Universidade
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FELIPE PINHEIRO DE SOUZA

EFEITO DA MICROALGA *Schizochytrium* sp. E DO ß-1,3/1,6-GLUCANA NOS PARÂMETROS HEMATO-IMUNOLÓGICOS, BIOQUÍMICOS E NA MICROBIOTA INTESTINAL DE TILÁPIAS-DO-NILO (*Oreochromis niloticus)*

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

Orientador: Prof. Dr. Nelson Mauricio Lopera-Barrero

Coorientador: Prof. Dr. Ulisses de Pádua Pereira

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RESUMO

Devido à intensificação na produção aquícola nos últimos anos, alternativas que permitam uma redução no estresse no cultivo e que sejam aliadas ao desenvolvimento sustentável, englobam as principais demandas atuais no mercado piscícola mundial. O objetivo do presente estudo foi avaliar a influência da microalga *Schizochytrium* sp. e do β-glucana nos parâmetros hemato-imunológicos, bioquímicos e na microbiota intestinal de tilápias-do-Nilo (*Oreochromis niloticus*). Para atender os objetivos, foram elaborados dois experimentos independentes. Experimento 1: Tilápias-do-Nilo (50g) foram distribuídas em oito tanques-rede (6 m^3) e alimentadas com dieta contento 1,2% de farinha de *Schizochytrium* sp. (SUP) e dieta controle (ração comercial sem *Schizochytrium* sp.) por 105 dias. Experimento 2: Tilápias-do-Nilo (8,3g) foram distribuídas em 15 aquários contento zero, 0,1 ou 0,3 mg L^{-1} de β-glucana (tratamento controle, 0,1 e 0,3, respectivamente). Após 15 dias, os animais foram desafiados com hipóxia por nove horas e a mortalidade foi avaliada. Em ambos os experimentos, sangue foi coletado da veia caudal para mensurações hematológicas e imunológicas, e a microbiota intestinal foi analisada por meio da amplificação e sequenciamento da região V3/V4 do rRNA16S. No experimento 1, amostras de intestino foram coletadas para análise histológica. No experimento 2, depois do desafio com hipóxia, uma nova coleta de sangue foi realizada para mensuração de parâmetros bioquímicos. Os resultados do experimento 1 demonstraram que a inclusão de 1,2% de *Schizochytrium sp*. na dieta não alterou os parâmetros hematológicos, exceto a contagem de hemácias e linfócitos, que foram menores em SUP ($p < 0,05$). A análise metagenômica revelou maiores valores do estimador de Chao ($p < 0,05$) (índice de riqueza taxonômica) e abundância de bactérias do filo Firmicutes em SUP. A análise de histologia intestinal não revelou diferenças significavas entre as dietas ($p > 0,05$). No experimento 2, após o desafio com hipóxia, a concentração de glicose sérica aumentou ($p < 0,05$) em todos os grupos, e aumento ($p < 0.05$) na contagem de eritrócitos e diminuição no volume corpuscular médio foi observado no grupo controle ($p < 0,05$). Os níveis de glicose retornaram aos valores basais sete dias após o desafio com hipóxia. Em 0,3, um aumento na concentração de hemoglobina corpuscular média foi encontrado após a hipóxia ($p < 0,05$). Proteínas totais, lactato, triglicérides e colesterol não diferiram entre os tratamentos ($p > 0.05$). A contagem de leucócitos totais e linfócitos foram superiores ($p < 0.05$) em 0,3 em relação a 0,1. As tilápias que receberam 0,1 mg L^{-1} de β-glucana na água apresentaram menor mortalidade ($p < 0,05$) sete dias após a hipóxia. Maior riqueza de Chao ($p < 0.05$) foi verificada em 0,1, com maior abundância de membros da família Vibrionaceae em relação ao grupo controle. Conclui-se que tanto a farinha de *Schizochytrium* sp fornecida dieteticamente quanto o β-glucana na água podem modular as concentrações de células sanguíneas em tilápias-do-Nilo, porém, de modos distintos. O fornecimento de 0,1 mg L^{-1} de β-glucana pôde amenizar os efeitos estressantes da hipóxia. Em ambos os experimentos, os ingredientes avaliados demonstraram ser capazes de modular a microbiota intestinal, ocasionando, principalmente, no aumento da riqueza microbiana.

Palavras-chave: Aquicultura. Estresse. Metagenômica. Peixe. Prebióticos.

SOUZA, Felipe Pinheiro de. **Effect of *Schizochytrium* sp. and β-1-3/1-6-glucan in hemato-immunological, biochemical parameters and in the intestinal microbiota of Nile tilapia (*Oreochromis niloticus*).** 2021. 119 pp. Thesis. (Doctorate degree in Animal Science) – Universidade Estadual de Londrina, Londrina, 2021.

ABSTRACT

Due to the intensification of aquaculture production in recent years, alternatives that allow a reduction in stress in cultivation and that are combined with sustainable development, encompass the main current demands in the world fish market. The aim of the present study was to evaluate the influence of *Schizochytrium* sp. and β-glucan in hemato-immunological, biochemical parameters and in the intestinal microbiota of Nile tilapia (*Oreochromis niloticus*). To meet the objectives, two independent experiments were developed. Experiment 1: Nile tilapia (50g) were distributed in eight net tanks (6 m^3) and fed with a diet containing 1.2% *Schizochytrium* sp. (SUP) and control diet (commercial feed without *Schizochytrium* sp.) For 105 days. Experiment 2: Nile tilapia (8.3g) were distributed in 15 aquariums containing zero, 0.1 or 0.3 mg L^{-1} of β-glucan (treatment control, 0.1 and 0.3, respectively). After 15 days, the animals were challenged with hypoxia for nine hours and mortality was assessed. In both experiments, blood was collected from the caudal vein for hematological and immunological analysis, and the intestinal microbiota was analyzed by amplifying and sequencing the V3 / V4 region of the rRNA16S. In experiment 1, intestine samples were collected for histological analysis. In experiment 2, after the challenge with hypoxia, a new blood collection was performed to measure biochemical parameters. The results of experiment 1 demonstrated that the inclusion of 1.2% of *Schizochytrium* sp. in the diet did not change the hematological parameters, except for the count of red blood cells and lymphocytes, which were lower in SUP ($p < 0.05$). Metagenomic analysis revealed greater values in Chao estimator ($p < 0.05$) (taxonomic richness index) and abundance of bacteria from the phylum Firmicutes in SUP. The analysis of intestinal histology did not reveal significant differences between diets ($p > 0.05$). In experiment 2, after the challenge with hypoxia, the concentration of serum glucose increased ($p < 0.05$) in all groups, and increase ($p < 0.05$) in the erythrocyte count and decrease in mean corpuscular volume in the control group ($p < 0.05$). Glucose levels returned to baseline seven days after the hypoxia challenge. In 0.3, an increase in the mean corpuscular hemoglobin concentration was found after hypoxia ($p < 0.05$). Total proteins, lactate, triglycerides and cholesterol did not differ between treatments ($p > 0.05$). The total leukocyte and lymphocyte count were higher ($p <0.05$) in 0.3 compared to 0.1. The tilapias that received 0.1 mg L^{-1} of β-glucan in water had lower mortality ($p < 0.05$) seven days after hypoxia challenge. Higher Chao richness ($p < 0.05$) was found in 0.1, with a greater abundance of members of the Vibrionaceae family compared to the control group. It is concluded that both *Schizochytrium* sp supplied dietary and β-glucan in water can modulate blood cell concentrations in Nile tilapia, however, in different ways. The supply of 0.1 mg L^{-1} of β-glucan was able to mitigate the stressful effects of hypoxia. In both experiments, the evaluated ingredients demonstrated to be able to modulate the intestinal microbiota, mainly inferring in the increase of the microbial richness.

Key-words: Aquaculture. Fish. Metagenomics. Prebiotics. Stress.

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LISTA DE ABREVIATURAS E SIGLAS

7AHC	*7 days after hypoxia challenge* (7 dias após desafio com hipóxia)
AHC	*After hypoxia challenge* (Após desafio com hipóxia)
AMOVA	*Analysis of molecular variance* (Análise de variância molecular)
ANOVA	*Analysis of variance* (Análise de variância)
BHC	*Before hypoxia challenge* (Antes do desafio com hipóxia)
CF	*Condition factor* (Fator de condição)
CON	*Control diet* (dieta controle)
DHA	Ácido docosahexaenoico
EDTA	*Ethylenediaminetetraacetic acid* (Ácido etilenodiamino tetra-acético)
EG	*Eosinophilic granulocytes* (Granulócitos eusinofílicos)
EPA	Ácido eicosapentaenóico
FAO	*Food and Agriculture Organization*
FAT	*Fatty acid* (Ácido graxo)
FOS	Frutoligossacarídeos
GC	*Goblet cells* (Células caliciformes)
GOS	Galactoligossacarídeos
Hb	*Hemoglobin* (Hemoglobina)
Ht	*Hematocrit* (Hematócrito)
LC-PUFAs	Ácidos graxos poliinsaturados de cadeia longa
LP	*Lamina propria* (Lâmina própria)

LPS	Lipopolissacarídeos de membrana
MCH	*Mean corpuscular hemoglobin* (Hemoglobina corpuscular média)
MCHC	*Mean corpuscular hemoglobin concentration* (Concentração de hemoglobina corpuscular média)
MCV	*Mean corpuscular volume* (Volume corpuscular médio)
MF	*Mucosal folds* (Vilosidades)
MOS	Mananoligossacarídeos
MUFAS	*Monounsaturated fatty acids* (Ácidos graxos monoinsaturados)
NGS	*Next generation sequencing* (Sequenciamento de próxima geração)
OTUs	*Operational taxonomic units* (Unidades taxonômicas operacionais)
PUFAS	Ácidos graxos poliinsaturados de cadeia longa
RBC	Red blood cells (hemácias)
SFA	*Saturated fatty acid* (Ácidos graxos saturados)
SM	*Subepithelial mucosa* (Mucosa subepitelial)
SNV	*Supranuclear vacuoles* (Vacúolos supranucleares)
SUP	*Supplemented diet with 1.2% Schizochytrium sp. meal* (dieta suplementada com 1,2% de farinha de *Schizochytrium* sp.)
WBCs	*White blood cells* (Leucócitos)
XOS	Xiloligossacarídeos

SUMÁRIO

1 INTRODUÇÃO

A crescente demanda de produtos alimentícios saudáveis tem impulsionado o crescimento da aquicultura nos últimos anos, destacando essa atividade frente a outros setores da produção animal (FAO 2020, Peixe BR, 2020). Estima-se que a produção global de peixes tenha atingido cerca de 179 milhões de toneladas em 2018 (abrangendo peixes marinos e de água doce, oriundos de pesca e aquicultura), sendo que, desse total, 156 milhões de toneladas foram usadas para consumo humano, equivalente a um abastecimento anual estimado de 20,5 kg per capita (FAO, 2020). As 22 milhões de toneladas restantes foram destinadas para usos não alimentares, principalmente para produção de farinha de peixe e óleo de peixe (FAO, 2020).

Entre os peixes mais produzidos no cenário mundial e nacional, destaca-se a tilápia-do-Nilo. Mundialmente, a produção de tilápias só é inferior à produção de carpas (FAO 2020), já no Brasil essa espécie lidera com 57% de toda a produção aquícola (Peixe BR, 2020). Apesar dessa grande produção, alguns gargalos na tilapicultura estão longe de serem extintos. Nos últimos anos, a intensificação do cultivo dessa espécie tem levado ao surgimento de alguns agravantes, como aumento do estresse dos peixes, que facilita a ocorrência de surtos bacterianos e parasitários, e resulta na diminuição da taxa de crescimento (Buck et al., 2017; Levy-Pereira et al., 2018; Suphoronski et al., 2019). Por essa razão, um manejo dietético adequado é fundamental para atender todas as demandas nutricionais, promover crescimento adequado, fortalecer o sistema imune, e minimizar os efeitos do estresse que possam agravar a produtividade nas pisciculturas.

Nesse sentido, estudos recentes mostram que a utilização de ingredientes funcionais pode ser muito benéfica na produção de peixes, já que podem promover aumento das defesas imunológicas (Levy-Pereira et al., 2018), resistência a doenças (Suphoronski et al., 2019) e até na melhora na composição do filé (Sarker et al., 2016a). Neste último estudo é abordado a importância do uso de fontes alternativas na alimentação de tilápias-do-Nilo, como a microalga estudada (*Schizochytrium* sp), como substituto ao óleo de peixe na ração (fonte de ácidos graxos), que é um recurso limitado na natureza, extraído de peixes pescados no oceano. E isso se torna ainda mais importante tendo em vista a possibilidade de produção em escala comercial de algumas algas que podem compor a dieta de peixes, como a *Nannochloropsis oculata* e a *Schizochytrium* sp (Sarker et al., 2016a; Sarker et al., 2020). A utilização dessas duas algas em dietas de tilápias já demonstrou ser viável economicamente, sendo potenciais fontes de proteínas (*N. oculata*) e lipídeos (*Schizochytrium* sp) (Sarker et al., 2020).

Outra demanda voltada ao desenvolvimento sustentável da aquicultura diz respeito ao uso de aditivos ou ingredientes funcionais como potenciais profiláticos, servindo como alternativas ecológicas ao uso de antibióticos, como os prebióticos. Em suma, os prebióticos são carboidratos não-digestíveis, que fornecem substrato que as bactérias benéficas do intestino são capazes de fermentar, promovendo efeitos positivos à saúde do hospedeiro (Saad, 2006). Frutoligossacarídeos (FOS), mananoligossacarídoes (MOS), galactoligossacarídeos (GOS), lipopolissacarídeos de membrana (LPS) e glucanas são alguns dos exemplos dos prebióticos mais utilizados na aquicultura (Sado, 2008). Entre seus principais efeitos fisiológicos em peixes, destacam-se a modulação da microbiota (Hoseinifar et al., 2019), estimulação do sistema imune (Levy-Pereira et al., 2018) e aumento da resistência frente a condições estressantes, como desafio com bactérias patogênicas (Domenico et al., 2017).

Considerando o grande potencial aquícola brasileiro e a crescente demanda de produtos saudáveis, aliados a uma produção sustentável, é nítida a necessidade de estudos que avaliem os efeitos sistêmicos de aditivos prebióticos sobre os peixes mais cultivados no Brasil. Nesse sentido, a tilápia-do-Nilo, impulsionada pelo melhoramento genético, destaca-se pela alta produtividade e merece atenção nas pesquisas voltadas à nutrição e respostas metabólicas frente ao fornecimento desses aditivos.

2 REVISÃO DE LITERATURA

2.1 TILÁPIA-DO-NILO (*OREOCHROMIS NILOTICUS*)

Natural do continente africano, especificamente das bacias dos rios Nilo, Níger, Chade e dos lagos do centro-oeste, a tilápia-do-Nilo se popularizou em mais de 100 países localizados em regiões tropicais e subtropicais do mundo (Verani et al., 1980; Vicente et al., 2014). Sua popularidade mundial é decorrente de diversas características favoráveis da espécie, tais como rápido crescimento em sistemas intensivos, adaptabilidade em diferentes ambientes, boas características organolépticas, filé sem espinhos intramusculares, entre outras (Lopera-Barrero et al., 2011; Vicente et al., 2014).

Classificação taxonômica da tilápia-do-Nilo (*Oreochromis niloticus,* Linnaeus 1758), segundo Myers et al. (2020):

Reino: Animalia
  Filo: Chordata
    Classe: Teleostei
      Ordem: Perciformes
        Família: Cichlidae
          Gênero: *Oreochromis*
            Espécie: *Oreochromis niloticus*

Figura 1. Exemplar de tilápia-do-Nilo (*Oreochromis niloticus*) adulta.

Fonte: O autor.

Dados da FAO (2020) indicam um rápido crescimento da produção mundial de tilápia-do-Nilo nos últimos anos, ficando atrás apenas da produção de duas espécies de carpas (Carpa capim, *Ctenopharyngodon idellus*; carpa prateda, *Hypophthalmichthys molitrix*). Nacionalmente, com a produção de 432.149 t no ano de 2019, a tilápia-do-Nilo representou 57% de toda produção piscícola brasileira, resultado 7,96% superior ao ano anterior (Peixe BR, 2020). Presente em todas as regiões do país, a produção de tilápia-do-Nilo foi maior no estado do Paraná, com 146.212 t, seguido pela produção de São Paulo (64.900 t), Santa Catarina (38.559 t), Minas Gerais (36.350 t) e Pernambuco (25.421 t) (Peixe BR, 2020).

Morfologicamente, a tilápia-do-Nilo apresenta um corpo achatado lateralmente, composto por escamas do tipo ciclóides, acinzentadas, que se sobrepõem umas as outras, de forma a cobrir o corpo todo do animal; possui, também, uma nadadeira dorsal dividida em duas partes: anterior espinhosa e posterior escamosa (Silva et al., 2015). Desde que existam condições adequadas, as tilápias podem se reproduzir ao longo de todo o ano, sendo que a maturidade sexual é atingida nas fêmeas em torno do quarto mês (SENAR, 2017). Em temperaturas na faixa de 27 a 29ºC, as fêmeas são capazes de produzir de 500 a 2000 ovos por desova (SENAR, 2017).

O pacote tecnológico existente na cadeia produtiva da tilápia-do-Nilo permite que essa espécie seja produzida em diferentes condições ambientais, as quais variam de acordo com o grau de tecnologia e intensidade de produção. De acordo com Turco et al. (2014), aumento na produção de tilápias em tanque-rede tem sido notada nos últimos anos. Segundo os autores, algumas vantagens desse sistema são destacadas, como o aproveitamento de ambientes aquáticos existentes, controle facilitado dos estoques e despesca, proteção contra predadores, reduzido custo de implantação, entre outras.

2.2 RESPOSTAS AO ESTRESSE EM PEIXES

Assim como os outros animais, os peixes estão frequentemente sujeitos a alterações nos meios interno e externo, cujas respostas fisiológicas compensatórias são estimuladas para manter a homeostase (estado de equilíbrio dinâmico do organismo) (Urbinati et al., 2014). De acordo com Barton (2002), essas alterações que podem levar a perda da homeostase, chamados de estressores, podem ser de origem física (operações de manejo, por exemplo), químicas (composição do meio aquático), ou biológicas (predadores, hierarquia social, e interações de macro ou microrganismos).

Segundo Urbinati et al. (2014) os mecanismos de resposta ao estresse são divididas em três etapas, sendo a primeira a resposta primária, onde há um reconhecimento do agente estressor; a resposta secundária, que é uma resposta adaptativa do organismo frente ao estressor; e a resposta terciária, que envolve a exaustão dos sistemas biológicos. A resposta primária envolve a ativação simpática do sistema nervoso autônomo e posterior liberação de catecolaminas (adrenalina e noroadrenalina) pelas células cromafins, localizadas na porção cefálica do rim. Além do eixo sistema nervoso autônomo simpático – células cromafins, outro eixo também é ativado no estresse, o eixo hipotalâmico – hipofisário – inter-renal (Urbinati et al., 2014). Essa ativação leva o hipotálamo a produzir o hormônio liberador de corticotrofina, que por sua vez estimula a liberação de corticotrofina pela hipófise. Esse último hormônio, ao ser liberado na corrente sanguínea, estimula as células interrenais a produzirem o cortisol, que juntamente com as catecolaminas, mediará a organização das respostas adaptativas ao estresse (Urbinati et al., 2014).

