical signs and mortality.

The probiotic feed was produced every 10 days. Approximately three bacterial colonies of *Enterococcus faecium* were placed in 600mL of broth MRS *Lactobacillus* Kasvi®, incubated under agitation for 48 hours and 28ºC. Then, 100mL was pulverized in 1kg feed, with 5mL universal vehicle and drying in 28ºC for 8-12 horas. Fish were fed four times a day. The group with probiotic in water had reduced water flow for 10 cm tall (volume about 27 liters) and added 100mL of broth MRS with *Enterococcus faecium*, during for two hours. Then, volume water was reestablished. This rocedure performed every 10 days. Probiotic concentration in each preparation was also verified by bacterial counting.

Blood sampling

Blood samples were collected at 38 days post treatment with probiotcs (16 samples per group). The fish were anaesthetized with benzocaine (0.1 g/L), and blood was collected by puncturing the caudal vessel in 3 mL syringes (21 G) containing 10% anticoagulant (ethylen- ediaminetetraacetic acid). The blood was used to measure the hematocrit (Hct; %) using the microhematocrit method (Ranzani, Pádua, Tavares-Dias and Egami, 2013), and red blood cells (RBC; 106/µL) were counted in a Neubauer chamber following dilution at 1:200 in Dacie solution. White blood cell (WBC; 103/µL) and total thrombocyte counts were calculated using an indirect method (Ishikawa, Ranzani-Paiva and Lombardi, 2008). For differential counting of leukocytes, the smears were stained with May-Grünwald/Giemsa/Wright stain. The hemoglobin concentration (Hgb; g/dL) was analyzed by the cyanmethemoglobin method (Collier, 1944) using commercial kits (Labtest, Lagoa Santa, MG, Brazil) to determine the hematimetric indices of the mean corpuscular volume (MCV; fL) and mean corpuscular hemoglobin concentration (MCHC; g/dL).

Innate immune analysis

The innate immune analysis was performed in all groups (five fish for each replicate) 38 days after treatment . Blood samples were collected without anticoagulant, allowed to coagulate, and centrifuged at 1400 g for 10 min to obtain the serum, which was stored at −20 °C.

Serum lysozyme activity was determined using a methodology adapted from that of Demers and Bayne, 1997. Briefly, the initial and final absorbances were measured by spectrophotometry while determining the serum lysozyme activity by the lysis of the Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma-Aldrich Chemical Co.). The reduction in the absorbances of the samples was converted into an estimate of the lysozyme concentration (µg mL-1).

Alternative complement pathway activity (ACH50) was determined following the method using rabbit red blood cells (RaRBC) as target cells for hemolysis (Sunyer and Tort, 1995). Briefly, serially diluted serums were mixed with rabbit eryth- rocyte (RaRBC) suspension and incubated at 25 °C for 1 h with occasional shaking. The extent of hemolysis was estimated by measuring the optical density of the supernatant at 490 nm (OD4,4). Serum dilutions resulting in greater than 90% or less than 15% lysis were excluded from the calculation and the serum dilution that resulted in 50% lysis of RaRBC was represented as ACH5O units/µL.

Microbiome analysis

After 38 days of treatment, six fish from each group were used for the bacterial microbiome analysis Each DNA sample was isolated from the stools of two fishes and pooled. The animals in each experimental group were euthanized by benzocaine overdosage. The stool of the entire intestinal tract was removed aseptically and maintained in ster- ile vials with refrigeration. The samples were immediately stored in a freezer at −80 °C until processing. For total DNA extraction, the commercial QIAamp DNA Stool Mini kit (QIAGEN, Hilden, Germany) was used accord- ing to the manufacturer’s instructions. Then, the V4 region of the 16S ribosome subunit gene was amplified with primers containing overlapping regions with Illumina platform

primers (Klindworth et. al., 2013). After verification of the amplicon quality, the samples were sent to Neoprospecta company for sequencing using the Illumina MiSeq (paired-end library) platform with the 250-cycle V2 kit. The next steps were followed according to Suphoronski et. al., 2019, using MOTHUR v.1.36.1 software75.

Statistical analysis

Through the software R data were submitted to normality and homogeneity tests and subsequently to analysis of variance (ANOVA), followed by Tukey test for comparison between arithmetic means, adopting a significance level of 5%. For quantitative variables that did not present normal distribution, the nonparametric Kruskal-Wallis test was used followed by the Dunn test with a significance level of p <5%. The cumulative mortality was analyzed using the Fisher exact test with a significance level of 5% using OpenEpi v. 3.01 (https://www.openepi.com/Menu/OE_Menu.htm). In metagenomic, to verify the abundance significance of taxon between groups, STAMP (Statistical Analysis of Metagenomic Profiles) was per- formed using parent level 1 and profile level 6 to analyze the significance between two groups using the two-sided Welch's t-test (Donovan, Gene, Philip and Robert, 2014).

Results

Probiotic bacteria selection, genome assembly and identification of metabolic regions

The inhibition halos measured for picked strain were 16 mm (*Escherichia coli),* 15 mm (*Staphylococcus* sp.) e 10 mm (*Streptococcus* sp.), as shown in Figure 2. And the inhibition zone for Francisella noatunensis subsp. orientalis F1 was 18 mm.The complete genome of *Enterococcus faecium* strain comprises a single circular chromosome 2,625.745 bp in length and two plasmids with 206.375 bp and 80.816 bp adding 2.912.936 bp total, with 37.96% G+C content, 18 rRNA operons, 68 tRNA genes and 131 pseudogenes (Table 3). It was found in the phylogenetic tree the ancestors of the bacterium evaluated as *Enterococcus casseliflavus* (Figure 3). We

observed that in the heat map there is a high similarity of *Enterococcus faecium* evaluated with other strains of the same species, but it was not 100% similarity with other strains. In the anti-SMACH analysis, we observed as secondary metabolite Enterocin A with 100% similarity in region 10 and polysaccharide in region 3 with 82% similarity (Figure 4). In ResFinder, two resistance genes were found in one of the plasmids, being for aminoglycosides and macrolides.