Conforme salientado, as respostas secundárias baseiam-se nas adaptações fisiológicas provocadas pela liberação de hormônios na primeira fase do estresse. A liberação de catecolaminas causa aumento da taxa de ventilação e estimula o fluxo branquial para fornecer oxigênio aos tecidos, paralelamente, induz a liberação de glicose hepática via glicogenólise para suprimento de energia (Lima et al., 2006). Um aumento no número de eritrócitos e hemoglobina no sangue também pode observado, devido à contração esplênica, como resposta secundária para atender o aumento da demanda de oxigênio (Lai et al., 2006). A liberação de cortisol é importante para manter níveis elevados de glicose no sangue, pois participa do processo glicogenolítico (juntamente com as catecolaminas), e na estimulação da gliconeogênese (Urbinati et al., 2014).

Caso a condição estressante perdure por um longo período de tempo, ocorre um direcionamento de energia para as funções vitais do peixe, como respiração, natação e osmoregulação, diminuindo o aporte de energia para o crescimento e respostas imunológicas, levando a uma imunossupressão. Essa é a resposta terciária ao estresse, a qual pode deixar o peixe mais predisposto a doenças, reduzir a capacidade reprodutiva, piorar o desempenho zootécnico e em casos graves levar a morte (Urbinati et al., 2014).

Nesse sentido, estudos com alimentos funcionais e/ou imunoestimulantes tem sido demandados na tentativa de minimizar efeitos estressantes em peixes. Por essa razão, o conhecimento da fisiologia do estresse e como mensurar essas respostas é importante em pesquisas que buscam a validação ou desenvolvimento de produtos que possam melhorar a saúde dos peixes frente a condições estressantes e trazer ganhos produtivos na piscicultura.

2.3 ALIMENTOS FUNCIONAIS NA AQUICULTURA

Peixes são animais pecilotérmicos, portanto, são diretamente afetados pelas condições ambientais. Características como o comportamento, integridade estrutural, saúde, funções fisiológicas, reprodução e crescimento são influenciados não só pelos sistemas de produção, como também pelos hábitos alimentares (Cyrino et al. 2010). Portanto, o manejo alimentar, assim como o controle da qualidade da água e saúde dos peixes são fundamentais para otimizar o crescimento e ganho produtivo nas pisciculturas.

A melhora do desempenho e resistência a doenças em produções de tilápia-do-Nilo tem se tornado um grande desafio nos últimos anos, e isso se torna ainda mais importante ao levar em conta a intensificação da produção dessa espécie (Berto, 2013). Por essa razão, a utilização de ingredientes ou produtos que possam melhorar a saúde e imunidade desses animais é uma grande demanda do mercado piscícola atual. De acordo com Cyrino et al. (2010), a alimentação excessiva ou o uso de rações não balanceadas reduzem a absorção de nutrientes pelos peixes, o que pode resultar em excesso de matéria orgânica nos sistemas de produção, sendo esse processo agravado em situações intensivas, podendo gerar perdas econômicas consideráveis por mortalidade ou atraso no crescimento. Paralelamente, o uso indiscriminado de antibióticos tem levado ao aparecimento de cepas bacterianas resistentes. Por essa razão, a busca por ingredientes menos nocivos e prejudiciais ao meio ambiente, e que possam melhorar a saúde e imunidade dos peixes, tem sido incentivada e é alvo de diversos estudos recentes na tilapicultura (Buck et al., 2017; Levy-Pereira et al., 2018; Amphan et al., 2019; Suphoronski et al., 2019)

De acordo com Ribeiro et al. (2012), um alimento pode ser considerado funcional se for verificado que o mesmo pode afetar beneficamente uma ou mais funções do organismo, tendo valores nutricionais adequados e ser relevante tanto para o bem-estar e saúde, quanto para a redução do risco de enfermidades. Segundo os mesmos autores, os alimentos funcionais apresentam compostos bioativos que podem apresentar funções biológicas distintas, podendo ser consumidos em dietas convencionais.

Os ingredientes funcionais podem ter diferentes fontes (animal ou vegetal) e influenciar em diferentes áreas do organismo (sistema gastrointestinal, no sistema cardiovascular, no metabolismo de substratos, no crescimento, no desenvolvimento e diferenciação celular e no comportamento das funções fisiológicas) (Ribeiro et al., 2012), concomitantemente. Na tilapicultura, tais ingredientes já demonstraram ser multifuncionais em seus efeitos fisiológicos, os quais incluem aumento das defesas imunológicas, resistência a doenças, melhora na composição do filé, entre outros, trazendo consigo, além desses benefícios, um foco sustentável almejado na produção animal. Estudo recente indicou que um aditivo dietético (A-live) baseado em extratos de plantas da família *Alliaceae* pode proporcionar maior resistência em tilápias frente a desafio com *Francisella orientalis* (Suphoroski et al., 2019). Sarker et al. (2016) demonstraram que a substituição de óleo de peixe pela microalga *Schizochytrium* sp. promove melhora da deposição de ácidos graxos poli-insaturados de cadeia curta no filé, sendo uma alternativa sustentável para substituição do óleo de peixe na ração de tilápias. Mananoligossacarídeos isolados da levedura *Saccharomyces cerevisiae* podem promover aumento na contagem de leucócitos, linfócitos, monócitos e na concentração de lisozima sérica de juvenis de tilápia-do-Nilo, sendo um potencial aditivo profilático em substituições aos antibióticos (Levy-Pereira et al., 2018).

2.3.1 Utilização de Algas na Alimentação de Peixes

Existem cerca de 221 espécies de algas no mundo, sendo que cerca de 145 espécies (66%) são utilizadas para alimentação (Zemke e Ohno, 1999). As algas, em geral, são ingredientes relativamente baratos, abundantes, fáceis de cultivar e com características nutricionais desejáveis (alta proteína, presença de ácidos graxos poli-insaturados, minerais, vitaminas, etc.), que fazem dela um componente alternativo para rações (Watagnet, 2013). No entanto, as algas ainda são pouco reconhecidas e exploradas na nutrição aquícola, razão pela qual ainda serão necessários estudos que permitam sua adequada utilização (Sarker et al., 2020). Trabalhos pioneiros, como o realizado por Fujimoto e Kaneda (1980), e mais recentes (Mesko e Jacob-Lopes, 2012; Tenório, 2015) demonstraram que as algas podem ser fontes de proteínas, carboidratos, lipídios, fibras, minerais e vitaminas.

As algas estão na base da cadeia alimentar aquática, perfazendo importantes recursos alimentares para os animais aptos a consumi-las. Devido às atividades funcionais proporcionadas pelo consumo de algumas algas, as quais incluem mediação do metabolismo lipídico, resposta imune e hematológica, expressão de genes, além de influenciar desempenho zootécnico, diversos estudos têm sido realizados avaliando a inclusão desses ingredientes na dieta de peixes (Garcia et al., 2012; Norambuena et al., 2015; Santos et al., 2015; Reverter et al., 2016; Hoshino et al., 2017). Além disso, com a expansão dos sistemas aquícolas intensivos, fontes alternativas de proteína e ácidos graxos, como determinadas algas, são essenciais para reduzir a dependência de ingredientes como farinha de peixe e óleo de peixe (Van Hoestenberghe at al., 2014; Vizcaíno et al., 2014). Nesse sentido, a viabilidade econômica desses ingredientes alternativos substitutos também deve ser investigada. Em estudo recente realizado por Sarker et al. (2020), rações contendo as algas *Nannochloropsis oculata e Schizochytrium* sp. como substitutos de farinha de peixe e óleo de peixe, respectivamente, apresentaram taxas de conversão econômica ($/kg de tilápia) muito similares às obtidas pela ração com os ingredientes convencionais (taxa de conversão econômica de 0,95 $/kg de tilápia com ração com *N. oculata* e *Schizochytrium* sp × 1,03 $/kg de tilápia com ração controle com farinha e óleo de peixe). Os resultados desse último estudo corroboraram a viabilidade econômica e o potencial de incorporação de algas na ração de peixes como fontes de proteínas e lipídeos.

Além disso, outros estudos constataram que a inclusão de algas na dieta de tilápias-do-Nilo pode influenciar a resposta hematológica frente a estresse agudo. Garcia et al. (2012) observaram que o fornecimento de dieta com 10 g kg^{-1} de suplemento com base nas algas marinhas *Laminaria digitata* e *Ascophyllum nodosum,* aumentou a concentração de trombócitos circulantes após estresse térmico. Por outro lado, Mentoza Rodriguez et al. (2017) verificaram que a inclusão de 5 g kg^{-1} de Carragenina, um extrato de algas marinhas vermelhas, durante sete semanas, proporcionou aumento da concentração de imunoglobulinas totais em *Sciaenops ocellatus*, evidenciando o potencial dessa substância frente a estimulação da resposta imune. Em outro estudo, Hoshino et al. (2017) constataram que a inclusão de 1 g kg^{-1} de produto composto por farinha de levedura de cerveja, sódio, aluminossilicato de cálcio e a alga *Chlorella vulgaris* na dieta de *Arapaima gigas* foi capaz aumentar a contagem de trombócitos e promover a diminuição da glicose, triglicérides e colesterol plasmático. Reverter et al. (2016) observaram que *Platax orbicularis* alimentados com plantas polinésias e com a alga *Asparagopsis taxiformis* apresentaram maior ganho de peso e maior expressão de genes relacionados com a resposta imune (Lys G e TGF-b1) em relação ao grupo controle.

Sob outra perspectiva, alguns estudos já demonstraram que a composição da dieta também pode influenciar a diversidade da microbiota intestinal em peixes, à qual está relacionada com ação do sistema imunológico, aquisição de nutrientes e concorrência com patógenos (Tarnecki et al., 2017), sendo que as algumas algas já demostraram esses efeitos. Lyons et al. (2017), em estudo avaliando a inclusão da microalga *Schizochytrium limacinum* na dieta de *Oncorhynchus mykiss*, observaram que a composição das comunidades microbianas do grupo tratado com a alga e do grupo controle eram semelhantes, no entanto, o grupo tratado possuía maior diversidade de táxons em relação ao grupo controle. Os autores justificaram esses resultados sugerindo uma flexibilidade no microbioma dos peixes tratados em resposta a digestão da microalga.

Além da capacidade de afetar a estrutura e diversidade da microbiota intestinal, outras pesquisas identificaram que a presença de algas na ração pode exercer influência sobre a histologia intestinal. Hussein et al. (2014) encontraram alterações histológicas (aumento da vacuolização dos enterócitos, aumento da lâmina própria com infiltração de leucócitos e deslocamento de núcleos dos enterócitos) em intestino posterior de tilápias-do-Nilo alimentadas com produtos comerciais a base de algas (Alga50, Algamaxx) enriquecidas com fosfato dicálcico. Paralelamente, Vizcaíno et al. (2014) observaram correlação positiva entre o comprimento das vilosidades intestinais e o nível de inclusão da alga *Scenedesmus almeriensis* na dieta de *Sparus aurata*. Além disso, os autores constataram aumento das atividades enzimáticas intestinais nos grupos que receberam a alga.

Levando em conta a grande diversidade de espécies de algas no mundo com potencial para inclusão em dietas de peixes, conforme demonstrado pelos estudos acima, fica evidente que incorporação destes produtos tem se tornado uma promissora alternativa na nutrição de peixes. Também é importante destacar que poucas pesquisas brasileiras avaliaram a fundo o efeito dessas algas de maneira sistêmica como tem sido realizado no exterior. Além disso, considerando o grande potencial aquícola brasileiro e a alta produtividade tilápia-do-Nilo, impulsionada pelos programas de melhoramento genético e controle do ambiente de cultivo, essa espécie merece destaque nas pesquisas envolvendo tecnologias voltadas à nutrição.

2.3.1.1 *Schizochytrium* sp.

A alga *Schizochytrium* sp. é um tipo de microalga pertencente ao reino Stramenopilia, filo Heterokonta, classe Thraustochytrida, ordem Thraustochytriales, família Thraustochytriaceae, gênero *Schizochytrium* e espécie *Schizochytrium* sp. (Leipe et al., 1994).

Em comparação com outras algas da família

Thraustochytriaceae, a *Schizochytrium* sp. tem rápida taxa de crescimento em culturas de microalgas (Ganuza et al., 2008). Se trata de um microrganismo heterotrófico, unicelular esférico (Ganuza et al., 2008; Sarker et al., 2016) (Figura 2).

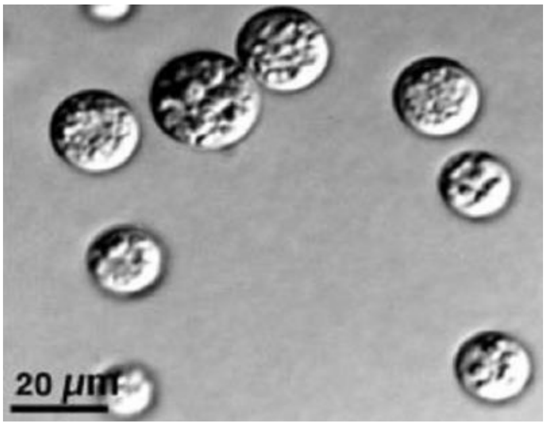

Figura 2: Morfologia da alga *Schizochytrium* sp

Fonte: Takao et al. (2005)

Como principais características morfológicas, a *Schizochytrium* sp. possui uma rede ectoplásmica (redes com filamentos interconectados), com a formação de zoósporos ovoides e aplanósporos. As células possuem tamanho entre 10-20 μm (Yokoyama et al. 2007). Suas características permitem uma produção constante, realizada em processo de fermentação sob condições controladas, e por isso pode se tornar um produto alimentício cuja produção não é limitada pelos estoques naturais (Ganuza et al., 2008).

Essa alga tem sido considerada uma fonte promissora de ácidos graxos essenciais, sendo comercializada como produto seco ou em formato de óleos, possuindo atividade antioxidante (Atienza et al., 2012; Sarker et al., 2016a). Além disso, é capaz de produzir grande quantidade de biomassa com significativo conteúdo lipídico (Ganuza et al., 2008; De La Peña et al., 2016). Essa alga possui alto valor nutricional, evidenciado pelos níveis de ácido docosahexaenoico (DHA) que podem representar até 50% dos ácidos graxos presentes (Barclay e Zeller, 1996), perfazendo uma fonte rica de ácidos graxos poli-insaturados de cadeia longa (LC-PUFAS) (Hadley et al., 2017).

2.3.1.2 Utilização de *Schizochytrium* sp. na alimentação de peixes

Trabalhos recentes demonstram que a incorporação da alga *Schizochytrium* sp na dieta têm proporcionado diversos benefícios fisiológicos na produção de peixes, as quais incluem ganho de peso (Santos et al., 2015) e deposição de ácidos graxos (Sarker et al., 2016a), devido suas propriedades funcionais, as quais estão incluídas a elevada quantidade de PUFAs, DHA e Ácido eicosapentaenóico (EPA).

Do ponto de vista zootécnico, a utilização de *Schizochytrium* sp. pode promover aumento da sobrevivência e melhorar a conversão alimentar em alevinos de tilápias-do-Nilo sob condições laboratoriais (Santos et al., 2015). Em juvenis da mesma espécie, Sarker et al. (2016a) avaliaram níveis de substituição do óleo de peixe por células inteiras secas da microalga *Schizochytrium* sp., e observaram que a substituição total do óleo de peixe pela microalga proporcionou maior ganho de peso, melhorou o coeficiente de eficácia proteica e a conversão alimentar. De acordo com Tsappis (2014), o fornecimento crescente (0, 2, 4 e 8 g kg^{-1}) do produto comercial com base na farinha de *Schizochytrium limacinum* (ALL-G Rich) na ração de tilápias durante 13 semanas, promoveu aumento crescente da concentração do DHA no filé conforme o nível de inclusão. Já Jorge (2016) constatou que o fornecimento de 30 g kg^{-1} de farinha de *Schizochytrium* sp. na dieta de tilápias adultas por 60 dias aumentou a quantidade de ácidos graxos ômega 3 na cabeça, vísceras e espinha.

Recentemente, Sarker et al. (2016b) compararam a inclusão de três tipos de alga (*Spirulina*, *Chlorella* e *Schizochytrium*) em dieta de tilápias e observaram que a dieta com *Schizochytrium* sp. apresentou o maior conteúdo de lipídios e ácidos graxos insaturados, e maior coeficiente de digestibilidade aparente. Kousoulaki et al. (2016) constataram melhoria na eficiência de retenção de EPA + DHA em *Salmo salar L.* que receberam dietas com níveis crescentes de *Schizochytrium* sp*.* Mais recentemente, tilápias-do-Nilo que receberam *N. oculata* e *Schizochytrium* sp. como substitutos de farinha de peixe e óleo de peixe, respectivamente, apresentaram maior ganho de peso e deposição de DHA no filé (Sarker et al., 2020). Outro recente estudo com salmão do atlântico (*Salmo salar*) demonstrou maior digestibilidade de PUFAs no grupo que recebeu a dieta com *Schizochytrium* sp. em relação ao grupo que recebeu a dieta controle (com óleo de peixe) (Hart et al., 2021). Shi et al.

(2020) demonstraram que a inclusão dessa microalga na dieta é capaz de aumentar a taxa de sobrevivência frente ao desafio por *Edwardsiella piscicida*, modular a microbiota intestinal e o perfil metabólico de zebrafish (*Danio rerio*). Outro estudo demonstrou que essa espécie de alga pode ser uma interessante opção de suplemento alimentar em tilápias devido à alta produção de carotenoides que apresentam atividades antioxidantes e imunomoduladoras (Atienza et al., 2012).

2.3.2 Prebióticos na Aquicultura

De acordo com Saad (2006), os prebióticos são carboidratos não-digestíveis, incluindo diversos oligossacarídeos que fornecem carboidratos que as bactérias benéficas do intestino são capazes de fermentar. Segundo o autor, eles podem inibir a multiplicação de patógenos, garantindo benefícios adicionais à saúde, sendo que a velocidade de fermentação e a atividade desses carboidratos são fatores primordiais para a saúde intestinal do hospedeiro. A utilização de prebióticos na aquicultura tem se tornado cada vez mais frequente e estimulada por ser uma alternativa ambientalmente sustentável ao uso de antibióticos.

Um dos efeitos mais desejáveis dos prébióticos, além da modulação benéfica da microbiota, é sua estimulação do sistema imune. Na aquicultura, os prebióticos mais utilizados como imunoestimulantes são derivados de bactérias e leveduras, como o muramil-dipeptídeo (MDP), os lipopolissacarídeos de membrana (LPS), as bacterinas de diversas espécies, os glucanas (origem fúngica, leveduras ou vegetal) e os oligossacarídeos, dos quais fazem parte os frutoligossacarídeos (FOS), mananoligossacarídeos (MOS), galactoligossacarídeos (GOS) e xiloligossacarídeos (XOS) (Sado 2008).

O papel imunomodulatório dos MOS já foi verificado em tilápias-do-Nilo, onde se observou aumento nas concentrações de leucócitos e lisozima sérica nos animais que receberam esse ingrediente nas concentrações de 8 e 15 g kg^{-1} na ração por 45 dias, demonstrando ser um potencial aditivo profilático nessa espécie (Levy-Pereira et al., 2018). Estudo avaliando o efeito de GOS na microbiota intestinal do peixe barata Cáspia (*Rutilus rutilus caspicus)* revelou que a concentração de 20 g kg^{-1} de GOS na dieta é capaz de aumentar os níveis de bactérias ácido lácticas na microbiota dessa espécie (Hoseinifar e Rudabeh, 2015). Pacus (*Piaractus mesopotamicus*) alimentados com 2 g kg^{-1} de FOS na dieta por 60 dias apresentaram aumento na concentração de eritrócitos, segundo estudo realizado por Deon (2019).

Portanto, nota-se claramente efeitos fisiológicos benéficos diretos acerca da utilização de prebióticos em diversas espécies piscícolas, conforme demonstrado por esses estudos recentes. Aliado a isso, algumas ferramentas de análises que surgiram e foram aperfeiçoadas nos últimos anos, como o NGS (*Next-Generation Sequencing* – Sequenciamento de Próxima Geração), têm facilitado e disponibilizado informações detalhadas sobre como os prebióticos podem modular o microbioma intestinal dos peixes.

Também, constata-se que uma oferta crescente de produtos comerciais tem surgido para atender essa demanda de ingredientes "*eco-friendly*", ou seja, ingredientes que causam menos danos ao ambiente em comparação a ingredientes equivalentes (Holzer, 2018), entre os quais se destacam os prebióticos, probióticos e simbióticos. Por consequência, estudos são necessários para avaliar a viabilidade de produção e aplicação desses novos produtos na aquicultura, especificamente as concentrações, tempo e vias de administração e efeitos fisiológicos.

2.3.2.1 β-glucana

O grupo dos glucanas abrange todos os polímeros de glicose, incluindo a celulose (β1,4-glucana), e apresentam um grande número de variações, tais quais o peso molecular, a presença ou ausência de modificações, isômeros α ou β e tamanho da cadeia principal (Goodridge et al. 2009) (Figura 3).

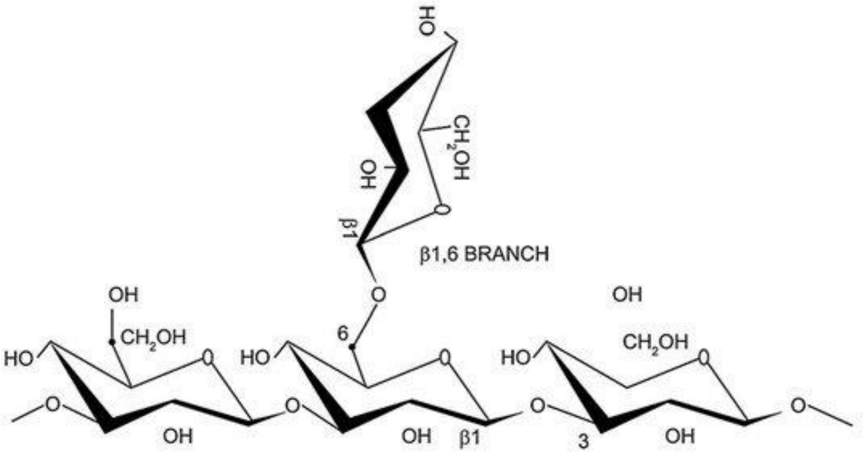

Figura 3: Cadeia 1,3 glicosídica linear de monômeros β-D-glicose ligados por uma ligação glicosídica 1,6
Fonte: Bashir e Choi (2017)

Produtos naturais como fungos contendo β-glucanas têm sido consumidos por, provavelmente, milhares de anos, especialmente na China e no Japão por seu papel na melhora da saúde geral (Ali et al., 2009). Ao discutir as funções imunomoduladoras dos glucanas, comumente considera-se o β 1,3/1,6-glucana purificado de paredes celulares de fungos, sendo que fontes comuns de β-1,3-glucanas incluem leveduras (tipicamente *Saccharomyces cerevisiae*), cogumelos (*Sclerotium glucanicum* e outros), bactérias (curdlan de *Alcaligenes faecalis*) e algas marinhas (laminarina de *Laminaria digitata*) (Goodridge et al. 2009). Especificamente, os β-glucanas aumentam a quimiotaxia e adesão de neutrófilos, participam da ativação de macrófagos, células T *helper* e *natural killer* (NK), e na promoção da diferenciação e ativação de linfócitos T, além de amplificarem a morte fagocítica de células tumorais opsonizadas e combinarem-se com anticorpos monoclonais para aumentar sua atividade tumoricida (Ali et al., 2009). Por essa razão, além da função imunomodulatória, os β-glucanas também são usados como um agente de prevenção de doenças, bem como uma parte da terapia anticâncer ou anti-inflamatória (Bashir e Choi, 2017).

Por ser um ingrediente “seletivamente fermentável”, permitindo mudanças específicas na microbiota, o β-1,3/1,6 glucana também possui importante função prebiótica (Hamaker e Tuncil, 2014), inclusive já demonstrada em peixes (Jung-Schroers et al., 2016).

Os β-glucanas, de maneira geral, têm sido vastamente estudados como ingredientes funcionais na nutrição humana e animal nas últimas décadas, e na aquicultura não é diferente. Ainda assim, nota-se que com o avanço tecnológico de procedimentos analíticos nos últimos anos, pesquisas mais aprofundadas sobre os efeitos dessas moléculas a nível sistêmico no organismo têm sido realizadas em tilápias-do-Nilo, possibilitando resultados inovadores sobre sua regulação metabólica.

2.3.2.2 Utilização do β-glucana na aquicultura

O uso do β-glucana na piscicultura é uma das principais alternativas aos antibióticos, cujo uso indiscriminado resultou no surgimento de diversos patógenos resistentes, se tornando uma das maiores preocupações de pesquisadores e aquicultores (Meena et al., 2013).

A maioria dos estudos sobre os efeitos do β-glucana em peixes ainda diz respeito à modulação imunológica, devido à característica dessa molécula de se ligar a diferentes tipos de receptores de leucócitos, estimulando a resposta imune inata (Ali et al., 2009). Ao avaliar a inclusão de β-1,3/1,6-glucana na dieta de camochilo (*Trachinotus ovatus*), Huu et al. (2016) encontraram aumento no número de leucócitos, diminuição na contagem de *Vibrio* intestinal e maior taxa de sobrevivência após teste de resistência à salinidade (1 ppt por 4 horas) em Pampo-verdadeiro (*Trachinotus ovatus*) que receberam 0,5 e 1 g kg^{-1} do ingrediente na dieta. Jundiás (*Rhamdia quelen*) alimentados por 21 dias com dieta contendo 0,1 e 1 g kg^{-1} de β-1,3/1,6-glucana apresentaram maior atividade do sistema complemento, maior sobrevivência frente ao desafio com *Aeromonas hydrophila* e menor contagem de colônias dessa bactéria no sangue (Domenico et al., 2017).

Tilápias-do-Nilo alimentadas com 0,5 g kg^{-1} de β-glucana e expostas a deltametrina na água apresentaram maior atividade fagocítica e maior concentração de lisozima sérica em relação às tilápias que foram expostas ao químico, mas que não receberam o β-glucana (Dawood, 2020a). Nesse mesmo estudo, foi constatado que a inclusão do ingrediente na dieta amenizou os danos histológicos no intestino, fígado e baço dos animais que receberam deltametrina. *Oreochromis mossambicus* alimentadas com 2, 5 e 10 g kg^{-1} de β-glucana e desafiadas com concentrações sub letais de amônia, apresentaram maiores valores séricos de atividade das enzimas catalase e glutationa peroxidase em relação ao grupo controle (grupo desafiado com amônia, mas que não recebeu β-glucana) (Divya et al., 2020). Os resultados desse estudo indicam uma maior proteção antioxidante nos animais que receberam o ingrediente. Isso é um achado muito importante, já que, em situações estressantes, a produção de radicais livres é aumentada (Divya et al., 2020).

Não é somente no sistema imunológico que o β-glucana demonstra poder modulatório. A microbiota intestinal de peixes também pode ser influenciada por este ingrediente, conforme demonstrado em estudo realizado por Jung-Schroers et al. (2016), onde a microbiota intestinal de carpas (*Cyprinus carpio*) alimentadas com dieta contendo 10 g kg^{-1} de β 1,3/1,6-glucana apresentou maiores valores nos índices de Shannon–Wiener e Simpson (índices indicadores de diversidade microbiana) em relação a microbiota do grupo controle (0 g kg^{-1}). Os autores salientam que uma diversidade aumentada na microflora intestinal pode resultar em um ambiente mais estável e menos suscetível a ação de microrganismos patogênicos. Esses resultados são reforçados por estudo realizado em Robalos europeus (*Dicentrarchus labrax*), que demonstrou que o fornecimento de 1 g kg^{-1} de β-1,3/1,6-glucana na dieta durante oito semanas aumenta a riqueza de táxons bacterianos na microbiota intestinal (Carda-Dieguez et al., 2014). Também relacionado à saúde intestinal, estudo que avaliou a toxicidade do inseticida organofosforado Clorpirifós sobre a morfologia do tecido intestinal em tilápias-do-Nilo, revelou que os animais que receberam o inseticida e não receberam a suplementação com β-1,3/1,6-glucana na dieta apresentaram enterite catarral, com degeneração e descamação da parte apical das vilosidades intestinais, com infiltração linfocítica difusa; já os animais que receberam 1 g kg^{-1} de β-1,3/1,6-glucana na dieta apresentaram vilosidades com as estruturas preservadas, assim como no grupo controle (grupo que não recebeu inseticida e β-1,3/1,6-glucana) (Dawood et al., 2020c).

Outras funções metabólicas dos β-glucanas são estudadas devido ao seu potencial como fibra dietética. Segundo Ali et al. (2009), o potencial de redução do colesterol pelos β-glucanas pode ser explicado pela propriedade desse ingrediente de aumentar a viscosidade do trato gastrointestinal, estimulando a produção de ácidos biliares. Além disso, a capacidade do β-glucana de se ligar nos ácidos biliares estimula a produção de mais desses ácidos derivados do colesterol, sendo esse processo realizado endogenamente ou capturado na circulação (Bell et al., 1999). Ademais, uma redução da insulina sérica devido ao aumento da produção de ácidos graxos de cadeia curta, que são produtos da fermentação dos β-glucanas pelas bactérias intestinais, pode ser mais um efeito de modulação em resposta a ingestão do ingrediente (Bell et al., 1999). No entanto, estudos na aquicultura que avaliaram a influência dos β-glucanas no perfil lipídico ou energético ainda são escassos. Carpas alimentadas com 0 (controle), 1, 10 e 20 g kg^{-1} de β-1,3/1,6-glucana por oito semanas não apresentaram diferenças significativas na concentração de glicose sérica (Kuhlwein et al., 2014). Tilápias-do-Nilo que receberam 0,1 g kg^{-1} de β-glucana na dieta por 60 dias não apresentaram diferenças significativas nas concentrações de glicose, triglicérides e colesterol séricos em relação ao grupo controle (Dawood et al., 2020b). Por outro lado, Pilarski et al. (2017), ao avaliarem duas moléculas de β-glucana com diferentes graus de pureza (BG1 mais puro, BG2 menos puro), encontraram que tilápias que consumiram a dieta com BG2 apresentaram menores valores de glicose sérica e maior peso final em relação ao grupo BG1 e controle, enquanto a dieta BG1 apresentou maior potencial de modulação imunológica.

Conforme discutido por Bell et al. (1999) e Ali et al. (2009), e reforçado pelos estudos acima citados, algumas características dos β-glucanas como a fonte, solubilidade, pureza, espécie de peixe avaliada, entre outras, determina o potencial de ação prebiótica ou imunomoduladora dessas moléculas. Paralelamente, de acordo com revisão realizada por Meena et al. (2013), a maioria dos estudos com β-glucana na aquicultura são realizados com a inclusão do prebiótico via dietética, e poucos estudos avaliaram outras formas de administração, como via imersão na água (banhos) ou injetável. Por essas razões, pesquisas ainda são necessárias para elucidar os efeitos do β-glucana maneira detalhada e averiguar sua viabilidade em produções em larga escala de peixes.

2.4. MICROBIOTA INTESTINAL DE PEIXES

Sabe-se que a microbiota intestinal desempenha importante função no metabolismo dos nutrientes, vitaminas, regulação da resposta imune, competição com microrganismos patogênicos, entre outros (Eichmiller et al., 2016; Vatsos, 2016), compreendendo em um “órgão oculto” (Eichmiller et al., 2016), cujo entendimento pode ser explorado para fins produtivos na aquicultura.

A expansão de tecnologias como o NGS nos últimos anos permitiu a caracterização mais detalhada de comunidades microbianas e suas interações com os hospedeiros (Tarnecki et al., 2017). Em diversas espécies de peixes, essa caracterização permitiu avaliar efeitos da dieta, do ambiente, da interação com patógenos, entre outros fatores, sobre a composição microbiana (Eichmiller et al., 2016; Tarnecki et al., 2017; Suphoronski et al., 2019)

Em tilápias-do-Nilo, a microbiota “core”, ou seja, o grupo comum de bactérias presentes no intestino desses animais é aparentemente formado por táxons dos filos Firmicutes, Fusobacterias e Proteobacteria, conforme demonstrada por alguns estudos recentes (Fan et al., 2017; Suphoronski et al., 2019). No entanto, nos mesmos estudos, é constatada maior variação taxonômica quando se compara níveis mais específicos, como famílias e gêneros. Isso ocorre devido às diferentes condições experimentais, fase de vida do peixe, dietas testadas, entre outros fatores, indicando uma capacidade de adaptação da microbiota intestinal dessa espécie frente a diferentes condições de cultivo. Aliado a isso, conforme salientado por Tarnecki et al. (2017), a pressão pública contra o uso de antibióticos na piscicultura nos últimos anos mudou o foco de “matar patógenos” para “promover microrganismos benéficos”, e, como resultado, muitos esforços foram adotados para entender os efeitos dos prebióticos e probióticos utilizados na piscicultura.

No entanto, embora a tecnologia NGS permita descrever detalhadamente o microbioma das principais espécies de peixes com potencial produtivo, e os efeitos modulatórios da dieta na composição microbiana, ela ainda é recente, e muitos estudos são necessários para compreender as complexas interações entre essas comunidades (Tarnecki et al., 2017).

Estudos paralelos envolvendo metabólitos funcionais produzidos por esses microrganismos, bem como análises transcriptomicas, também impulsionadas pelo avanço do NGS, poderão trazer novas e valiosas informações sobre essas interações, auxiliando no melhor uso desses ingredientes para incrementar o manejo nutricional na tilapicultura.

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4 HIPÓTESE

A microalga *Schizochytrium sp*. fornecida dieteticamente na concentração concentração de 12 g kg^{-1} (1,2%) e a administração de β-1,3/1,6-glucana na água de cultivo (0,1 e 0,3 mg L^{-1}) podem ter efeitos benéficos nos parâmetros hemato-imunológicos, bioquímicos e na microbiota intestinal de tilápias-do-Nilo. Além disso, o fornecimento de β-1,3/1,6-glucana na água pode diminuir indicadores de estresse em tilápias-do-Nilo após desafio com hipóxia.

5 OBJETIVOS

5.1 OBJETIVO GERAL:

Objetivou-se avaliar os efeitos da microalga *Schizochytrium* sp. e do β-glucana nos parâmetros fisiológicos de tilápias-do-Nilo (*Oreochromis niloticus*).

5.2 OBJETIVOS ESPECÍFICOS

1) Avaliar o efeito da farinha da microalga *Schizochytrium* sp. sobre os parâmetros hematológicos e concentração de lisozima sérica de tilápias aos 105 dias de cultivo em tanques-rede.
2) Analisar os efeitos da farinha da microalga *Schizochytrium* sp. sobre a histologia e microbiota intestinal de tilápias-do-Nilo.
3) Avaliar os efeitos do β-glucana na água de cultivo sobre os parâmetros de crescimento, hematológicos, imunológicos, bioquímicos e na microbiota intestinal de tilápias-do-Nilo.
4) Analisar os indicadores de estresse (parâmetros hematológicos, bioquímicos e mortalidade) de tilápias-do-Nilo desafiadas com hipóxia após 15 dias de tratamento com β-glucana na água.

6 ARTIGO A – PUBLICADO NO PERIÓDICO PLOS ONE – EFFECTS OF DIETARY SUPPLEMENTATION WITH A MICROALGA (*SCHIZOCHYTRIUM* SP.) ON THE ON THE HEMATO-IMMUNOLOGICAL, AND INTESTINAL HISTOLOGICAL PARAMETERS AND GUT MICROBIOTA OF NILE TILAPIA IN NET CAGES (https://doi.org/10.1371/journal.pone.0226977)

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Abstract

Nutritional improvements in intensive aquaculture production systems is necessary for the reduction of stress, maximum utilization of nutritional components, and expression of the genetic potential of fish. The objective of this study was to evaluate the hemato-immunological, and histological parameters and gut microbiota of Nile tilapia fed with the microalga *Schizochytrium* sp. Males of Nile tilapia were distributed among eight net cages (6 m^3), and fed for 105 days with two diets: control (CON), without *Schizochytrium* sp., and supplemented (SUP), with 1.2% *Schizochytrium* sp. in the diet. The final weight, mortality, hematocrit, total erythrocyte count (RBC), hemoglobin, hematimetric indices, white blood cell count (WBC), total protein, and serum lysozyme were measured. Alterations in intestinal morphology were evaluated. The gut microbiota was evaluated with next-generation sequencing. No significant differences ($p>0.05$) were found in the final weight and mortality between diets. Regarding the hematological parameters, a difference ($p<0.05$) was detected only in RBC, with there being lower values in the SUP, although this group also showed a tendency toward having an increased mean corpuscular hemoglobin level. There were no differences ($p>0.05$) in total protein and serum lysozyme concentrations or in WBCs between diets, except for lymphocytes, which presented lower values ($p<0.05$) in the SUP, suggesting immunomodulation by the polyunsaturated fatty acids present in the microalga. There was no difference ($p>0.05$) in the intestinal morphology between diets. Metagenomic data indicated greater richness (represented by the Chao index) and a higher abundance of the bacterial phylum Firmicutes in the gut microbiota of the tilapia fed with the SUP diet, demonstrating that the digestion and use of the components of the microalga could influence the microbial community. The results indicated that the microalga had modulatory effects on blood cells and the intestinal microbiota, without affecting the structure and integrity of the intestinal villi.