Experimental Design and Basal Diet

The mean probiotic concentration was 5.53 x 10^6 CFU g-1. The medium final body weight and weight gain and were demonstrated in Table 4. No significant differences were observed in weight gain (g) ($p > 0.05$, Table 4), however, medium final body weight (g), specific growth rate (SGR) and weight gain (%) were significantly higher in G1 group compared with other groups, incluid NC.

Blood sampling and innate immune analysis

There were no significant differences ($p > 0.05$) between the fish treatments and control group in their measurements of the Hematocrit, Hemoglobin, Erytrocytes, MCV and MCHC parameters. Thrombocytes counts, on the other hand, showed significantly larger values in the G1 than in the G2 and NC. However, there was a difference in MCH comparing NC with G2. The differential leukocyte cell counts did not show significant differences between treatments for total leukocytes, neutrophils, or monocytes. No eosinophils and basophils were observed in either of the treatments tested (Table 5). The serum lysozyme concentrations and complement activity mean, also did not differ significantly between the treataments groups and NC ($p > 0.05$) (Table 5).

Microbiome analysis

A total of 9.038.053 sequences were obtained for all groups, and 309 OTUs (operational taxonomy units) were identified. A rarefaction curve showed that

sequencing was sufficient to sequence most of the bacterial species present in the fish gut, suggesting that the read count of the trial was representative of the intestinal bacteriome in all groups (Figure 3). In Table 6 displays the total number of sequences obtained in each group. Moreover, Mothur software was used to calculate the Shannon index, which compares the diversity of species in each group. However, no significant differences were observed. The boxplot shows that G1 and NC groups presented the lowest level of species diversity, while the G2 and G3 groups had higher level of diversity (Figure 6). Suggesting that the groups receiving the probiotic water had greater diversity. The abundance of bacterial species calculated by Mothur software is shown in Figure 7. The abundance plot displays the most abundant bacteria in each sample: Cetobacterium, Vibrionaceae_unclassified, Plesiomonas, Gammaproteobacteria_unclassified, Streptophyta_unclassified, Bacteroidales_unclassified and Enterobacteriaceae_unclassified. Cetobacterium was most abundant in all groups, but the G1 and NC groups obtained great porcentage of Cetobacterium, comparing with groups G2 and G3. However Vibrionaceae_unclassified was more abundant in groups G2 and G3.

Mortality after infection

The cumulative mortality of fishes infected *Francisella noatunensis* subsp. *orientalis* F1 was: CP= 65,12%; G1=68,18%, G2=68,18% e G3=54,35%, yet there was no statistical difference between the groups (Figure 8). However, in the cumulative mortality of fishes infected *Streptococcus agalactiae* S13 showed statistical difference between CP and other groups that received probiotic in feed and/or water. Mortality was: PC=88,29%; G1=75,56% e G2 e G3=73,33% (Figure 9).

Discussion

Use of probiotics in aquaculture is now increasingly being considered as an eco-friendly approach to mitigate health related problems. Disease preventing abilities of probiotics are achieved through enhancement of immunity and exclusion of pathogens (Das, Nakhro, Chowdhury and Kamilya, 2013). To use a bacterium as a probiotic, tests

are needed to verify its potential (Repally, Perumal, Dasari, Palanichamy and Venkatesan, 2018). *In vitro* tests performed on isolated Nile tilapia bacteria, *Enterococcus faecium*, showed their potential, forming halos against pathogenic bacteria. Similar results were found by Reda, Selim, El-Sayed and El-Hady (2017), where he found antibacterial activity in Nile tilapia intestinal bacteria against the pathogens *Aeromonas sobria, Aeromonas hydrophila, Pseudomonas aeruginosa, Pseudomonas putida and Staphylococcus aureus* .

However, too it is important to know the bacterial genome that is being used as probiotic and with the use of bionformatics it is possible to discover possible mutations, resistance genes and antibacterial metabolites, which can define the bacteria as a probiotic potential. We observed from the heatmap and phylogenetic tree that strain LAC7.2 was not 100% similar to other strains. This results can influence the probiotic effect, being better, worse or absent. In the analysis of secondary metabolites performed in silico the main finding was enterocin, as well as in the work of Aymerich et. al., 1996, which also observed bactericidal power of this metabolite.

Two resistance genes were found in one of the plasmids, being for aminoglycosides and macrolides, but we don't know if these genes are functional or not. The antimicrobials released by Brazilian legislation today for use in fish farming are Florfenicol and Tetracyclines, that is, they do not belong to the class of antimicrobials that the bacterium in question has resistance.

Regarding fish performance, group G1 had a significant increase in the final average weight and specific growth rate, but the average weight of all groups did not differ. Safari, Adel, Lazado, Caipang and Dadar (2016) , evaluating juvenile rainbow trout *Oncorhynchus mykiss* that received different doses of *Enterococcus casseliflavus* for eigth weeks found that the highest dose groups (10^8 CFU $g^{(-1)}$) of feed [T2] and 10^9 CFU $g^{(-1)}$) of feed [T3]) significantly improved growth parameters. Then consider that increasing the probiotic dose may provide an improvement in results.

In analyses blood, thrombocyte counts were higher in the G1 group (probiotic in feed), with estatistic diference G2 (probiotic in water) and NC. The thrombocytes are important in the organic defense mechanism, this is demonstrated by their appearance in coagulation and inflammatory processes as well as by their phagocytic activity during infections (Jatobá et. al. 2011). Thus, this result suggests that fish that received

probiotics in the feed a more stimulated immune system than those that received in water. In the other haematologycal parameters there was no difference between the groups.

Proliferation of cytokines and stimulation of natural killer lymphocytes, increased production of antibodies, phagocytic rate, and lysozyme activity are responses of modulation of the immune system from probiotic supplementation. (Matsuzaki and Chin 2000). Several studies have shown that the use of probiotic in fish increases these immunological indices (Jatoba et. al., 2011; Pereira et. al., 2016; Ruiz et. Al., 2019). However, in this study there was no difference in complement activity mean and lysozyme.

The microbial community of the gastrointestinal tract is known to stimulate immune system development and promote competition with pathogenic micro-organisms. Moreover, they are fundamental for the integrity of intestinal villi and to ensure proper nutrient metabolism for fish (Hooper and Littman, 2015). There are few studies that talk about the evaluation of the intestinal community after probiotic supplementation from metagenomic analysis. In the present study, we observed that the genus Cetobacterium varied its abundance in the groups, groups G1 and NC presented higher percentage, while G2 and G3 smaller. Thus, we also observed higher percentages of unclassified Vibrionaceae and Plesiomonas in groups G2 and G3, in the less abundant NC Plesiomonas. Suggesting that groups that received the probiotic only in water or water and race have greater diversity. At work, Standen et. al., 2015 using a commercial probiotic (AquaStar®), which contained various bacteria, found different populations in the gut microbiota after eight weeks, and in NC, *Bacillus, Cetobacterium* and *Mycobacterium* were the dominant genera and while *Bacillus, Enterococcus* and *Pediococcus* were the largest constituents in fish fed probiotics. Has previously been shown that fish gut communities vary within species because of factors such as dietary input, season, developmental stage and the surrounding habitat (Sullam et. al., 2013).

Mortality caused after *Streptococcus agalactiae* S13 infection was significantly lower in all groups receiving the probiotic than in the positive control group. As observed in other studies where the probiotic used stimulated the immune system during infection with pathogenic bacteria (Elala and Ragaa, 2015; Safari et. al, 2016).

However, mortality with *Francisella noatunensis* subsp*. orientalis* F1 had no difference between groups. In the present work we evaluated the administration of only one probiotic bacteria in Nile tilapia. Lee et. al., 2017 carried out a study comparing the administration of different probiotics (*Bacillus subtilis* WB60 and *Lactobacillus plantarum* KCTC3928), but in isolation. Few studies have verified the action of symbiotics, using probiotics associated with other probiotics or prebiotics. Devi et. al, 2019, using a symbiotic diet found that immune responses in the fish evaluated were earlier, compared with the groups that received only probiotic or prebiotic.

It is important to note that the use of autoctone probiotics can benefit not only the fish itself, but the aquatic community, as it is a bacterium that is already present in the environment. Unlike isolated probiotics in other regions / countries that can negatively influence the local aquatic community. And yet the use of probiotics is directly related to unique health, because if we use less antimicrobials in animal production we will be collaborating so that there is no increase in superbugs. Therefore, further studies with autochthonous probiotic bacteria should be carried out in different concentrations and dosages to better assess their potential in fish.

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Data Availability Statement

The authors declare that all information about this work are promptly available by contacting the corresponding author by email upaduapereira@uel.br. Also, all the raw data (file of reads in fastq format) are available in SRA database (www.ncbi.nlm.nih.gov/sra).

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Tables

Table 1: Genome sequencing project information

Property	Term
Finishing quality	Finished
Libraries used	One paired-and library (mean size 300 bp, DNA insert size of ~300bp)
Sequencing platforms	Illumina MiSeq
Fold coverage	377.0x
Assemblers	NCBI Prokaryotic workbench v12.0.2
Gene calling method	NCBI Prokaryotic Genome Annotation Pipeline
Genebank ID	CP045012.1 (chromosome) CP045013.1 (plasmid pI) CP045014.1 (plasmid pII)
Genbank Date of Release	October 18, 2019
BIOPROJECT	PRJNA224116
Source Material Identifier	LAC7.2
Project relevance	fish

Table 2: Division of groups before and after the challenge

Before the challenge		
Group	Treatment	Tanks
G1	probiotic in feed	2
G2	probiotic in water	2
G3	probiotic in feed and water	2
NC	no probiotic	3
After challenge		
Group	Treatment	Tanks
G1A	probiotic in feed and challenge with Francisella noatunensis subsp. orientalis F1	1
G1B	probiotic in feed and challenge with *Streptococcus agalactiae* S13	1
G2A	probiotic in water and challenge with Francisella noatunensis subsp. orientalis F1	1
G2B	probiotic in water and challenge with Streptococcus agalactiae S13	1
G3A	probiotic in feed and water and challenge with Francisella noatunensis subsp. orientalis F1	1
G3B	probiotic in feed and water and and challenge with Streptococcus agalactiae S13	1
NCA	no probiotic and challenge with *Francisella noatunensis* subsp. *orientalis* F1	1
NCB	no probiotic and challenge with *Streptococcus agalactiae* S13	1
NCC	o probiotic and no challenge with bacteria	1

Table 3: Genome *Enterococcus faecium* annotated by RAST server

Attribute	**Value**
Gene (total)	2.931
CDs (total)	2.841
Genes (coding)	2.710
Genes (RNA)	90
rRNAs	6, 6, 6 (5S, 16S, 23S)
tRNAs	68
ncRNAs	4
Pseudo Genes (Total)	131

Table 4. Growth performance of Nile tilapia from different treatments *Different letters (a and b) indicate significant difference between the treatments ($p < 0.05$); *G1: Probiotics in feed; G2: Probiotics in water; G3: Probiotics in feeds and water; NC: negative control.