Introduction

In recent decades, world aquaculture has undergone a marked expansion due to the increased production of some species, such as Nile tilapia (*Oreochromis niloticus*), which stands out as one of the most produced fish in the world [1]. In the year 2016, world production of Nile tilapia reached 4.2 million tons, representing 8% of the total fish produced in the world, with it being the fish produced at the highest levels in several countries [1]. In Brazil, the production of this species represented approximately half of all aquaculture production in 2017, which was approximately 559 thousand tons [2]. However, due to the rapid expansion and intensification of Nile tilapia production, the high densities adopted in farming systems have become a challenge, since they are a stress factor for the fish that can cause economic losses [3–5].

Given the above, Nile tilapia farming in intensive systems, such as net cages, has increased significantly. This growth has mainly been due to the excellent productivity per unit of space and ease of assembly of the cage structure, as well as the control of the stock and harvest it allows [6,7]. Because of the high density adopted in these tanks, the use of food that improves fish health and immunity is essential to minimize the effects of stress and allow the genetic potential of the fish to be fully expressed. In addition, there is a global demand for the use of low-cost natural compounds in animal feed that have physiological benefits and pose no risk to the environment [8].

Algae, being the bases of many natural aquatic food chains, represent an important food resource that contains several compounds with functional activities that can aid in animal and human nutrition [9]. Among the algae used for animal feed, the microalga *Schizochytrium* sp. stands out. This alga is particularly well-known for having a high content of polyunsaturated fatty acids (PUFAs), in particular docohesaexanoic acid (DHA) [10], which is known to have diverse human and animal health benefits. Studies have shown that the inclusion of *Schizochytrium* sp. in the diet can increase the concentration of fatty acids in Nile tilapia [11] and channel catfish (*Ictalurus punctatus*) [12], modulate the gut microbiota of rainbow trout (*Oncorhynchus mykiss*) [13], and increase the final biomass of Nile tilapia [14]. However, no studies have evaluated the effects of this microalga on fish farmed in intensive systems like net tanks.

Recently, technologies such as next-generation sequencing (NGS) have allowed there to be a rapid expansion in research related to fish nutrition. It is known that the microbiota of the digestive tract play important roles in the production of vitamins, nutrient distribution, regulation of innate immunity, and maintenance of intestinal tissue integrity [15,16], which can be modulated by different diets and farming conditions [17]. However, no study to date has been conducted in an integrated manner to evaluate the influence of dietary microalgae on intestinal histology and microbiota, in addition to on the general health and immunity of Nile tilapia. For these purposes, information on hematological and immunological parameters are useful to indicate the conditions of cellular defense and homeostasis during stressful periods, and previous studies have already shown that they can be modulated by the inclusion in the diet of algae, such as *Laminaria digitata* and *Ascophyllum nodosum* [18], *Ulva clathrata* [8] and *Gracilaria verrucosa*, which has also been shown to to be capable to increase the resistance of tilapia to pathogens [19]. In addition, PUFAs have been shown to influence immune response through modulation of leukocyte cells [[20,21]], although immunological studies evaluating the effects of providing PUFA-rich sources on fish feed are still scarce. Therefore, hematological and immunological assays on the effects of microalgae *Schizochytrium* sp may provide important information on a possible modulation of the immune response.

Therefore, the objective of the present study was to analyze the influence of the addition of the microalga *Schizochytrium* sp. to the diet on the hemato-immunological, and histological parameters and intestinal microbiota (assessed via NGS) of Nile tilapia cultivated in net cages.

Materials and methods

The methodologies employed during this experiment were approved by the Ethics Committee on the Use of Animals of the State University of Londrina (CEUAUEL, nº18363.2017.07).

Experimental design

This study was conducted in a fish farm from the Fish Experimental Station of the Maringá State University (UEM/Codapar) located in the Corvo River, near the city of Diamante do Norte and at the Rosana Hydroeletric plant (22°39'25.20" S and 52°46'52.78" W) in the state of Paraná, Brazil. Were used 800 Nile tilapia juveniles of the GIFT lineage that had been sexually reversed into males, with initial weights of approximately 50 g. The fish were distributed among eight net cages (6 m^3) arranged in a completely randomized design, consisting of two treatments with four replicates each, with 100 fish per replicate for a total of 400 fish per treatment. The net cages were arranged in a single line while intercalating the treatments to avoid any effects of the position of the cage in the river.

To meet the study objectives and experimental conditions proposed, two treatments were used: one with a control diet (CON) (commercial feed without supplementation), and one using a diet supplemented with 1.2% microalgal (*Schizochytrium* sp.) meal (SUP) (guarantee levels per kilogram: Ether extract 500 g, DHA 140 g. Source: manufacturer – ALL-G RICH™, Alltech, Lexington, KY, USA).

The microalgal meal was added to the extruded commercial feed, with soybean oil being used as the vehicle (1.6% of the diet in total). The control diet received only soybean oil in the same amount. The feeds were each mixed for five minutes in a concrete mixer (M-120 Maqtron). The procedures described by [22] were used for the calculation of the dry matter, crude protein, mineral matter, and crude energy (Parr Instrument Co. AC6200) in the ether extract of each feed (Table 1). Lipid extraction from the feed was performed by the Bligh-Dyer method [23]. The methyl esters of fatty acids were quantified according to the method of [24], in which the methyl ester of tricosanoic acid (Sigma, USA) was used as a standard. The theoretical values of the correction factor of the flame ionization detector (FID) were used to obtain the concentrations of fatty acids [25] (Table 1).

Table 1. Compositions of the control (CON) and alga-supplemented (SUP) diets provided to Nile tilapia.

Centesimal composition	**CON**	**SUP**
Gross energy (kcal/kg)	4.28	4.37
Dry matter (%)	92.33	92.46
Crude protein (%)	34.53	33.22
Ether extract (%)	5.41	6.10
Mineral matter (%)	11.51	11.52
Lipid composition (mg/100 g of diet sample)	**CON**	**SUP**
PUFA	1574.28	1970.87
MUFA	1352.37	1330.44
SFA	1113.69	1558.03
FAT (%)	4.44	5.34
n-6/n-3	250.08	12.95
n-6	1568.01	1829.57
n-3	6.27	141.30
DHA	Nd	131.81

PUFA = total polyunsaturated fatty acid; MUFA = total monounsaturated fatty acid; SFA = total saturated fatty acid; FAT = total fatty acid content; n-6 = total n-6 fatty acid; n-3 = total n-3 fatty acid; DHA = docosahexaenoic acid; nd = not detected. Composition: Soybean meal, ground whole corn, wheat meal, meat and bone meal, fish meal, degummed soybean oil. Vitamin and mineral mix (guarantee levels per kilogram): Calcium 15 g, Phosphorus 4000 mg, Sodium 2000 mg, Copper 15 mg, Manganese 40 mg, Zinc 60 mg, Cobalt 0.75 mg, Iodine 0.75 mg, Selenium 0.3 mg, Vitamin A 7000 UI, Vitamin D3 2000 UI, Vitamin E 90 UI, Vitamin K3 12 mg, Vitamin B1 20 mg, Vitamin B2 20 mg, Vitamin B6 20 mg, Vitamin B12 mcg, Niacin 100 mg, Calcium Pantothenate 50 mg, Folic Acid 5mg, Biotin 0.15, Vitamin C 300 mg, Choline Chloride 1350 mg, Inositol 30 mg. Source: manufacturer – Poli Nutri (Osasco, SP, Brazil).

An acclimatization period of 15 days was carried out, and then followed by the experimental period, which had a duration of 105 days (15 weeks). Monthly biometric analyses were performed to control the amount of feed provided. Fish were fed four times a day in the initial growth phase (5% of biomass, fish of 50-100g), two times a day in the growth phase (4% of biomass, fish of 100-500g), and two times a day in the final phase (2% of biomass, fish of 500g until end of the trial). Mortality was measured throughout the entire experimental period. The water temperature was evaluated daily in the morning and afternoon.

The final weight (g), standard length (cm) (from the anterior end of the head to the beginning of the caudal fin insertion), and total length (cm) (from the anterior end of the head to the end of the caudal fin) [26] of the fish was measured on the day of collection (105th day). Condition factor (CF;%) was determined according [27], with the formula CF (%) = [weight of fish (g)/ (length of fish (cm))^3] × 100.

Hematological profile and total protein and serum lysozyme content

Samples of blood (1 mL) were collected from the caudal vein of the fish in plastic microtubes containing dipotassium ethylenediaminetetraacetic acid (K2EDTA, Hemstab, Lagoa Santa, MG, Brazil) as an anticoagulant for the preservation of the samples destined for use in hematological tests. Fourteen fish were used per diet group (CON and SUP), and blood was collected after the fish had been anesthetized with clove oil (0.1 ml L^{-1}) (Eugenol, Ibipora, PR, Brazil). The determination of the total hemoglobin (Hb) concentration in the blood was performed by the hemoglobin cyanide method using the commercial Labtest® kit (Lagoa Santa, MG, Brazil). Erythrocyte counts (RBC x 10^6 μL^{-1}) were made with a Neubauer camera after dilution (1:200) in Dacie's solution [28]. For the calculation of the globular volume (VG in %) by the microhematocrit technique and the determination of the hematimetric indices MCV (mean corpuscular volume - fL), MCHC (mean corpuscular hemoglobin concentration - g dL^{-1}), and MCH (mean corpuscular hemoglobin - pg), the methodologies described by [29] were followed. Leukocyte differential counts were performed using the indirect method with May-Grünwald-Giemsa-Wright staining [29]. Briefly, in each blood sample, 2000 cells were counted, including erythrocytes, thrombocytes, and leukocytes. The number of leukocytes ($\times 10^3$ uL^{-1}) (lymphocytes, neutrophils, and monocytes) and thrombocytes ($\times 10^3$ uL^{-1}) were calculated using a rule of three, while considering the number of cells counted in the neubauer camera.

To measure total serum proteins (g dL^{-1}) and serum lysozyme (μg mL^{-1}) concentrations in blood samples, 1 mL of blood (without anticoagulant) was centrifuged for 10 min at 2500× *g* for serum separation. Total serum protein was measured by the colorimetric biuret method (Analisa®, Belo Horizonte, MG, Brazil). Serum lysozyme activity was determined using a methodology adapted from that of [30]. 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}). Both analyses were performed using a digital spectrophotometer (Coleman 33D).

Intestinal histology

For this analysis, a medullary section was performed on the animals previously anesthetized for blood collection. Samples were collected from the distal intestine of 16 animals per treatment. The collected material was fixed in 10% buffered formaldehyde for 24 h, and then inserted into 70% ethanol. The samples were next submitted to serial dehydration in ethanol, sectioned into 4-5 μm sections, stained with Alcian blue (pH = 2.7) and hematoxylin and eosin (H&E), and covered with coverslips.

A semi-quantitative score was developed based on the proposed score of [31] for Atlantic salmon (*Salmo salar* L.) (Table 2). Briefly, five parameters were independently classified: 1) flattening of mucosal folds (MF); 2) width of the lamina propria (LP); 3) presence of supranuclear vacuoles (SNV); 4) abundance of goblet cells (GC); and 5) degree of infiltration of eosinophilic granulocytes (EG) into the lamina propria (LP) and subepithelial mucosa (SM). Each of these parameters was scored on a scale from 1 to 3, with lower score value representing a more normal condition and greater integrity of the villi and intestinal cells. The slides were visualized under an Opticam 0500R microscope coupled to a camera. The images were captured using OPTHD software (version x64 3.7.8).

Table 2. Description of the semi-quantitative score used to evaluate different histological parameters in the Nile tilapia intestine.

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

Adapted from the score used by Uran et al. (2008) for Atlantic salmon (*Salmo salar* L.)

All statistical analyses were performed using the software R version 3.3.3 [32]. Data were presented as means ± standard error. The zootechnical indexes of the animals, results of hematological analyses, lysozyme concentration, total serum protein, and histological parameters were compared between diet groups using Student's *t*-test, with a significance level of 5%. The data were submitted to Levene's test to verify the homogeneity of their variances, and the Shapiro-Wilk test to evaluate the normality of their residuals. The data that did not meet the assumptions of homogeneity and normality were instead analyzed with the Wilcoxon-Mann-Whitney non-parametric test.

Gut microbiota

For the analysis of the gut microbiota, the intestinal digesta was initially expelled after the opening of the ventral surface of the abdomen, and then the intestinal loops were removed and sectioned into two portions: proximal and distal. By applying gentle pressure to the intestinal loops, the feces were expelled and stored (with microbiota not adhered) in sterile 15 mL Falcon tubes. The intestinal loops were then carefully washed with sterile 0.9% saline solution and stirred at 200 rpm, and the resulting supernatant was removed and separated for collection of the adherent bacteria. The intestinal contents (feces + supernatants) were then placed into sterile Falcon tubes (15 mL) containing sterile 0.9% saline solution. For each diet (CON and SUP), a three-fish digesta pool was established in duplicate. The tubes were placed in a Styrofoam box with dry ice until arrival at the laboratory, where they were stored in a freezer at -80ºC.

The commercial PowerSoil® DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) was used for the extraction of bacterial DNA from the digesta samples, according to the recommendations of the manufacturer. The V3-V4 region of the 16S subunit gene of the bacterial ribosome was amplified with primers from the Illumina platform. The quality of the generated amplicons was verified, and they were then sent to Neoprospecta (Santa Catarina, Brazil) to be sequenced (paired-end library) on the Illumina MiSeq platform with the 250-cycle V2 kit.

Briefly, the samples were normalized to 5 ng uL^{-1}, and then polymerase chain reaction (PCR) was performed with Illumina TruSeq adapters (Illumina, San Diego, CA) under the following conditions: 95ºC for 5 min, 25 cycles of 95ºC for 45 s, 55°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 2 min. Subsequently, a second PCR was performed with the index sequences under the following conditions: 95°C for 5 min, 10 cycles of 95°C for 45 s, 66°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 2 min. The final product of this PCR was purified with the aid of AMPureXP beads (Beckman Coulter, Brea, CA), and the samples were grouped into sequencing libraries. The libraries were sequenced on a MiSeq system, using the standard Illumina primers provided in kit V2, with 250 cycles at each end.

Bioinformatic analyses were performed using the Mothur software (v.1.36.1) following the methodologies described by [33] and [34], with some modifications. Briefly, contigs were assembled based on the ‘fastq’ files of the ‘read1’ and ‘read2’ produced for each sample. Ambiguities in the sequences were removed, and the sequences were later aligned with the SILVA database. Homopolymers, nucleotide redundancies, nonspecific amplifications, and chimeras were removed with the aid of the VSEARCH algorithm. The sequences were then classified and grouped into OTUs (operational taxonomic units) for the taxonomic comparison of the sequences. To reduce the error caused by non-uniformity in the number of sequences, a subsample was taken from the sample with the lowest number of sequences for the normalization of the data. The Chao, Simpson, Inverse Simpson, and Shannon diversity indices were then calculated, and their mean values were submitted to t-test (significance level of 5%). To compare the structure of the microbial communities between the two treatments, an analysis of molecular variance (AMOVA) was performed. A Venn diagram was generated to demonstrate the intersection of the microbial assemblages among the analyzed pools.

Results

Water temperature, mortality, and zootechnical indexes

The mean water temperature was 25.99 ± 3.58°C. There was no statistically significant difference between the mortality of fish fed the SUP and CON diets ($p > 0.05$). The final weight of tilapia was 732.69 ± 41.17 g in the SUP diet group and 678.90 ± 39.56 g in the CON diet group, which did not significantly differ between diets ($p = 0.35$). The standard and total lengths were numerically higher in SUP, but without significant difference ($p > 0.05$). The standard length means (cm) were 25.19 ± 0.43 cm and 24.41 ± 0.41 cm, for SUP and CON ($p = 0.20$), respectively; and total lengths means were 30.33 ± 0.50 cm and 29.63 ± 0.46 cm ($p = 0.32$) (SUP and CON, respectively). The condition factor (CF %) was similar between treatments (2.54 ± 0.04 for SUP, and 2.53 ± 0.04 for CON), with no statistical difference ($p = 0.87$).

Hematological profile, total serum protein and serum lysozyme

There were no significant differences ($p > 0.05$) between the fish in the SUP and CON diet groups in their measurements of the VG, Hb, MCV, MCH, and MCHC parameters. Erythrocyte counts (RBC×10^6 μL^{-1}), on the other hand, showed significantly lower values ($p = 0.02$) in the SUP than in the CON group (Table 3). The differential leukocyte cell counts (×10^3 μL^{-1}) did not show significant differences between diets for total leukocytes (WBC), neutrophils, or monocytes. However, a significant difference ($p = 0.04$) in lymphocyte counts was observed, with there being lower values in the fish on the SUP diet. No eosinophils and basophils were observed in either of the treatments tested. Thrombocyte counts and total serum protein and serum lysozyme concentrations, also did not differ significantly between the diet groups ($p > 0.05$) (Table 3).

Table 3. Hematological parameters (mean ± standard error), total serum protein, and serum lysozyme concentrations of Nile tilapia fed with a diet containing 1.2% *Schizochytrium* sp. meal (SUP) or a control diet (CON, commercial feed without supplementation) for 105 days.

<table>
<tr><th>Diet</th><th>RBC (x10^{6} µL^{-1})</th><th>Hb (g dL^{-1})</th><th>Ht (%)</th><th>MCH (pg)</th><th>MCV (fL)</th><th>MCHC (g dL^{-1})</th></tr>
<tr><td>CON</td><td>2.27 ±0.06</td><td>9.67 ±0.11</td><td>33.57 ±1.52</td><td>43.18 ±1.56</td><td>149.11 ±7.49</td><td>29.91 ±1.91</td></tr>
<tr><td>SUP</td><td>2.06 ±0.05</td><td>9.62 ±0.11</td><td>30.64 ±1.29</td><td>47.16 ±1.33</td><td>149.20 ±5.15</td><td>31.94 ±1.04</td></tr>
<tr><td>p-value</td><td>0.02*</td><td>0.73</td><td>0.15</td><td>0.06</td><td>0.99</td><td>0.36</td></tr>
<tr><td></td><td>WBC (×10^{3} µL^{-1})</td><td>Lymphocytes (×10^{3} µL^{-1})</td><td>Neutrophils (×10^{3} µL^{-1})</td><td>Monocytes (×10^{3} µL^{-1})</td><td colspan="2">Thrombocytes (×10^{3} µL^{-1})</td></tr>
<tr><td>CON</td><td>46.91 ±4.13</td><td>28.92 ±3.10</td><td>13.88 ±2.90</td><td>4.10 ±0.77</td><td colspan="2">50.24 ±4.32</td></tr>
<tr><td>SUP</td><td>37.42 ±2.06</td><td>21.07 ±1.96</td><td>11.81 ±2.44</td><td>4.54 ±0.91</td><td colspan="2">49.52 ±4.15</td></tr>
<tr><td>p-value</td><td>0.05</td><td>0.04*</td><td>0.59</td><td>0.72</td><td colspan="2">0.90</td></tr>
<tr><td></td><td colspan="3">Total serum proteins (g dL^{-1})</td><td colspan="3">Lysozyme (µg mL^{-1})</td></tr>
<tr><td>CON</td><td colspan="3">3.58±0.05</td><td colspan="3">16.22±1.17</td></tr>
<tr><td>SUP</td><td colspan="3">3.57±0.07</td><td colspan="3">16.94±2.21</td></tr>
<tr><td>p-value</td><td colspan="3">0.96</td><td colspan="3">0.77</td></tr>
</table>

RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell.

*$p < 0.05$: significant at the 5% level.

Intestinal histology

Regarding intestinal morphology, there were no statistically significant differences in any of the evaluated parameters between the diet groups (Table 4). Numerically, the greatest (but non-significant) differences were found in the number of goblet cells and the presence of supranuclear vacuoles, which both had higher scores in fish in the CON than the SUP diet group (Table 4). S1 Fig demonstrates each of the parameters evaluated by the developed score system, and illustrates the morphological and cellular alterations observed for each characteristic evaluated.

Table 4. Histological scores (mean ± standard error) of different aspects of the intestinal morphology of Nile tilapia fed with a diet supplemented with 1.2% *Schizochytrium* sp. meal (SUP) or a control diet (CON).

Diet	MF	LP	SNV	GC	EG
CON	2.12 ±0.15	2.06±0.17	2.13±0.09	2.12±0.18	2.06±0.19
SUP	2.18±0.13	2.25±0.17	1.87±0.13	1.81±0.16	2.00±0.20
p-value	0.80	0.44	0.26	0.21	0.84

MF, mucosal folds; LP, lamina propria; SNV, supranuclear vacuoles; GC, goblet cells; EG, eosinophilic granulocytes.

Gut microbiota

A total of 272,941 contigs were generated in the sequence reads. After quality control, a total of 258,918 contigs were generated, which were aligned in the SILVA database to access information on the OTUs present in the samples. A subsample of 21,999 reads per sample was used for the normalization of the data, which generated the rarefaction curve of the samples shown in Fig 1. The subsample yielded coverage higher than 99.9%, indicating good representativeness of the total microbial population. After the subsampling, a total of 51 OTUs (operational taxonomic units) were obtained at the genus level, with 37 genera found in both treatments.

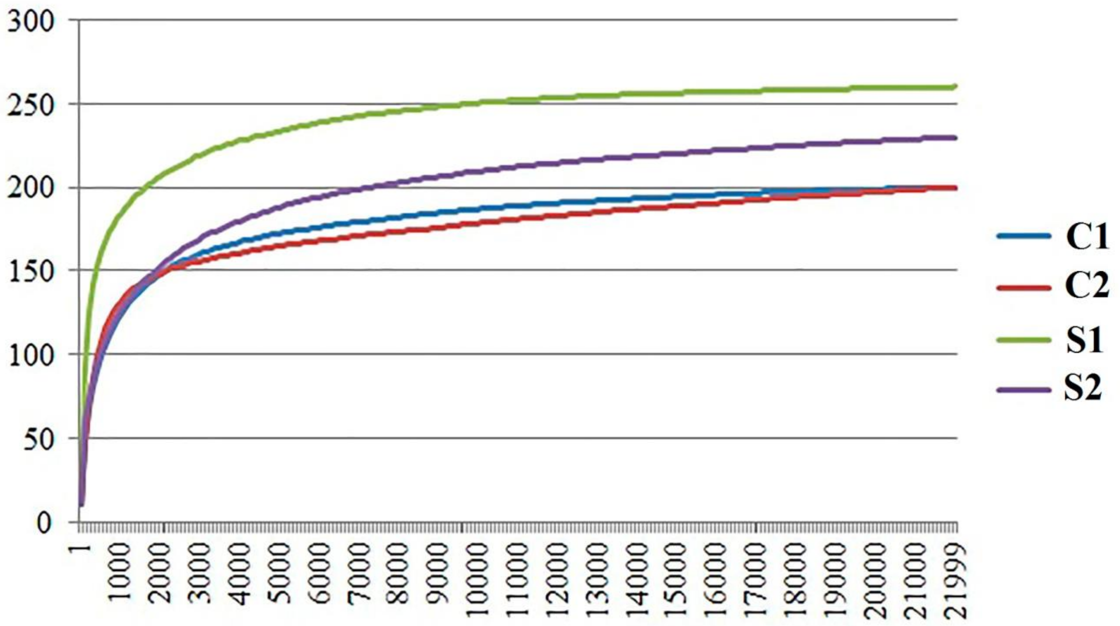

Fig 1. Rarefaction curve of each sample showing the number of reads (x-axis) in relation to the number of OTUs (y-axis). Control group: C1, C2; Supplemented group: S1, S2.

The mean alpha diversity indices and their standard errors are shown in Table 5. Significantly higher values of the Chao richness index were observed in the SUP diet group than in the CON group ($p < 0.05$). Although this group also presented higher values of the Inverse Simpson and Shannon indices, these differences were not statistically significant ($p > 0.05$). The values of the Simpson index also did not differ between diets ($p > 0.05$).

Table 5. Mean ± standard error of the Chao, Simpson, Inverse Simpson, and Shannon diversity indices of the gut microbiota of Nile tilapia fed the control diet (CON) or that supplemented with 1.2% *Schizochytrium* sp meal (SUP).

Diet	**Chao**	**Simpson**	**Inverse Simpson**	**Shannon**
CON	20.75±7.50	0.82±0.06	1.22±0.14	0.45±0.11
SUP	26.00±0.00	0.57±0.12	1.82±0.55	0.97±0.12
p-value	0.02*	0.21	0.27	0.08

*p < 0.05: significant the 5% level.

The relative abundances of different bacterial OTUs at the phylum and class levels in each treatment are shown in Fig 2. Members of 10 different phyla were found, with the phyla Firmicutes, Fusobacteria, and Proteobacteria being predominant (Fig 2A). In the SUP diet group, there was a predominance of the phylum Firmicutes, whereas in the CON group the relative abundances of Firmicutes and Fusobacteria were balanced. Proteobacteria was the third most abundant phylum for both diets tested.

At the class level (Fig 2B), there was a predominance of the class Clostridia (Firmicutes) in the SUP diet group, and of Fusobacteriia (Fusobacteria) and Clostridia (Firmicutes) in the CON group. The SUP diet group had a higher relative abundance of Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria (Proteobacteria) in relation to that in the CON diet group. In turn, the presence of several exclusive classes in the CON group, such as Erysipelotrichia and Negativicutes (Firmicutes), Bacteroidia (Bacteroidetes), and Caldilineae (Chloroflexi), was found (Fig 2B). However, the SUP diet group had only one exclusive class, Bacillus (Firmicutes). Representatives of other classes, such as Planctomycetia (Planctomycetes) and Spartobacteria

(Verrucomicrobia), were found only at low relative abundances in both diet groups.

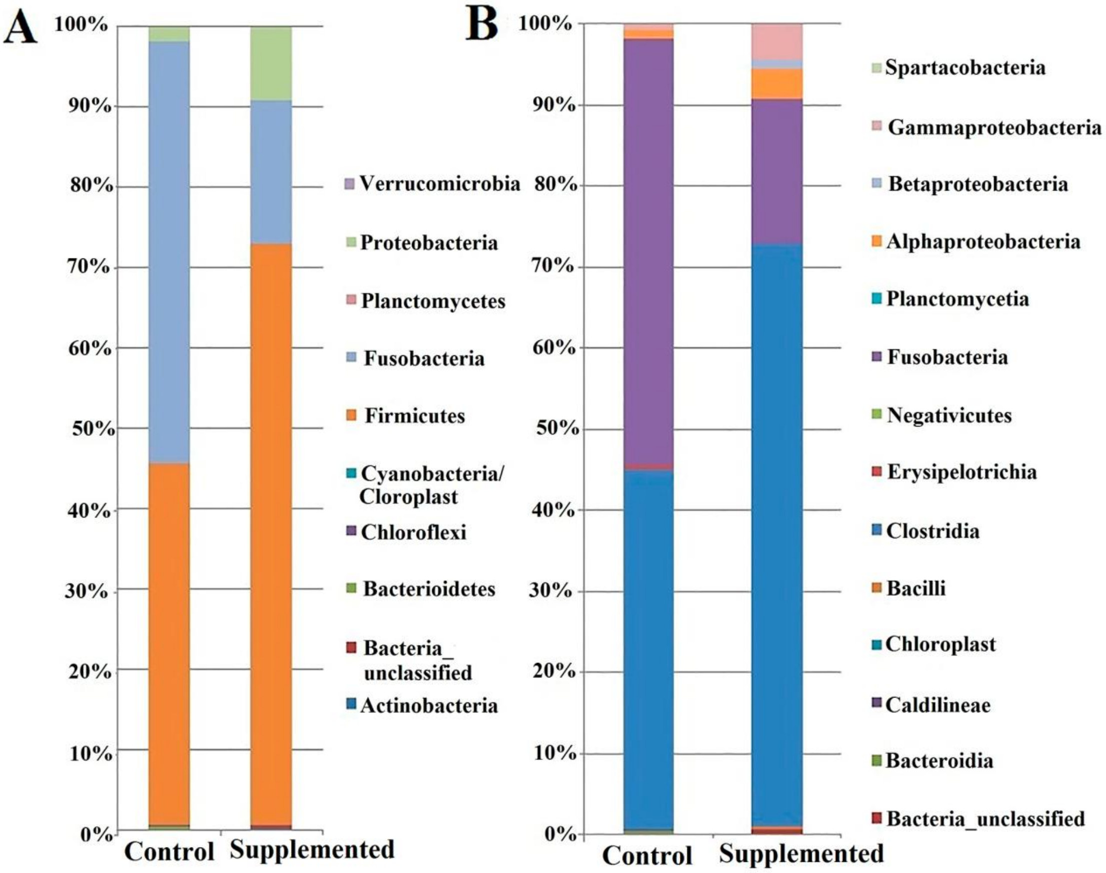

Fig 2. Mean relative abundances of different OTUs of gut bacteria in Nile tilapia fed with a diet containing 1.2% *Schizochytrium* sp. meal (SUP) or a control diet (CON). Results are shown at the levels of bacterial (A) phyla and (B) classes.

Of the 51 genera identified, the relative abundances of the 15 orders and genera with the highest numbers of sequences are displayed in S1 Table 1. The genus *Romboutsia* (phylum Firmicutes) and *Cetobacterium* (Fusobacteria) were the most abundant in both treatments (128,238 and 117,108 sequences in total, respectively), followed by *Fusobacteriace*_unclassified (3254), *Pseudomonas* (2195), and *Methylocystis* (1731). The *Romboutsia* genus was the one that presented the highest relative abundance in the SUP diet group (71.76%), while in the CON group, the genus

Cetobacterium was the most abundant one (51.83%).

The analysis of molecular variance (AMOVA) demonstrated that there was greater variation within treatments than between them, and thus found a non-significant structuring between diets ($p > 0.05$). The Venn diagram found a total of 51 OTUs, with 14 exclusive genera for each treatment and 23 shared genera (S2 Fig).

Discussion

The mean temperature of the water during the experimental period was compatible with the thermal comfort zone of Nile tilapia [6], and was similar to that in a previous experiment conducted in Rio do Corvo [4]. The zootechnical indexes of tilapia did not differ significantly between treatments ($p > 0.05$), and the final weight was consistent with the slaughter weight of this species when farmed in net cages.

Recent studies have been carried out to evaluate the effects of diet on the hematological, immunological, and histological parameters or the gut microbiota of Nile tilapia [5,8,35–41], which have contributed to our understanding of the effects of dietary constituents on different physiological aspects of this species. However, no study to date has evaluated these variables in an integrated manner and/or in real farming situations. Despite the absence of significant effects of the diet tested in this study on the final weight and mortality of tilapia, its influence on hematological parameters and the gut microbiota revealed that the addition of 1.2% *Schizochytrium* sp. meal to the diet may influence important aspects of the health of Nile tilapia.

Wide variation in the hematological parameters of Nile tilapia is found in the literature due to a variety of factors, such as alterations in temperature [42], the administration of vaccines [43], stress stimuli [44], and supplementation of diets with probiotics [37,38] and algae [8,18], among others. The values of the hematological parameters found in the present study were similar to those in the studies cited above.

The lower erythrocyte counts found in the SUP diet group may have been related to physiological processes resulting from microalgae ingestion, which may be related to stress minimization, as already shown in studies with other food additives provided for tilapia. In a study by [38], Nile tilapia at high stocking densities showed higher RBC counts in the control group than in the group receiving a probiotic (*Bacillus subtilis*) in their diet. The authors of that study emphasized that the supplied probiotic minimized the erythrocytosis related to the physiological stress caused by the high density, which increased the fish's oxygen supply. Similarly, [44] observed an increase in the number of RBCs in tilapia submitted to single and consecutive stress stimuli. Additionally, [41]found lower RBC values in tilapia that received phytogenic compounds and organic salts compared to the control group. The RBC count in the present study was slightly higher than that found by [41], who considered normal values for the species. Therefore, microalgae supply would hardly be related to anemia processes, corroborated by Ht (%) values above 20% [29].

It is known that animals held at high densities, such as in net cages, can present elevated stress levels [3], which can lead to an increase in the number of red blood cells in the blood to meet the physiological oxygen demand [38]. Glucocorticoids, such as cortisol, promote adaptive changes in cells and tissues in response to stressors, and erythrocytosis is one of the compensatory stress responses [38]. As observed by [38] by providing *Bacillus subtilis*, it is possible that the inclusion of microalgae in the present work has minimized erythrocytosis related to the physiological stress. However, even though lower RBC values were found in the SUP diet group in this study, significant differences were not found ($p > 0.05$) in the Hb concentration and hematimetric indices between the diets tested. The greater numerical difference in the hematimetric indices, such as the MCH ($p = 0.06$), indicated the possibility that the amount of hemoglobin present in each erythrocyte was increased [29] in the SUP group, which would demonstrate a physiological compensation of oxygen supply due to lower RBC values, increasing the ability of red blood cells to carry hemoglobin. Future studies with stress markers such as cortisol, glucose, lactate and serum electrolytes may clarify whether microalgae can reduce tilapia stress in net cages.

The differential analysis of WBC counts is an important indicator of fish health and immunity status that provides information on cell defense conditions [38]. The use of dietary algae such as *Gracilaria verrucosa* has been shown to be able to modulate leukocyte cells and increase the survival rate of tilapia following *Streptococcus agalactiae* challenge [19]. In the present study, no statistically significant difference was observed ($p > 0.05$) in the counts of any leukocytes between diets, except for lymphocytes. Counts of thrombocytes, cells responsible for the formation of protective barriers that also possess phagocytic activity [29,45], also did not differ significantly between diets ($p = 0.90$). In a study evaluating the effect of the alga *Sargassum ilicifolium* in the diet of a sturgeon (*Huso huso*), it was found that supplying algae at the concentration of 10% of the diet increased the number of WBCs, with an increase in the numbers of neutrophils and a decrease in the number of lymphocytes [46]. In sea bream (*Sabanejewia aurata*) fed with the alga *Asparagopsis taxiformis* and its extract, significant decreases ($p < 0.05$) in RBC counts, Hb, Ht, and WBC counts were found [47]. That study's authors suggested that the inclusion of algae in the diet had negative effects on hematological parameters, which could be related to a dose/response effect.

Although there was an apparent decrease in numbers of WBCs ($p = 0.05$) suggested by changes in lymphocyte counts, the findings of the present study indicated that the inclusion of the alga in the diet did not drastically influence the immune response of Nile tilapia, considering that the great majority of leukocytes, in addition to the concentrations of total serum protein and lysozyme, did not differ between treatments. [46] observed an increase in serum total proteins and lysozyme activity in sturgeon fed with the alga *Sargassum ilicifolium* at concentrations of 7.5 and 10% in relation to the control group. In contrast, Atlantic salmons fed fourteen weeks with the macroalga *Palmaria palmata* at concentrations of 5, 10 and 15% in the diet showed no significant differences in serum total protein values and lysozyme activity compared to the control group [48], as in the present study. These results demonstrate that the immune responses of algae-fed fish can be variable, depending on the algae species, concentrations used, and even fish eating habits, which can affect the ability to digest and efficiently absorb nutrientes [48].

With regard to lymphocytes, PUFAs have been shown to reduce proinflammatory responses, decreasing the ability of T lymphocytes to proliferate [21]. The probable mechanism of this modulation is based on the increased number of suppressor T lymphocytes that occurs with the administration of DHA, which negatively regulates the other lymphocytes [20,21,49]. The presence of high amounts of n-3 PUFAs in the diet supplemented with the microalga *Schizochytrium*, was possibly the reason for the reduction in lymphocyte concentrations seen in the SUP group. More specific immunological assays, such as tests of the expression of CD4 + lymphocytes, FoxP3, and interleukins, could clarify the lymphocyte modulation of diets rich in PUFAs in fish in more detail.

The microbial community occupying the gastrointestinal tracts of fish plays a crucial role in the development, physiology, and health of these animals, since it stimulates the development of the immune system and promotes competition with pathogenic microorganisms, in addition to also being fundamental in preserving the structure and integrity of the intestinal villi to ensure the adequate acquisition and metabolism of nutrients by the fish [15,17]. Regarding the analysis of the gut microbiota in the present study, the rarefaction curve constructed after subsampling demonstrated uniformity in the number of individuals detected in relation to the number of reads in the samples (Fig 1). The coverage being greater than 99.9% of all samples demonstrated that the sequences obtained were sufficient to represent most of the total microbial diversity, which strengthens the reliability of the results. Although the Chao richness index presented significantly higher values in the SUP diet group ($p < 0.05$), the treatments did not differ significantly in their values of the Simpson, Inverse Simpson, and Shannon indices, which indicate the alpha diversity of the bacterial community. This result was probably caused by the different abundances of bacterial species between the fish in the CON and SUP diet groups, which did not significantly influence species heterogeneity and equitability.

In a study of rainbow trout (*Oncorhynchus mykiss*) fed with the microalga *Schizochytrium limacinum* for 15 weeks, [13] observed higher numbers of OTUs and Chao index values in the supplemented group than in the control. These authors pointed out that these results may have been due to the adaptation of the gut microbiota to the digestion of the polysaccharides present in the microalga, which was sufficient to cause differences in diversity and richness, but not in the bacterial community structure. In the present study, although there was no difference in the number of OTUs between treatments (37 in each), the highest value of the Chao index found agreed with that found by [13], indicating a greater richness in the bacterial community in the fish that received the diet with the microalga, and the absence of effects on the microbial community structure of the diets tested.

The composition of the microbiota being predominantly represented by the phyla Firmicutes, Fusobacteria, and Proteobacteria corroborates the findings of other studies of the gut microbiota of Nile tilapia [35,50,51], although with different abundances of each phylum found. A slight increase in the relative abundance of Firmicutes was also observed in trout that received a diet containing *Schizochytrium limacinum* [13]. Increased bacterial richness and diversity, in addition to increased abundance of the phylum Firmicutes, has also been related to increased consumption of the proteins and carbohydrates in vegetable flour, which includes lactic acid bacteria (LAB) [52]. These bacteria are abundant in the gut microbiota of some fish species, and are associated with benefits to the intestinal epithelium and improved immune responses [15,53,54]. Even though bacteria of the order Lactobacillales were present only in the supplemented group in the present study, they were only the 15th most abundant bacterial taxon in this group (S1 Table), and were predominantly represented by the Clostridiales. Therefore, the LAB likely did not have a significant influence on the percentage of Firmicutes found in this study.