Groups	G1	G2	G3	NC
Medium Final Body Weight(g)	40.15±1.35[a]	34.5±1.03[b]	35.4±0.85[b]	34.25±0.90[b]
Weight Gain (g)	28.64±3.51	22.86±0.61	22.91±0.57	21.39±0.9
Weight Gain (%)	248.29±23.3[a]	199.08±3.35[b]	183.53±4.23[b]	174.54±0.85[b]
Specific growth rate (SGR)	3.27±0.24[a]	2.88±0.04[b]	2.74±0.05[b]	2.65±0.01[b]

Table 5. Blood general parameters (mean± standard error) of experimental groups of Nile tilapia supplemented with probiotics. *Different letters (a and b) indicate significant difference between the treatments ($p < 0.05$); *G1: Probiotics in feed; G2: Probiotics in water; G3: Probiotics in feeds and water; NC: negative control.

Groups	G1	G2	G3	NC
Hematocrit (%)	28.83±0.77	21.8±1.03	25.8±0.89	28.6±1.04
Hemoglobin (g/dL)	7.29±0.41	6.49±0.53	5.84±0.59	5.97±1.26
Erythrocytes (10^6/µL)	1.49±0.08	1.14±0.04	1.18±0.04	1.37±0.06
Thrombocytes (10^3/µL)	64.1±3.87[a]	33.1±1.33[b]	42.2±3.38[ab]	34.02±3.91[b]
Leukocytes (10^3/µL)	58.04±4.61	31.88±0.78	39.46±2.07	50.60±5.21
Lymphocytes (10^3/µL)	25.72±2.92	13.05±0.52	19.48±1.17	20.93±1.84
Neutrophils (10^3/µL)	28.81±3.34	17.75±0.71	18.45±1.57	28.28±3.48
Monocytes (10^3/µL)	3.5±0.47	1.07±0.27	1.52±0.26	1.38±0.29
MCV (fL)	204.02±12.37	196.32±15.25	206.83±12.56	216.01±12.58
MCH (g dL^{-1})	50.67±2.05	56.85±0.99	53.69±0.82	43.71±0.95
MCHC (g/dL)	27.14±0.27	32.64±2.66	24.25±2.1	21.7±1.56
Lysozyme (µg/mL)	3.33±0.59	4.96±1.13	3.16±0.71	4.94±0.80
Complement activity mean (µL for lysis of 50% of erythrocyte)	45.25±2.87	52.1±4.58	41.01±3.09	45.63±1.22

Table 6. Count of sequences of the most abundant species in experimental groups. G1: Probiotics in feed; G2: Probiotics in water; G3: Probiotics in feeds and water; NC: negative control.

Taxon	Numbers of sequences (%)			
	G1	G2	G3	NC
Cetobacterium	91709.5 (92.7)	36749 (55.2)	54798.5 (61.7)	67008 (83.6)
Vibrionaceae_unclassified	5054.5 (5.1)	25973 (39)	28610 (32.2)	11570 (14.4)
Plesiomonas	1191.5 (1.2)	2020 (3)	3779 (4.3)	515 (0.6)
Gammaproteobacteria_unclassified	260.5 (0.3)	1271 (1.9)	1083 (1.2)	459 (0.6)
Streptophyta_unclassified	539 (0.5)	15.5 (0)	111.5 (0.1)	1.5 (0)
Bacteroidales_unclassified	36.5 (0)	188.5 (0.3)	47 (0.1)	361 (0.5)
Enterobacteriaceae_unclassified	185.5 (0.2)	303.5 (0.5)	435,5 (0.5)	227 (0.3)
Total of reads	91709.5	66520.5	88864.5	80141.5

6 CONCLUSÃO

Alternativas ao uso de antimicrobianos, como demonstrados nesses trabalhos, são de suma importância na produção animal, pois além de auxiliar na imunidade e performance dos que a consumem, também impede que novas bactérias super-resistentes apareçam. Além disso a utilização de compostos orgânicos, fitogênicos e probiótico autóctones não interferem no micro-ecossistema presente no ambiente aquático, sendo assim produtos mais seguros dos que antimicrobianos.

Por isso mais estudos devem ser realizados, utilizando outras espécies de peixes, dosagens e concentrações diferentes dos produtos e também a administração dos produtos em pisciculturas regionais verificando dessa forma melhor os seus efeitos, dentro na produção animal.