There is wide variation in the bacterial genera present in the intestinal microbiota among teleosts. However, the genus *Cetobacterium*, which was abundant in both treatments in the present study, is highly representative of the gut microbiota in other fishes, such as in some carp species [16], channel catfish [55] , rainbow trout [13], and even species of tilapia [35,51]. This bacterial genus is apparently commensal and encompasses the core microbiota of these species. Its importance is related to its capacity to synthesize vitamin B_{12}, antimicrobial metabolites, and volatile fatty acids, mainly acetic acid [56,57]. *Romboutsia* is another widely abundant genus of bacteria that was found mainly in the SUP group in the present study, which is adapted to an environment rich in carbohydrates and exogenous sources of amino acids, and is able to use these before other bacterial taxa by different metabolic pathways [58]. Carbon sources, such as glucose, fructose, maltose, trehalose, xylose, and sorbitol, can be used by different strains of this bacterium [58,59], which can synthesize acetic acid, ethanol, isso-butanoic acid, and isso-valeric acid via glucose fermentation [60].

The presence of saccharides, such as xylose and glucose, in addition to galactose and mannose, in the cell wall of the microalga *Schizochytrium* [61,62] may have contributed to the energetic supply of bacteria of the genus *Romboutsia*, which would have favored the proliferation and increased abundance of these bacteria. However, the absence of *in vivo* studies on the use of these nutrients and the modulation of gut microbiota hampers the ability for precise conclusions to be made about the influence of these compounds on this bacterial genus or other abundant ones. Investigations into the mechanisms of energy utilization by different gut bacteria of fish could more clearly elucidate the mechanisms of the modulation of gut microbiota by exogenous sources of nutrients.

Another genus abundant in both treatments, but mainly in the treated group, *Pseudomonas* has already been classified as one of the dominant genera in fish microbiota [63,64]. This genus is also found in freshwater aquatic environments and is rarely reported as pathogens in these animals, being a potential antagonist of pathogenic microorganisms from bacterial and fungal origin [64]. Less abundant and exclusive bacteria from the CON group such as bacteria from the genus *Turicibacter* and family *Caldilineaceae* have been reported in fish microbiota in natural environments [65,66]. Another bacteria little present, but with greater abundance in the SUP group, *Ralstonia* has been found in greater abundance in sea bass fed on functional diets, and its dominance possibly justified by the presence of essential oils in the diet [67]. The *Escherichia-Shigella* group, although considered pathogenic to other hosts, apparently have commensal characteristics in fish [68]. It should be emphasized that this was the first study that evaluated the gut microbiota of tilapia cultivated in net cages, and thus it can lead to further research the culture environment. For this reason, the composition of the microbial community and the abundance of certain genera found differed from those in other experiments performed in laboratory conditions, although it should be noted that the 'core' microbiota of the studied species were apparently maintained. Therefore, the present results may serve as a basis for future research on experimental conditions in the field to help researchers better understand the intestinal microbial ecology of fish in different growing environments.

The intestinal epithelial cells act as a first line of defense against potentially harmful agents, while also ensuring the adequate nutrient utilization, immune defense, and growth of fish [69]. The absence of significant difference ($p > 0.05$) in the intestinal morphology between the fish fed the CON and SUP diets indicated that the presence of the microalga in the digestive tract and the modulation of the gut microbiota caused by it was not enough to modify the structure and integrity of the intestinal villi, which eliminates the possibility of enteritis processes caused by algae. The score developed may be useful for future histological approaches in studies of teleosts, particularly Nile tilapia.

In recent years, new natural compounds, such as algae and phytogenic extracts, have been extensively explored as viable economic alternatives for use in animal production, including aquaculture. Added to this, there is a growing global demand for research on compounds that pose no risks to the environment and the consumer, but which can have physiological benefits to the cultured animals, such as promoting the better utilization of nutritional components and the maintenance of general health. In conclusion, the results of the present study indicate that the microalga *Schizochytrium* sp. has the capacity to modulate blood cells, including red blood cells and lymphocytes, and also has the potential to manipulate the intestinal microbial community, without having effects on the structure and integrity of the intestinal villi. Further studies with different concentrations of *Schizochytrium* sp., under other farming conditions, and using different increment of transcriptomic analyses could profoundly clarify the modulatory capacity of this microalga on the physiology of Nile tilapia.

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Supporting Information

S1 Fig. Intestinal morphology of Nile tilapia (*Oreochromis niloticus*). GC, goblet cells; LP; lamina propria; EG, eosinophilic granulocytes; SNV, supranuclear vacuoles; LV, lymphatic vessel. (A) Epithelium and whole villi, with no signs of flattening; fine and complete LP; and GC in small amounts. (B) Increase in CG; LP with slight thickening; diffuse reduction of SNV. (C) GC increased. (D) Shrinkage of villi; LP with increased size; SNV present and aligned. (E) 'Crumbling' (disruption) of villi; LV present; absence of SNV. (F) Increased presence of EG in LP; SNV present and aligned. (A, B, C, D, E: Alcian blue staining; F: H&E staining). Scale bar = 50 µm.

S2 Fig. Venn diagram showing the intersection between the bacterial diversity in the gut microbiota of the control (CON) and supplemented (SUP) diet groups.

S1 Table. Relative abundances (at order and genus level) of the 15 most abundant gut bacteria of Nile tilapia fed with a diet containing 1.2% *Schizochytrium* sp. meal (SUP) or a control diet (CON).

7 ARTIGO B – PUBLICADO NO PERIÓDICO AQUACULTURE REPORTS – EFFECT OF B-GLUCAN IN WATER ON GROWTH PERFORMANCE, BLOOD STATUS AND INTESTINAL MICROBIOTA IN TILAPIA UNDER HYPOXIA

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Declarations of interest: none

ABSTRACT

In the present study, the effects of β-glucan administration in water on growth performance and blood status (hematological indexes, immunological and biochemical parameters) were evaluated in Nile tilapia fingerlings (*Oreochromis niloticus*) under hypoxia-induced stress. In parallel, effect of this prebiotic (beta-glucan) was evaluated on gut microbiota diversity (16S rRNA gene) under normal oxygenation condition. Three treatments were implemented according to beta-glucan water supplementation: 0 (control), 0.1 and 0.3 mg L^{-1}. Blood was collected before hypoxic challenge (BHC) and 9 hours after hypoxic challenge (AHC). No significant differences were observed according to growh performance parameters. A significant increase in erythrocyte number and decrease in mean corpuscular volume ($P < 0.05$) were identified in control group when were under hypoxia. The plasma glucose level was higher in the 0.3 group than in the 0.1 group BHC; however, for all groups, the glucose level increased markedly AHC, and then receded seven days AHC (7AHC, $P < 0.05$). Total protein, serum lysozyme, plasma lactate, triglyceride, and cholesterol concentrations did not differ between groups. Comparison of the two β-glucan concentrations indicated that the 0.3 mg L^{-1} concentration promoted an increase in lymphocyte count. The 0.1 group showed the highest survival rate 7 days after hypoxia. Metagenomic analysis revealed a greater number of operational taxonomic units (OTUs) in the 0.1 and 0.3 groups. The Chao richness value was higher in the 0.1 group than in the control group ($P < 0.05$), with a greater abundance observed for members of the *Vibrionaceae* family. It is concluded that the concentration of 0.1 mg L^{-1} was able to increase tolerance to hypoxia, increasing the survival rate and regulating glucose levels. The concentration of

0.3 mg L^{-1} modulated hematological responses under stress and increased the lymphocyte count before hypoxic challenge.

Key-words: fish, *Oreochromis niloticus*, metagenomics, prebiotics, stress

1. Introduction

Nile tilapia (*Oreochromis niloticus*) farming has undergone rapid growth in recent years, in parallel with an expansion in global aquaculture. Data from (FAO, 2018) indicate that farmed tilapia production in 2016 was only lower than that of carp species (grass carp, *Ctenopharyngodon idellus*; silver carp, *Hypophthalmichthys molitrix*; and common carp, *Cyprinus carpio*), reaching 8% of global finfish production. Thus, in Brazil, tilapia is the most farmed species, accounting for approximately half of all fish production in the country in 2017 (IBGE, 2017). In general, fish farming is associated with an intensification of fish culture, that can affect fish welfare and then productive performance (Oliva-Teles, 2012; Stentiford et al., 2012). Coupled to crowding conditions, there are many practices related to handling or harmful environmental factors such as hypoxia and impaired water quality, that can lead to a stressing condition in cultured fishes making fishes prone to infectious diseases (Oliva-Teles, 2012).

Hypoxia is one of the most important and common challenges in fish farming, and may have multifactorial causes, including season, water composition and flow, and fish density (Abdel-Tawwab et al., 2019; Pinedo-Gil et al., 2019; Xiao, 2015; Yang et al., 2017). Fish under hypoxia respond to compensate for the oxygen restriction displaying physiological, biochemical changes associated to stress response that in the long term can affect to growth rate and immune response in fishes. In tambaqui (*Colossoma macropomum*), for example, an increase in plasma glucose was found in animals submitted to hypoxia (Chagas and Val, 2006). For Rainbow trout (*Oncorhynchus mykiss*), an increase in cortisol and plasma lactate was observed in animals at low oxygen concentrations in water (Pinedo-Gil et al., 2019).

The indiscriminate use of antibiotics to control pathogenic microorganisms in aquatic systems is an important concern for both scientists and fish farmers, owing to the risk of the development of antibiotic resistance among these microorganisms (Meena et al., 2013). Consequently, natural compounds with antimicrobial and immunostimulant properties are increasingly being investigated as alternatives (Buck et al., 2017; Pinedo-Gil et al., 2019; Quezada-Rodríguez and Fajer-Ávila, 2017; Suphoronski et al., 2019). In addition, the use of these compounds is also beneficial for the environment, and, consequently, for fish health, since they do not present risks to the environment, public health and food safety as antibiotics, and can favour the immunity and general health of fish (Suphoronski et al., 2019).

Beta-glucan is a polysaccharide immunostimulant bound by Beta-glycosidic linkages between glucose chains (Meena et al., 2013). The use of β-glucan in aquaculture has been shown to be effective in promoting greater resistance in fish under stressful conditions, especially to challenges by bacterial pathogens, through stimulation of innate immune responses (Meena et al., 2013; Petit and Wiegertjes, 2016; Pilarski et al., 2017). However, little it is known about how this ingredient affects fish gut microbiota. The intestinal microbiomes of fish are known to comprise complex and dynamic communities, and play important physiological roles that help maintain host homeostasis (Llewellyn et al., 2014; Tarnecki et al., 2017). Consequently, the diversity of these communities and the intra- and interspecific relationships of the associated microorganisms are fundamental for the health and maintenance of intestinal activities, including nutrient absorption and metabolism; dysbiosis of these communities can lead to disease (Cho and Blaser, 2012; Jung-Schroers et al., 2016; Llewellyn et al., 2014) and compromise productive performance. A recent study has shown that β-glucan administration can increase gut microbial richness of turbot (*Scophthalmus maximus*) (Miest et al., 2016), as well as the diversity of intestinal microflora of carp (*Cyprinus carpio*) (Jung-Schroers et al., 2016); however, a different study reported a reduction in the gut microbiota richness of mirror carp fed a β-glucan-supplemented diet (Kuhlwein et al., 2013). To date, to the best of our knowledge, no studies have evaluated how β-glucan administration affects the intestinal bacterial community in tilapia. Sequencing of the 16S rRNA gene enables the accurate and comprehensive characterization of intestinal communities, while next-generation sequencing (NGS) technology allows for detailed, rapid, and cost-effective profiling of this community (Tarnecki et al., 2017).

In addition, studies on the effect of β-glucan in fish under oxygen-restricted conditions are still scarce, and nonexistent in the case of the Nile tilapia. Zeng et al. (2016) demonstrated that administration of this polysaccharide can lead to decreased mortality and modulation of anaerobic metabolism in yellow croakers (*Pseudosciaena crocea*) exposed to hypoxia. Pinedo-Gil et al. (2019) observed that, after six hours of hypoxia-induced stress, rainbow trout who had been administered 8% dietary β-glucan-rich barley presented lower plasma glucose levels than the control group. Administration of another immunostimulant, ascorbic acid, has also been reported to decrease glucose levels in tambaquis (*Colossoma macropomum*) exposed to acute hypoxia (Chagas and Val, 2006). The use of cinnamon oil (*Cinnamomum* sp.) (plant with immunomodulatory properties) at a concentration of 0.15%, was able to increase the concentration of total plasma proteins in Nile tilapias subjected to hypoxia (Santos et al., 2016). These studies have shown that some compounds can help, even if indirectly, energy mobilization as a response to hypoxia-induced stress. However, further studies are needed to elucidate how β-glucan modulates systemic physiological responses, such as changes in hematological and biochemical parameters, under oxygen-restricted conditions.

Currently, the most suitable routes for β-glucan administration in fish remain undefined. Reviews on the use of this polysaccharide have indicated that the most frequently used route is dietary inclusion, followed by administration in water (immersion or bath) or by injection (Meena et al., 2013; Petit and Wiegertjes, 2016). Although the more effective in intraperitoneal β-glucan injection(Petit and Wiegertjes, 2016), dietary and water inclusion are cheaper and more practical methods since each application covers a greater number of fishes (Petit and Wiegertjes, 2016; Udayangani et al., 2017; Zhang et al., 2009). According to this, recent research using β-glucan administered in water has shown promising results in fish, such as upregulation of immunity-related genes (Udayangani et al., 2017; Zhang et al., 2009), increased survival against bacterial diseases (Udayangani et al., 2017), recovery from skin wounds (Przybylska-Diaz et al., 2013), and modulation of leukocyte responses (Sado et al., 2016). This is important for omnivorous filter-feeding fish such as tilapia, which can passively ingest and retain particles suspended in water (Sanderson et al., 2001, 1996; Turker et al., 2003). Owing to the limited number of studies addressing this route of administration, further research is required to clarify the possible effects of β-glucan on Nile tilapia.

The objective of the present study was to evaluate how β-glucan administered in water can improve growth performance, hematological, immunological, biochemical parameters as well as gut microbiota in Nile tilapia under a compromised condition such hypoxic water in order to improve this species culture.

2. Materials and methods

2.1. Animals and experimental design

All animal procedures were approved by the Ethics Committee on Animal Use of the State University of Londrina (approval number CEUA/UEL-5129.2019.01). All experiments were performed in accordance with the relevant guidelines and regulations.

Male Nile tilapia (*n* = 120) fingerlings (~8,30 g) were purchased from a local supplier and transported in plastic bags (containing one part water, where the fish were packed, and two parts pure oxygen which was injected into the plastic bag before it was sealed) to the acclimatized laboratory (27 ~ 28 °C) of the Center of Study and Research in Aquaculture and Genetics (NEPAG) of the State University of Londrina (UEL). In the laboratory, the fish were placed in 18 42-L aquariums under constant aeration and siphoned twice a day (50% of the total volume of water) to remove debris, and then refilled with chlorine-free water. Water parameters, such as temperature (25.70 ± 0.59 °C), dissolved oxygen (9.7 ± 0.51 mg L^{-1}) (Hanna Instruments, Barueri, SP, Brazil), and pH (7.34 ± 0.06) (pH meter Akso, São Leopoldo, RS, Brazil), were measured daily. Ammonia indices were measured three times a week by colorimetric assay and remained below toxic levels throughout the experiment. The photoperiod was 12:12 h (light/dark).

After 10 days of acclimatization, the Nile tilapia fingerlings were weighed (8.76 ± 0.15 g, average initial weight ± SE) and randomly distributed in 15 aquariums (8 fish per aquarium). Three experimental groups were assayed according to β-glucan administration in water: a control group without β-glucan, 0.1 and 0.3 mg β-glucan L^{-1} The treatments were assayed in fivefold (5 aquariums per group) for 15 days.

Preparation of the β-1,3/1,6-glucan stock solution was based on the methodology proposed by Przybylska-diaz et al. (2013) and Schmidt et al. (2016). Briefly, MacroGard (from *Saccharomyces cerevisiae,* >60% purity, Biorigin, Lençóis Paulista, SP, Brazil) was dissolved in MilliQ water, under constant stirring for 1 h on a magnetized stirrer at 90 °C, to a final concentration of 2.1 g L^{-1}. After each water change in the experimental facility, the solution was applied to the aquarium water in the experimental units, except to the control aquariums, to achieve a final concentration of 0.1 or 0.3 mg L^{-1}.

All the fish were fed the same extruded feed for early-stage tilapia (36% crude protein, 7% ether extract, 5% fiber in dry matter 2.6-mm diameter) twice daily according to 4% of biomass.

2.1.1. Hypoxic challenge

On day 15 fish were sampled and growth parameters were measured. After that, blood samples were collected for hematological, immunological and gut samples for microbiota assays. After that, the oxygen supply of each aquarium was interrupted and the dissolved oxygen (mg L^{-1}) was measured, as shown in Figure 1. Blood was collected (n = 10 fish per treatment) 9 hours after hypoxia onset (Figure 1), after the dissolved oxygen concentration had decreased by approximately 2.5 mg L^{-1} (in 7 hours) (Figure 1).

After hypoxic challenge and collection of biological samples, the remaining fish were randomly distributed at a density of six fish per aquarium and the oxygen was reconnected. The provision of β-glucan in water was interrupted, and the survival rate (%) was evaluated for seven days.

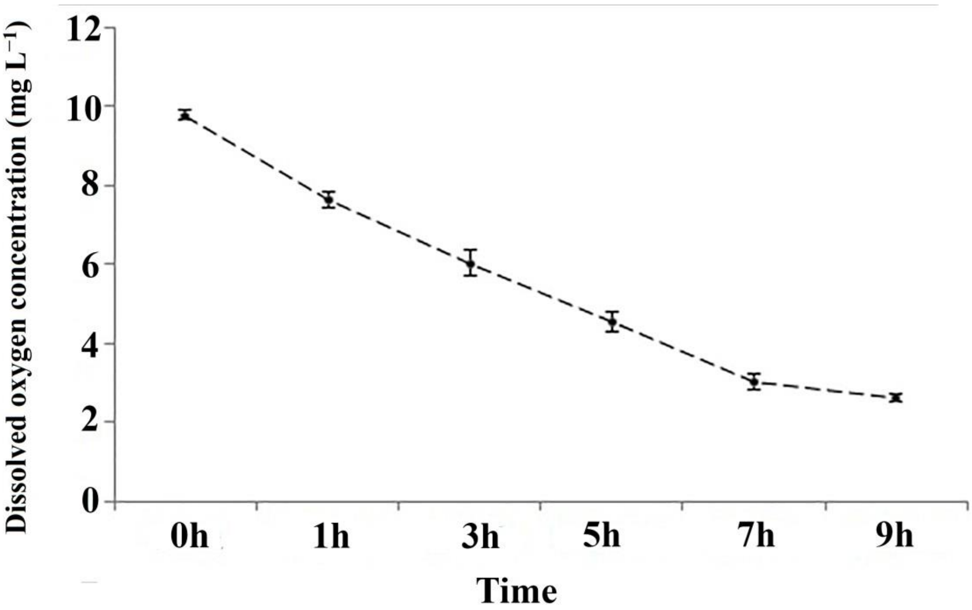

Figure 1. Changes in the Dissolved oxygen concentration (mg L^{-1}) of water aquariums stocked with Nile tilapia.

2.2. Growth performance

The final weight, standard length (from the anterior end of the head to the beginning of the caudal fin insertion) and total length (from the anterior end of the head to the end of the caudal fin) were measured to determine growth performance parameters. Weight gain (g) (final weight – initial weight), daily weight gain (g fish^{-1} day^{-1}) ([(final average weight – initial average weight)/experimental period]), specific growth rate (% day^{-1}) [(Ln (final weight) - Ln (initial weight))/experimental period x 100], and apparent feed conversion (feed intake/weight gain) were determined (Dawood et al., 2017; Oliveira et al., 2019; Souza et al., 2017). The condition factor (CF; %) was determined according to Dawood et al. (2017), using the formula CF (%) = [weight of fish (g)/length of fish (cm)^3] × 100.

2.3. Sampling procedure

Blood samples were collected before hypoxic challenge (BHC, 0 h in Figure 1) and immediately after hypoxic challenge (AHC, 9 h in Figure 1). The samples were collected from the caudal vein of the fish with 3-mL syringes containing EDTA (ethylenediaminetetraacetic acid) to preserve the samples for use in the hematological, plasma glucose and lactate analysis. Ten fish were used per treatment (two fish per aquarium), and blood was collected after the fish had been anesthetized with benzocaine (0.1 g L^{-1}). Glucose quantification was performed 15 days after β-glucan treatment (BHC), immediately after induction of hypoxia (AHC), and on day 7 after hypoxic challenge (7AHC). Plasma lactate concentrations were measured BHC and AHC, for this analysis, were used five pooled samples per treatment, two fishes per pool.

For serum separation for measurement of immunological parameters (total serum proteins and lysozyme, analyzed BHC and AHC), and biochemical (triglycerides and cholesterol, analyzed only BHC, blood samples were collected in syringes without EDTA as an anticoagulant (two fish per aquarium, ten fish per treatment), and centrifuged for 10 min at 1400 × *g*.

After blood collection before hypoxic challenge (BHC), six fish from each experimental group were euthanized with an overdose of benzocaine (0.4 g L^{-1}), and the intestinal digesta was expelled after the ventral surface of the abdomen had been opened. The stools were removed aseptically from the entire intestinal tract, and immediately stored at −80 °C. Stool collection was based on a study of Suphoronski et al. (2019). For extraction of bacterial DNA, three pools of feces were used for each treatment, with two fish per pool.

2.4. Hematological and immunological parameters

Hematocrit (Ht in %) in the blood samples was measured by the microhematocrit method (Ranzani-Paiva et al., 2013), while total hemoglobin (Hb g dL^{-1}) concentrations were quantified by the hemoglobin cyanide method (Collier, 1944) using the commercial Labtest® kit (Lagoa Santa, MG, Brazil). Red blood cell (RBC, 10^6 μL^{-1}) counts were performed using a Neubauer camera after dilution (1:200) of the blood in Dacie’s solution (Blaxhall and Daisley, 1973). The hematimetric indices calculated were MCV (mean corpuscular volume), MCHC (mean corpuscular hemoglobin concentration), and MCH (mean corpuscular hemoglobin) (Ranzani-Paiva et al., 2013). The indirect May–Grünwald–Giemsa–Wright staining method was used for differential leukocyte counts (Ranzani-Paiva et al., 2013), which were performed only before hypoxic challenge (BHC).

Total serum protein concentrations were quantified by the colorimetric method (Analisa, Belo Horizonte, MG, Brazil) using a digital spectrophotometer (Coleman 33D, Santo André, SP, Brazil). Five pooled serum samples per treatment (one pool of two fishes per aquarium) were used for assessment of serum lysozyme activity according to Ellis (1990). Briefly, solutions of chicken egg lysozyme standards (L6876; Sigma–Aldrich Chemical Co., Saint Louis, MO, USA) were prepared to generate a standard curve. Serum lysozyme activity was determined by lysis of the gram-positive bacterium *Micrococcus lysodeikticus* (Sigma–Aldrich Chemical Co.) using 90-µL serum aliquots; initial and final absorbance of the samples were assessed by spectrophotometry at 492 mn. A reduction in absorbance was converted into an estimate of lysozyme concentration (µg mL^{-1}) according to the linear equation of the standard lysozyme curve.

2.5. Biochemical parameters

Triglycerides and cholesterol concentration were assayed using enzymatic colorimetric assays (Analisa, Belo Horizonte, MG, Brazil) (mg dL^{-1}). The absorbance was determined at 505 nm for triglycerides and 500 nm for cholesterol.

For quantification of plasma glucose concentrations immediately after collection a drop of blood was placed on a glucose test strip using the FreeStyle Optium Neo glucose meter (Maidenhead, Berkshire, England).

The lactate concentration was determined by enzymatic colorimetric assay (Interkit, Belo Horizonte, MG, Brazil) at 540 nm using a Coleman 33D digital spectrophotometer.

2.6. Gut microbiota

The commercial QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used for extraction of bacterial DNA, according to the recommendations of the manufacturer. DNA integrity was confirmed on 1% agarose gels.

The samples were sent to NGS Soluções Genômicas (Piracicaba, SP, Brazil) for sequencing (paired-end library) on an Illumina MiSeq platform. For this, primers for the V3-V4 regions containing adapters for Illumina MiSeq sequencing were used for PCR amplification of the 16S rRNA gene. A first PCR (16S rRNA V3-V4) was performed under the following conditions: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. A second PCR was subsequently performed with the index sequences under the following conditions: 95 °C for 3 min, followed by 12 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCRBio Ultra Mix (PCR Biosystems, London, United Kingdom) was used in both PCR reactions, while AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used for purification. The samples were grouped into sequencing libraries, and the amplicons were sequenced with an Illumina MiSeq platform using the paired-end, 250-cycle V3 MiSeq Reagent Kit.

Mothur software (v.1.36.1) was used for bioinformatic analyses, following the methodologies described by Kozich et al. (2013) and Schloss et al. (2009), with some modifications. Briefly, the sequences were aligned using the SILVA database, and filtered to remove homopolymers, nonspecific amplicons, redundancies, and chimeras using the VSEARCH algorithm. The sequences were classified into operational taxonomic units (OTUs) for taxonomic comparison. To decrease bias, a subsample consisting of 18,058 sequences per sample, was selected to normalize sequence numbers, and the Chao Richness, Shannon, and Simpson indices were calculated.

2.7. Statistical analysis

For analysis of growth performance, hematological, immunological, biochemical, and metagenomic data (Chao Richness, Shannon, and Simpson indices), analysis of variance (ANOVA) was performed, followed by Tukey’s test if significant differences were found ($P < 0.05$). Analyses that did not meet the assumptions of residual normality or homogeneity were subjected to nonparametric Kruskal–Wallis analysis, followed by Dunn's test. To compare the data obtained BHC and AHC, a *t*-test was applied using the nonparametric Wilcoxon–Mann–Whitney test for data that did not meet the assumptions of normality and homogeneity. Statistical analyses were performed using R statistical software (R Core Team, 2017), with a significance level of 5%. The survival rate was analyzed using Fisher’s exact test, with a significance level of 5%, using OpenEpi v. 3.01 (https://www.openepi.com/Menu/OE_Menu.htm).

Mothur software (v.1.36.1) was used to performed the analysis of molecular variance (AMOVA) for statistical comparison of microbial community structure. A nonparametric tool (Lefse) was used to determine which OTUs were differentially represented between the treatments. A Venn diagram was generated to display microbial assemblages common to the three groups.

3. Results

3.1. Growth performance and survival rate

No significant differences were observed in growth performance (Table 1). In general there was a trend with data numerically higher in the 0.3 mg L^{-1} group particularly in weight gain values.

According to survival rate (Figure 1) no differences were observed before hypoxia (BHC) according to beta-glucan water suplementation; however, the survival rate was significantly higher in the 0.1 group seven days after hypoxia (7AHC), based on Fisher's exact test ($P < 0.05$).

Table 1. Effect of incorporation of different concentration of beta-glucan in water on growth performance parameters in tiliapia (*Oreochromis niloticus*).

	Control	0.1	0.3
Total length (cm)	8.99 ± 0.11	9.04 ± 0.12	9.14 ± 0.77
Standard length (cm)	7.21 ± 0.09	7.24 ± 0.10	7.38 ± 0.10
Final weight (g)	13.52 ± 0.52	13.88 ± 0.58	13.93 ± 0.58
Weight gain (g)	4.86 ± 0.13	5.00 ± 0.14	5.18 ± 0.09
Weight gain %	56.11 ± 1.37	56.51 ± 2.66	59.42 ± 1.79
Daily weight gain (g fish day^{-1})	0.30 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
Specific growth rate (% g day^{-1})	2.78 ± 0.06	2.79 ± 0.11	2.91 ± 0.07
Apparent feed conversion	1.27 ± 0.03	1.24 ± 0.03	1.20 ± 0.02
Condition factor (%)	1.82 ± 0.02	1.83 ± 0.02	1.79 ± 0.02

0.0 (control), 0.1 (0.1 group), and 0.3 mg L^{-1} (0.3 group). Mean ± standard error

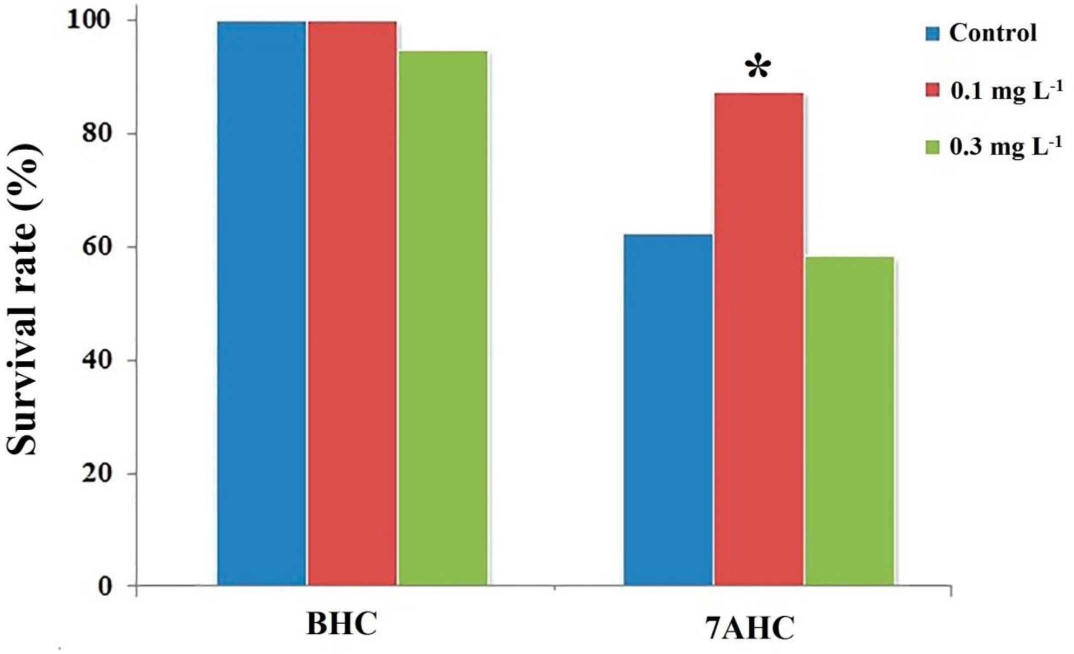

Figure 2. Effect of hypoxia on survival rate of Nile tilapia (*Oreochromis niloticus*) in water containing different concentration of β-glucan at 0.0 (control), 0.1 and 0.3 mg L^{-1} The asterisk indicates a significant difference between treatments under a same oxygen conditions according to Fisher's exact test ($P < 0.05$). BHC: before hypoxic challenge, 7AHC: seven days after hypoxic challenge.

3.2. Hematological profile, immunological and biochemical parameters

According to hematological determinations, no significant differences in RBC, VG, Hb, MCV, MCH, MCHC were displayed between the groups BHC (Table 2). After hypoxic challenge, only MCHC showed a significant increase ($P < 0.05$) in the 0.3 group when compared with the 0.1 group. When comparing the BHC with AHC time points, however, a significant increase ($P < 0.05$) in the RBC count, as well as a decrease in MCV, were observed in the control group. The MCHC values were higher in AHC compared to BHC in the 0.3 group, and the Hb values showed a tendency to increase ($P = 0.09$) AHC in the same group.

Table 2. Hematological profile of Nile tilapia (*Oreochromis niloticus*) in water containing different concentration of β-glucan, before and after hypoxic challenge.

	BHC			AHC		
	Control	0.1	0.3	Control	0.1	0.3
RBC ($\times 10^{6}$ μL^{-1})	1.32 ± 0.03 Aa	1.19 ± 0.09 Aa	1.43 ± 0.04 Aa	1.57 ± 0.03 Ab	1.38 ± 0.07 Aa	1.40 ± 0.10 Aa
Hb (g dL^{-1})	6.35 ± 0.19	6.02 ± 0.19	6.40 ± 0.16	6.95 ± 0.19	6.36 ± 0.29	7.86 ± 0.33
Ht (%)	24.10 ± 1.93	26.50 ± 1.96	29.60 ± 1.26	27.00 ± 0.91	26.89 ± 1.61	24.00 ± 1.51
MCH (g dL^{-1})	48.39 ± 1.48	52.56 ± 1.80	45.53 ± 1.75	45.63 ± 0.75	48.59 ± 3.40	62.53 ± 4.85
MCV (fL)	204.33 ± 5.37 Aa	226.06 ± 11.11 Aa	210.93 ± 8.9 Aa	172.7 ± 5.74 Ab	187.48 ± 11.23 Aa	179.28 ± 4.26 Aa
MCHC (g dL^{-1})	23.98 ± 0.88 Aa	23.59 ± 1.04 Aa	21.85 ± 0.63 Ab	27.21 ± 1.34 ABa	25.26 ± 0.63 Ba	37.86 ± 3.18 Aa

0.0 (control), 0.1 (0.1 group), and 0.3 mg L^{-1} (0.3 group). Mean ± standard error.

BHC: before hypoxic challenge; ACH: after hypoxic challenge. RBC: red blood cell; Hb: hemoglobin; Ht: hematocrit; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration.

Capital letters indicate significant differences ($P < 0.05$) between treatments in the same time point, and small letters indicate significant differences ($P < 0.05$) at different time points for the same treatment.

Analysis of differential leukocyte counts (Table 3) indicated that the lymphocyte number in the 0.3 group was significantly increased ($P < 0.05$) compared with that in the 0.1 group. No significant differences were observed in neutrophil and monocyte counts between treatments.

Table 3. Differential leukocyte counts of Nile tilapia (*Oreochromis niloticus*) exposed for 15 days to water containing different concentration of β-glucan.

Leucocyte cell counts ($\times 10^3$ μL^{-1})	Control	0.1	0.3
Leukocytes	115.28 ± 7.79 AB	75.98 ± 2.80 B	127.99 ± 8.23 A
Lymphocytes	65.95 ± 3.77 AB	44.65 ± 2.62 B	84.23 ± 4.31 A
Neutrophils	33.57 ± 2.48	22.91 ± 1.92	32.69 ± 3.89
Monocytes	15.76 ± 2.19	8.41 ± 0.81	11.07 ± 1.13

0.0 (control), 0.1 (0.1 group), and 0.3 mg L^{-1} (0.3 group). Mean ± standard error. Different letters indicate significant differences ($P < 0.05$) between treatments.

Plasma lactate, serum protein, or lysozyme concentrations showed no significant differences between treatments or between BHC and AHC period (Table 4).

Table 4. Plasma lactate, total serum protein, and serum lysozyme concentrations of Nile tilapia (*Oreochromis niloticus*) in water containing different concentration of β-glucan, before and after hypoxic challenge.

	BHC			AHC		
	Control	0.1	0.3	Control	0.1	0.3
Plasma lactate (mmol L^{-1})	2.01 ± 0.17	2.45 ± 0.12	2.53 ± 0.07	2.49 ± 0.11	2.57 ± 0.13	2.31 ± 0.09
Serum protein (g dL^{-1})	2.83 ± 0.06	2.77 ± 0.08	2.74 ± 0.08	2.89 ± 0.07	3.14 ± 0.12	2.98 ± 0.10
Serum lysozyme (µg mL^{-1})	11.76 ± 1.06	11.06 ± 0.84	11.57 ± 0.52	11.75 ± 0,85	12.76 ± 2,07	10.29 ± 1,35

0.0 (control), 0.1 (0.1 group), and 0.3 mg L^{-1} (0.3 group). Mean ± standard error.
BHC: before hypoxic challenge; ACH: after hypoxic challenge.

Serum triglyceride concentrations ranged from 373.38 (control) to 329.89 mg dL^{-1} (0.3 group), while cholesterol concentrations ranged from 123.94 (0.3 group) to 109.52 mg dL^{-1} (control) not displaying significant differences between treatments (Table 5).

Table 5. Serum triglyceride and cholesterol concentrations of Nile tilapia (*Oreochromis niloticus*) exposed for 15 days to water containing different concentration of β-glucan.

	Control	0.1	0.3
Triglycerides (mg dL^{-1})	373.38 ± 29.34	346.50 ± 31.03	329.25 ± 26.89
Cholesterol (mg dL^{-1})	109.52 ± 2.73	111.9 ± 3.34	123.94 ± 2.59

0.0 (control), 0.1 (0.1 group), and 0.3 mg L^{-1} (0.3 group). Mean ± standard error.

3.3. Plasma glucose concentration

Before hypoxic challenge, plasma glucose concentrations (Figure 3) ranged from 40.2 (0.1 group) to 55.9 mg dL^{-1} (0.3 group), and were significantly different ($P < 0.05$) between these two treatment groups (Figure 3). After hypoxic challenge, no significant differences were observed between groups, but there was a significant increase ($P < 0.05$) in all the groups after 9 hours of hypoxia (AHC) that seven days after returned to levels before hypoxia (BHC) except for the 0.3 group, which showed lower values ($P < 0.05$).