APÊNDICES

APÊNDICE 6: Figura 1

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1: Enterococcus_casseliflavus_strain_EC20	100	3	3	2	2	2	2	2	2	3	2	2	3	3	3	3	3	3	3	3
2: Enterococcus_mundtii_strain_DSM4838	3	100	73	6	6	6	2	2	2	6	6	6	6	6	6	6	6	6	6	6
3: Enterococcus_mundtii_strain_Pe103	3	80	100	7	7	7	2	2	2	7	6	7	7	7	7	6	7	7	7	7
4: Enterococcus_hirae_strain_R17	3	7	7	100	91	87	3	3	3	8	7	8	8	8	8	7	7	7	7	7
5: Enterococcus_hirae_strain_FDAARGOS_234	3	8	8	92	100	88	3	3	3	8	8	8	8	8	8	8	8	8	8	8
6: Enterococcus_hirae_ATCC9790	3	7	8	88	88	100	3	3	3	9	9	9	9	9	8	9	9	9	8	9
7: Enterococcus_faecalis_ATCC29212	2	2	2	2	2	3	100	88	87	5	4	5	4	4	4	4	5	5	4	3
8: Enterococcus_faecalis_strain_VE14089	2	2	2	2	2	2	80	100	96	4	3	4	4	4	3	4	3	3	2	3
9: Enterococcus_faecalis_strain_B594	2	2	2	2	2	2	82	98	100	3	3	3	3	3	3	3	3	3	2	3
10: Enterococcus_faecium_strain_ISMMS_VRE_1	4	8	7	8	8	8	6	5	5	100	83	83	84	83	83	84	88	88	82	83
11: Enterococcus_faecium_strain_2014_VREF_41	4	8	8	8	7	8	4	4	4	86	100	89	90	86	88	86	85	85	84	87
12: Enterococcus_faecium_Aus0085	4	7	7	8	8	9	5	4	4	85	88	100	94	92	89	88	87	87	84	85
13: Enterococcus_faecium_strain_6E6	4	8	7	8	8	9	5	5	5	87	91	95	100	92	90	88	87	87	86	88
14: Enterococcus_faecium_strain_VRE001	4	7	8	9	9	9	5	5	5	88	88	95	94	100	89	88	89	89	85	88
15: Enterococcus_faecium_strain_E1	4	8	8	8	8	8	5	5	5	88	91	93	92	90	100	90	89	89	86	89
16: Enterococcus_faecium_strain_AUSMDU00004142	5	9	8	8	8	9	5	5	5	90	90	92	91	89	91	100	90	90	88	89
17: Enterococcus_faecium_strain_UAMSEF_09	4	8	8	8	8	9	5	4	4	94	88	91	90	90	90	90	100	100	89	88
18: Enterococcus_faecium_strain_UAMSEF_20	4	8	8	8	8	9	5	4	5	94	88	91	90	90	90	90	100	100	89	88
19: Enterococcus_faecium_strain_LAC7.2	4	8	8	8	8	9	4	3	3	93	94	94	94	92	92	93	95	95	100	94
20: Enterococcus_faecium_DO	4	7	7	8	8	9	4	4	4	93	94	93	95	93	94	93	92	92	92	100

Figure 1: Heatmap based on whole genome of selected *Enterococcus faecium*. Heatmap of similarity among non-core regions of *Enterococcus faecium* genomes using Gegenees all in all fragmented comparison with high accuracy.

APÊNDICE 7: Figura 2

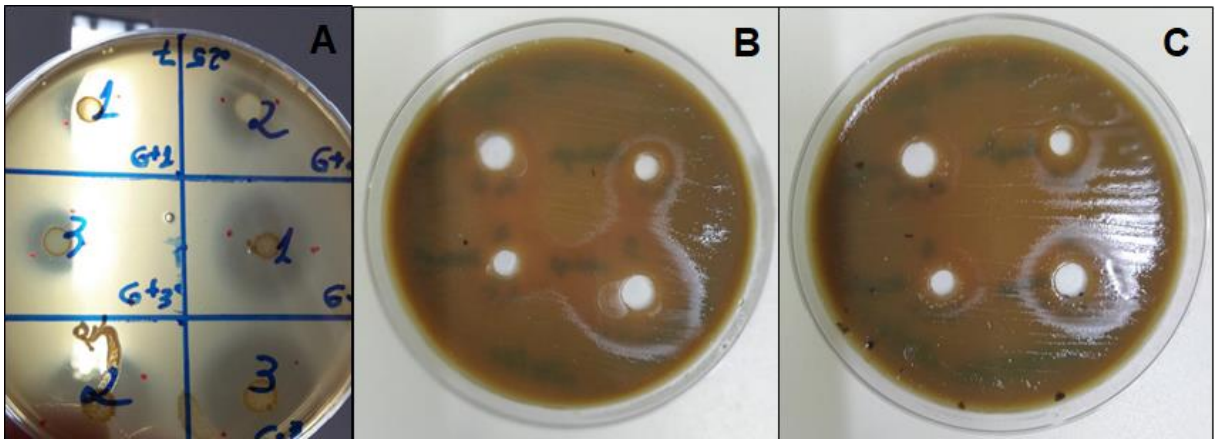


Figure 2: A: MRS agar plate containing probiotic bacteria seeding with overlay layer of Mueller-Hinton agar containing the bacterial pathogen, showing inhibition halos against *Staphylococcus* spp. **B:** Inhibition halos of 15-25 mm of the probiotic bacterium supernatant in heart cystine agar sown with *Francisella noatunensis* subsp. *orientalis.* **C:** Inhibition halos of 12-18 mm of the probiotic bacterium filtered supernatant in heart cystine agar sown with *Francisella noatunensis* subsp. *orientalis.*

APÊNDICE 8: Figura 3

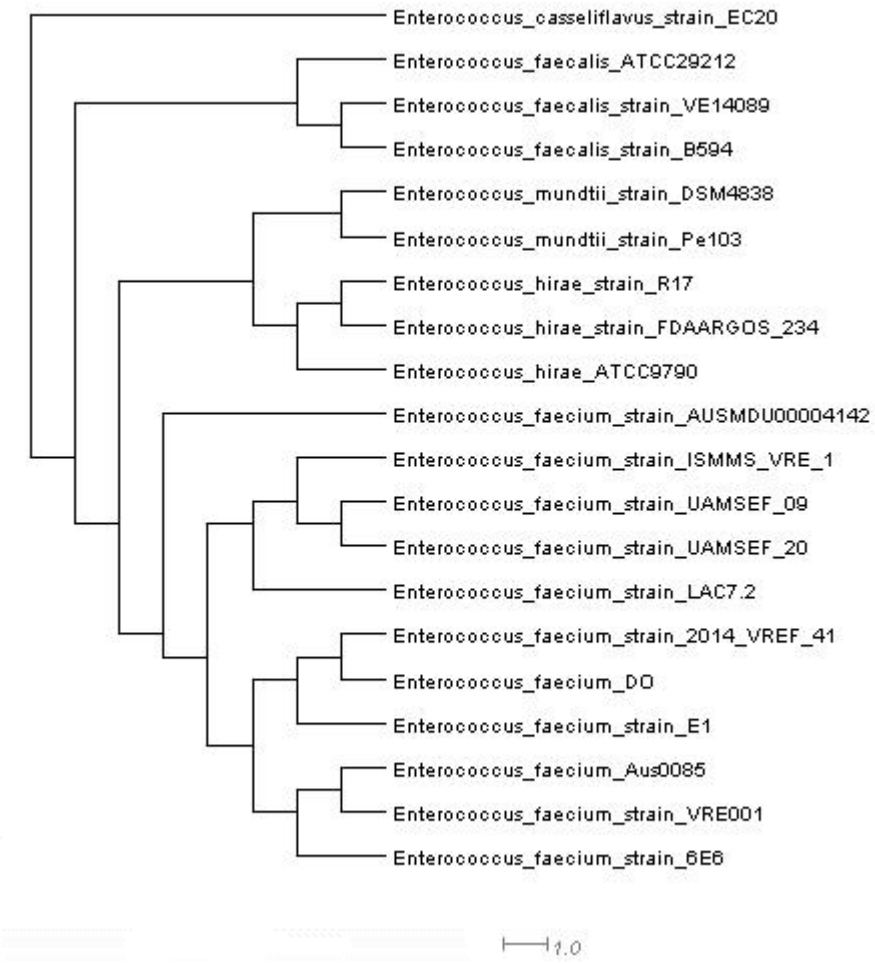
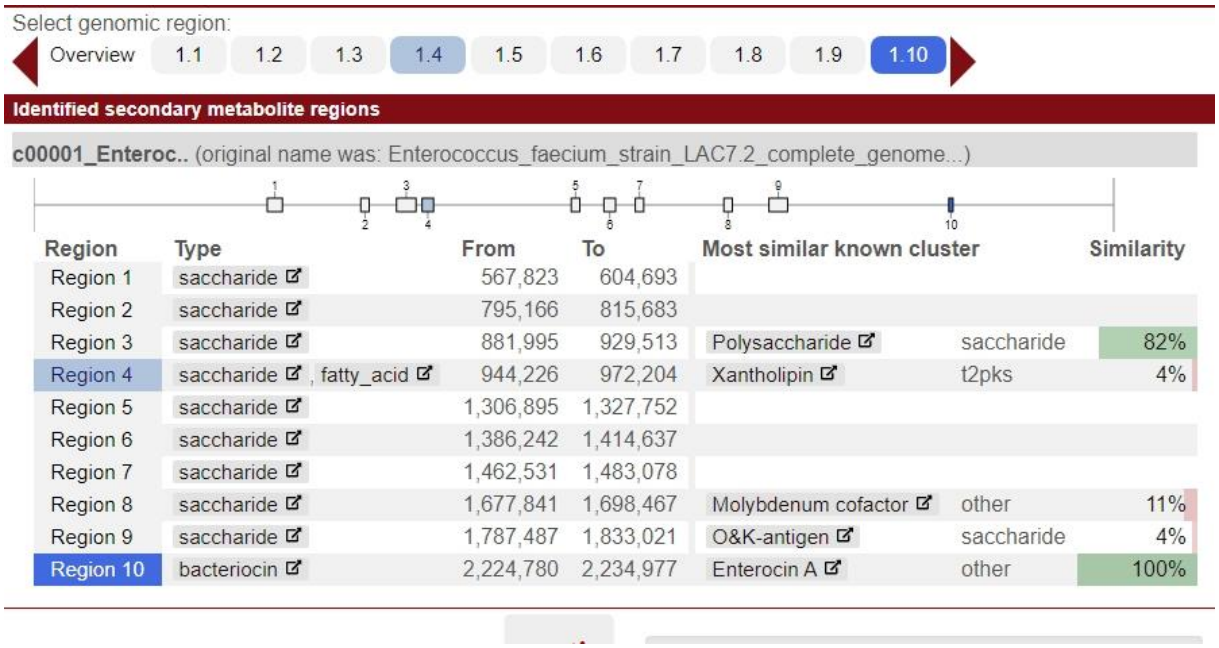


Figure 3*:* *Enterococcus faecium* phylogeny.

APÊNDICE 9: Figura 4



Region	Type	From	To	Most similar known cluster		Similarity
Region 1	saccharide	567,823	604,693			
Region 2	saccharide	795,166	815,683			
Region 3	saccharide	881,995	929,513	Polysaccharide	saccharide	82%
Region 4	saccharide, fatty_acid	944,226	972,204	Xantholipin	t2pks	4%
Region 5	saccharide	1,306,895	1,327,752			
Region 6	saccharide	1,386,242	1,414,637			
Region 7	saccharide	1,462,531	1,483,078			
Region 8	saccharide	1,677,841	1,698,467	Molybdenum cofactor	other	11%
Region 9	saccharide	1,787,487	1,833,021	O&K-antigen	saccharide	4%
Region 10	bacteriocin	2,224,780	2,234,977	Enterocin A	other	100%

Figure 4 *Enterococcus faecium* Anti-SMACH Analysis.

APÊNDICE 10: Figura 5

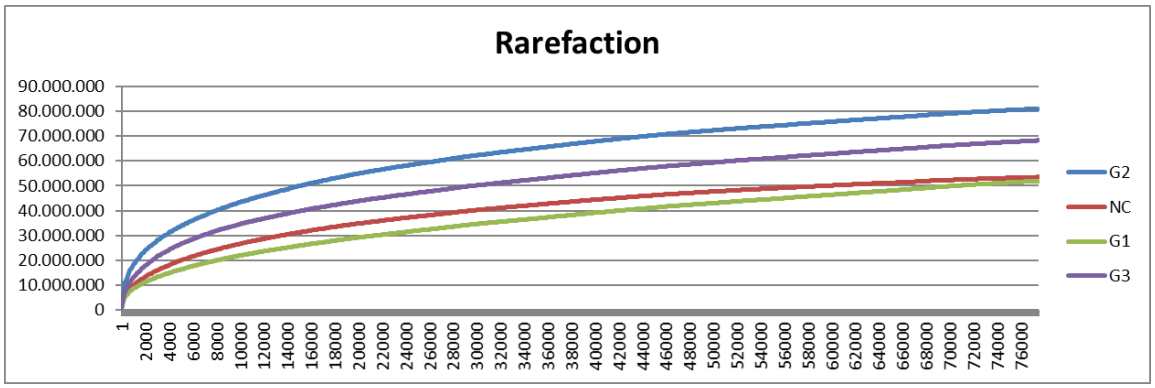


Figure 5. Rarefaction curve showing increasing species along the number of reads in different trial groups. G1: Probiotics in feed; G2: Probiotics in water; G3: Probiotics in feeds and water; NC: negative control.

APÊNDICE 11: Figura 6

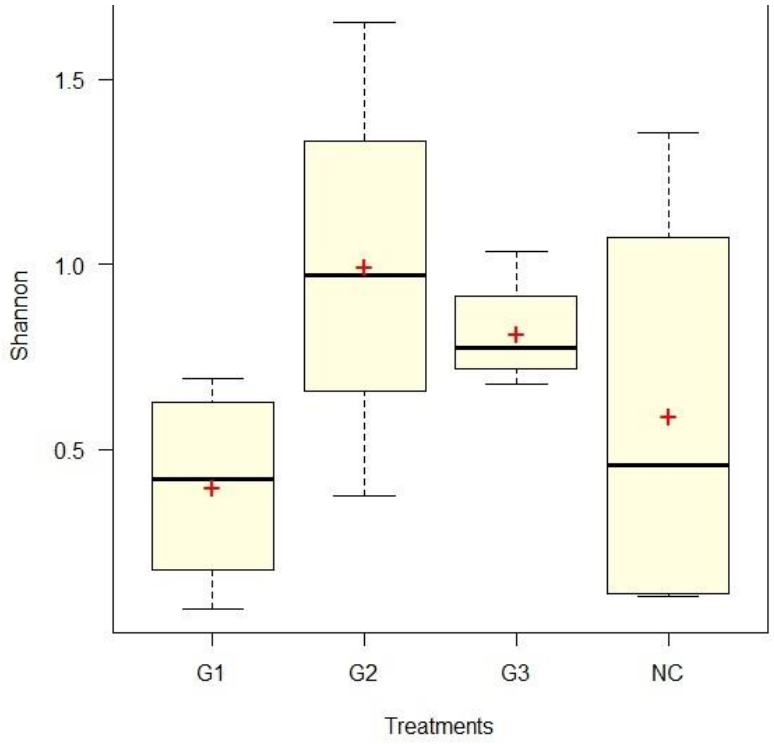


Figure 6: Shannon index in different trial groups after administration the probiotic *Enterococcus faecium*.

APÊNDICE 12: Figura 7

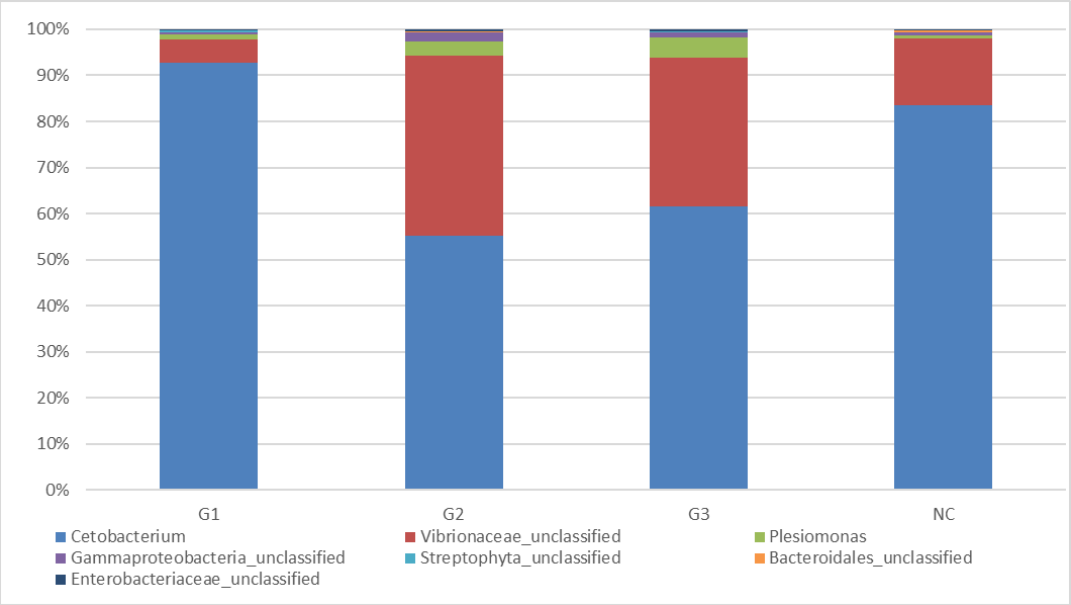


Figure 7: Abundance of experimental groups and information on the percentage of sequences in each group. G1: Probiotics in feed; G2: Probiotics in water; G3: Probiotics in feeds and water; NC: negative control.

APÊNDICE 13: Figura 8

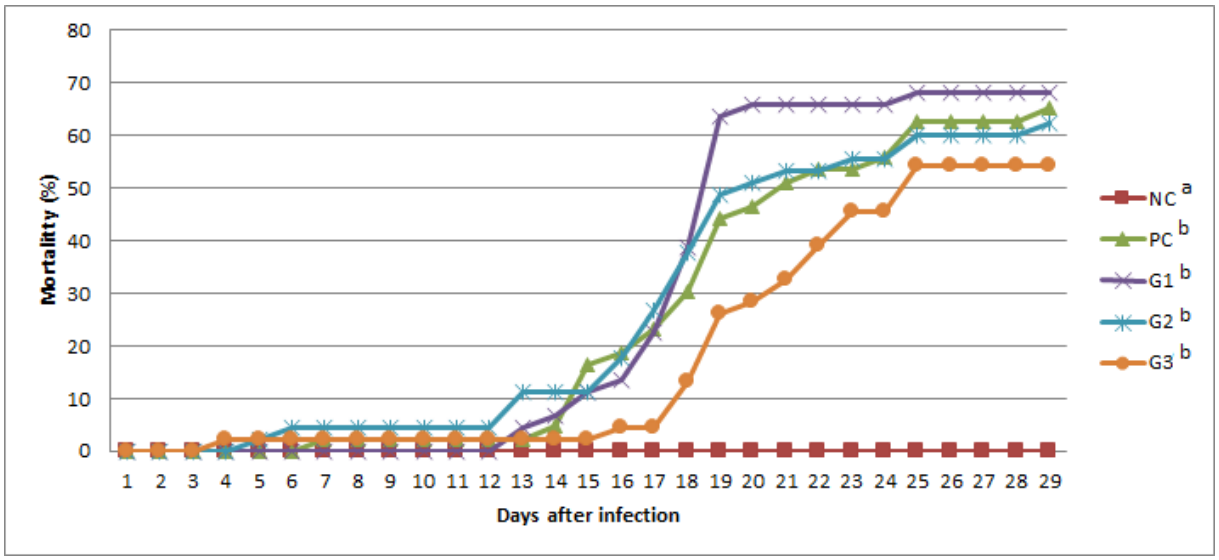


Figure 8: Cumulative mortality observed in the different groups after disease challenge by immersion with *Francisella noatunensis* subsp. *orientalis.* NC: negative control (no challenge with bacteria); PC: positive control; G1: Probiotics in feed; G2: Probiotics in water; G3: Probiotics in feeds and water.

APÊNDICE 14: Figura 9

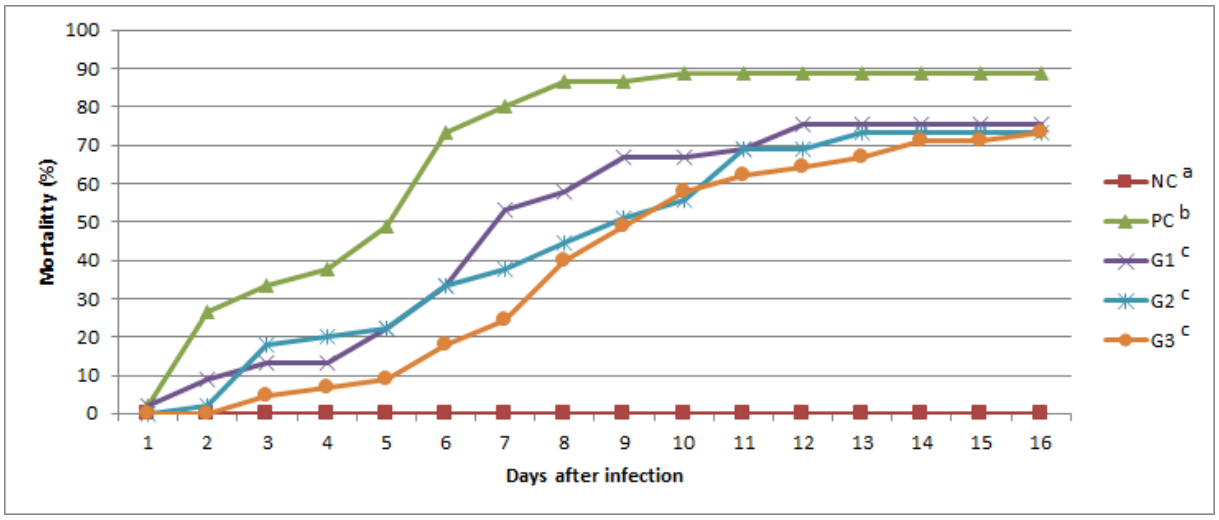


Figure 9: Cumulative mortality observed in the different groups after disease challenge by immersion with *Streptococcus .* NC: negative control (no challenge with bacteria); PC: positive control; G1: Probiotics in feed; G2: Probiotics in water; G3: Probiotics in feeds and water. CP= 88,29%; G1=75,56% e G2 e G3=73,33.