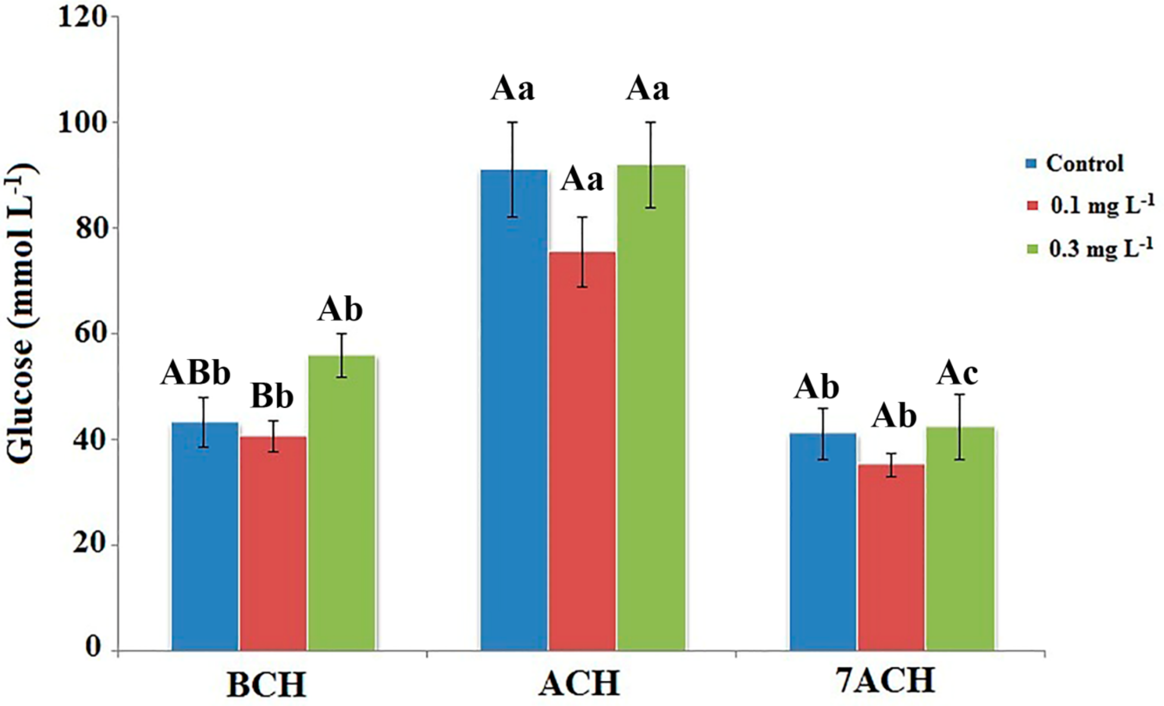

Figure 3. Plasma glucose concentrations of Nile tilapia (*Oreochromis niloticus*) in water containing β-glucan at 0.0 mg L^{-1} (control), 0.1 mg L^{-1} and 0.3 mg L^{-1}. BHC: before hypoxic challenge; ACH: after hypoxic challenge; 7AHC: seven days after hypoxic challenge. Capital letters indicate significant differences ($P < 0.05$) between treatments in the same time point, and lowercase letters indicate significant differences ($P < 0.05$) at different time points.

3.4. Gut microbiota

Processing of metagenomic data generated 1,278,841 contigs; after quality control, a total of 834,310 unique contigs were generated. The subsample coverage was higher than 99.9%, indicating good representativeness of the total microbial population. A total of 234 different genera belonging to 16 phyla were identified among all the samples.

The abundance of bacterial species calculated using Mothur software are shown in Figure 4A (phyla) and 4B (genera). Of the 16 phyla identified, Fusobacteria, Firmicutes, and Proteobacteria were the most abundant in all the treatments (Figure 4A). *Cetobacterium* and *Romboutsia* were the most abundant genera, followed by *Vibrionaceae_unclassified* (0.1 group) and *Plesiomonas* (control and 0.3 groups) (Figure 4B).

Chao richness and alpha diversity indices (Shannon and Simpson) are shown in Table 6. Although Shannon and Simpson index values were numerically higher in the β-glucan-treated groups, no significant differences were found in these indices between the three treatment groups. The largest numerical difference was observed in the Simpson index between the control group (0.49) and the 0.1 group (0.60) ($P = 0.1$). Chao richness, in turn, was significantly higher ($P < 0.05$) in the 0.1 group than in the control group.

Table 6. Chao Richness, Shannon, and Simpson diversity indices of the gut microbiota of Nile tilapia (*Oreochromis niloticus*) exposed for 15 days to different concentration in water of β-glucan.

Index	Control	0.1	0.3
Chao Richness	82.15 ± 3.24 B	127.83 ± 11.37 A	102.22 ± 11.31 AB
Shannon	0.82 ± 0.01	0.83 ± 0.05	0.90 ± 0.06
Simpson	0.49 ± 0.01	0.60 ± 0.03	0.51± 0.05

0.0(control), 0.1 (0.1 group), and 0.3 mg L^{-1} (0.3 group). Mean ± standard error. Different letters indicate significant differences ($P < 0.05$) between treatments.

AMOVA revealed that microbial community structure did not differ significantly between the groups. Lefse differential analysis showed a significant increase ($P < 0.05$) in the numbers of Vibrionaceae_unclassified in the 0.1 group (Figure 4). Finally, the Venn diagram (Figure 5) showed that the 0.1 and 0.3 groups shared the largest number of OTUs (85 OTUs), followed by the control and 0.1 (77), and control and 0.3 (72) groups. We identified 120, 141, and 147 OTUs in the control, 0.1, and 0.3 groups, respectively. A total of 60 OTUs were shared between the three treatments (Figure 5).

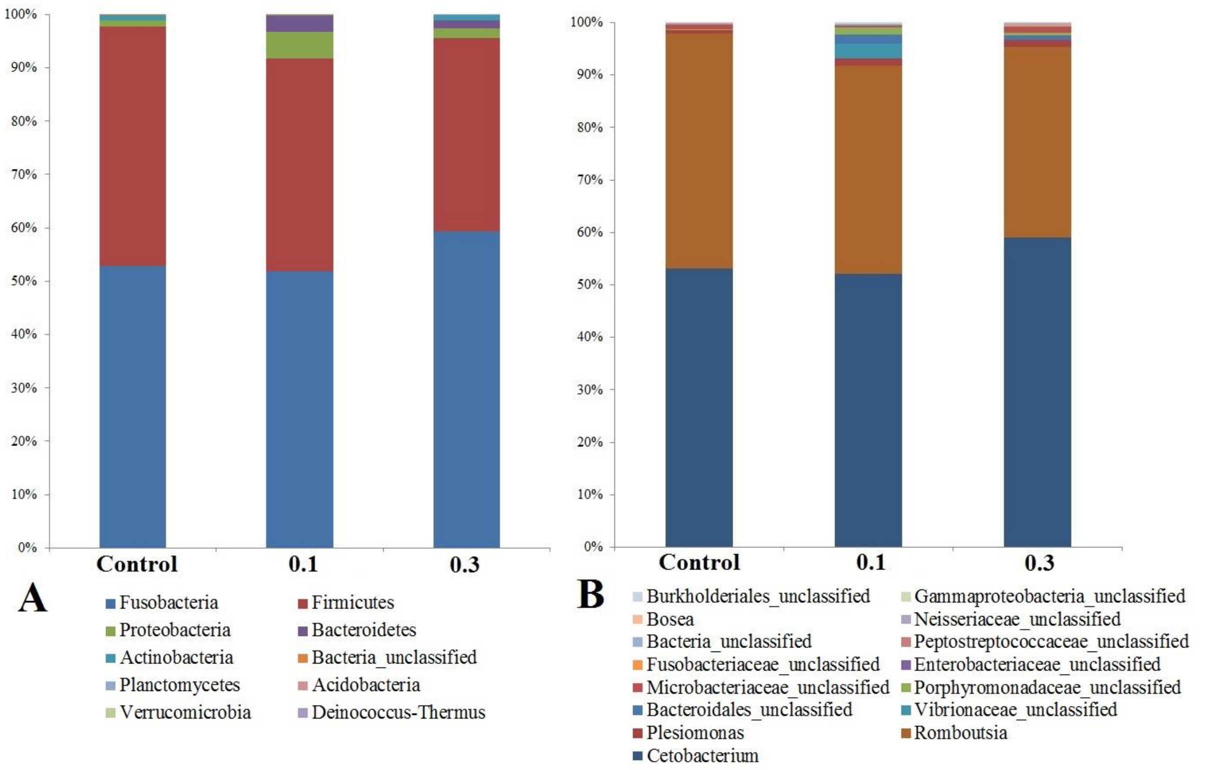

Figure 4. Mean relative abundance of different operational taxonomic units (OTUs) comprising the gut bacterial communities of Nile tilapia (*Oreochromis niloticus*) in water containing β-glucan at 0.0 (control), 0.1, and 0.3 mg L^{-1}. The 10 most abundant phyla (A) and 15 most abundant genera (B) are shown.

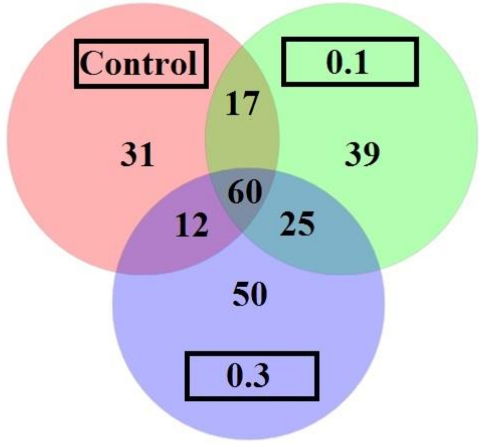

Figure 5. Venn diagram showing the intersection between the operational taxonomic units (OTUs) in the gut microbiota of Nile tilapia (*Oreochromis niloticus*) exposed for 15 days to water containing β-glucan at 0.0 (control), 0.1, and 0.3 mg L^{-}.

4. Discussion

Several studies have shown that β-glucan administration can generate differential responses in growth parameters, depending on the time and route of administration, dose, target species, life stage, and molecular characteristics of β-glucan, among other factors. By analyzing the effect of dietary inclusion of β-glucan with different purity grades (BG1, higher purity, and BG2, lower purity) in Nile tilapia Pilarski et al. (2017) found that treatment with BG2 led to a significant increase in weight gain, whereas weight gain with BG1 treatment did not differ significantly from that of the control group. Sealey et al. (2008) found a significant decrease in weight gain in rainbow trout fed with a diet containing high-β-glucan barley for three weeks, compared to that of the control diet; however, this decrease was not seen when the trout were fed with the same diet for nine weeks. Different growth results were also observed in fish administered β-glucan via water immersion. Weight gain in rainbow trout submitted to a β-glucan concentration of 0.1 mg L^{-1} in water did not differ from that of the control group. In contrast, tilapia larvae administered β-glucan (Macrogard) at a concentration of 100 mg L^{-1} presented increased weight and final length (Jesus et al., 2019). In the present study, although no significant differences were observed in growth parameters between treatments, β-glucan treatment groups presented a trend to higher growth parameter and lower feed conversion compared with the control group being this increase more accusated in 0.3 group. However, despite no significant differences were recorded the trends observed in feed conversion, condition factor, and weight gain, support that future studies with water-administered β-glucans, with a longer trial period and different β-glucan concentrations, may provide more detailed information on its effects on fish growth.

The hematological parameters values (RBC, Ht, Hb, MCH, MCV, MCHC) were similar to those reported in recent studies on tilapia (Quezada-Rodríguez and Fajer-Ávila, 2017; Suphoronski et al., 2019; Telli et al., 2014) and are, therefore, within the normal range for the species. Although no significant differences were found BHC, a significant increase in RBC values and a reduction in MCV ($P < 0.05$) were observed in the control group between the BHC and AHC sampling times. It was also observed a significant increase in MCHC in group 0.3 between the BHC and AHC periods, caused by the increase, even if not significant, in Hb between the periods. Changes in blood parameters may occur in fish challenged with acute hypoxia to maintain oxygen uptake and its transfer to tissues (Val et al., 2015). According Lai et al. (2006), an increase in RBC numbers occurs following the release of these cells by the spleen in response to increasing oxygen demand, which concomitantly increases the hemoglobin concentration. In addition, hypoxia stimulates the increase in erythropoietin in the kidneys, inducing an increase in erythropoiesis (Lai et al., 2006). In a study performed by Jimenez and Zaniboni-Filho, (2013), a significant increase in the RBC count and a decrease in MCV were found in piava fish (*Leporinus obtusidens*) submitted to severe hypoxia (1.74 mg L^{-1}) when compared with fish subjected to higher oxygen concentrations. Increased erythrocyte parameter values, such as Ht and Hb concentrations, have also been reported in fish such as cod (*Gadus morhua*) (Petersen and Gamperl, 2011), rainbow trout (Lai et al., 2006), and Curimatã (*Prochilodus nigricans*) (Val et al., 2015) under hypoxic challenge.

The dissolved oxygen concentration of water is critical for fish survival, as well as for the maintenance of their physiological functions; in turn, hypoxia is one of the most important and frequently occurring environmental variables in aquaculture (Pinedo-Gil et al., 2019; Yang et al., 2017). Responses that minimize tissue oxygen deficiency under physiological and behavioral stress are expected under hypoxic conditions (Gu and Xu, 2011; Pinedo-Gil et al., 2019; Yang et al., 2017; Zeng et al., 2016). In this study, β-glucan administration in water at 0.1 and 0.3 mg L^{-1} may have modulated the hematological response under stress, since, when comparing both groups with the control group, a decrease in MCV and an increase in RBC under hypoxia was observed only in the control group, indicating an increase in the synthesis of new red blood cells by the erythropoiesis process and/or an increase in the release of red blood cells by the spleen. Group 0.3, on the other hand, demonstrated a significant increase in MCHC not observed in the other groups, highlighting an adaptive response to stress. Due to the non-significant increase in hematological parameters between BHC and AHC, it is likely that the administration of β-glucan in water at 0.1 mg^{L-1} has softened the physiological responses in response to hypoxia, and in parallel, resulted in lower mortality after the challenge. In a study by Zeng et al. (2016), the mortality rate was decreased in a group of yellow croakers injected intraperitoneally with β-glucan and subjected to hypoxia, similar to that recorded in the present study. In a different study, dietary administration of β-glucan was also effective against salinity stress in red sea bream, resulting in reduced mortality in animals treated with the ingredient (Dawood et al., 2015). These results indicate that β-glucan administration to fish results in greater tolerance to stressful conditions that are not always related to pathogens.

The leukocyte count was significantly higher ($P < 0.05$) in the 0.3 group than in the 0.1 group. Increased white blood cell counts, especially characterized by lymphocytosis, have previously been reported in other studies on fish following β-glucan administration (Huu et al., 2016; Liranço et al., 2013). Lymphocytes, particularly B, play an important role in antibody-dependent immune responses by enhancing specific immunity response following infection (Huu et al., 2016; Ranzani-Paiva et al., 2013; Sunyer, 2014). Bacterial challenges could be further mitigated due to the protection afforded by the hematoimmunological properties of this ingredient when supplied in water, as has already been demonstrated with dietary administration. Other innate immunity-related parameters, such as lysozyme and total serum protein concentrations, were not affected by β-glucan concentration. However, it was observed an upward trend in total serum proteins, even if not significant, in groups 0.1 and 0.3 under hypoxia. It has already been found that β-glucan can modulate the production of immunoglobulins, increasing their concentrations in fish challenged with bacterial pathogens (Munir et al., 2018), however, further studies are needed to determine whether this prebiotic can modulate the concentration of total proteins, especially immunoglobulins, under other stressful conditions such as hypoxia.

Increased plasma lactate and glucose levels resulting from catecholamine-mediated glycogenolysis are commonly associated with stress in fish (Pankhurst, 2011). Factors such as hypoxia (Chagas and Val, 2006; Montoya et al., 2017; Pinedo-Gil et al., 2019) and bacterial infections (Suphoronski et al., 2019), for example, have already been shown to induce changes in plasma glucose levels as fish attempt to maintain tissue energy supply in response to stressors (Chagas and Val, 2006; Pérez-Jiménez et al., 2012; Pinedo-Gil et al., 2019). In the present study, this was evidenced by the significant increase observed in plasma glucose concentrations AHC ($P < 0.05$) with all the treatments (Figure 2), which was accompanied by a significant decrease when oxygen supply was resumed (7AHC).

Glucose levels BHC did not differ significantly between the treatment and control groups; nevertheless, glucose levels were significantly higher in the 0.3 group ($P < 0.05$) than in the 0.1 group. Recent studies have shown that β-glucan administration can generate different responses in plasma glucose concentrations. Mirror carp fed diets supplemented with 0.1, 1, and 2% Macrogard for eight weeks showed no significant differences in plasma glucose concentrations (Kuhlwein et al., 2014), and the same was observed in matrinxans (*Brycon amazonicus*) fed 0.1% of the additive for 15 days (Montoya et al., 2017). Pilarski et al. (2017) analyzed the effects of dietary administration of two β-glucan molecules (BG1 and BG2) on plasma glucose levels in tilapia, and found that administration of BG2, the less concentrated molecule, resulted in lower plasma glucose levels compared to those in both the control and BG1 groups. The authors suggested that provision of the BG2 species may have contributed to greater energy conservation through lower immunomodulation than BG1 (the more concentrated molecule). In the present study, the highest concentration of β-glucan administered (group 0.3) caused a greater immunostimulation in fish as evidenced by the leukocyte response, in relation to group 0.1. As demonstrated by Pilarski et al. (2017), administration of β-glucan at the concentration of 0.1 mg L^{-1} likely led to reduced energy expenditure and contributed to a greater tolerance to stressors. Additionally, the 0.3 group showed a significant decrease in plasma glucose levels ($P < 0.05$) at 7AHC compared to before BHC. This demonstrates that this concentration likely overstimulated metabolic responses BHC, which required greater energy expenditure; however, this would have been reversed when the β-glucan supply was interrupted. Nonetheless, the modulatory and protective mechanisms associated with β-glucan remain unclear, and further studies are needed to elucidate in detail the metabolic regulation mediated by this ingredient under stressful conditions.

Unlike the values obtained for glucose, plasma lactate concentration did not differ between treatments and between the BHC and AHC time points. However, as shown by Pinedo-Gil et al. (2019), plasma lactate levels in rainbow trout under acute hypoxic stress increased approximately threefold within the first 30 min of stress exposure, but then returned to baseline levels after 6 h. Consequently, sampling at the AHC time point may not have detected any earlier increase in lactate levels. Sampling at different time points may clarify whether β-glucan has a modulatory effect on plasma lactate levels.

According to Bell et al. (1999), lipid metabolism can be influenced by β-glucan intake. One of the mechanisms involved is related to the fermentation of this polysaccharide by intestinal bacteria, resulting in the production of short-chain fatty acids, which are captured by the portal vein and sent to the liver, decreasing the production of lipids. Other mechanisms are related to the binding of β-glucan to bile acids in the intestine, delayed gastric emptying and interference with fat absorption by increased intestinal viscosity (Bell et al., 1999). However, although there is a numerical and gradual decrease in serum triglycerides in groups 0.1 and 0.3 compared to the control group, no significant difference was observed between treatments. Other studies with fish that received dietary β-glucan also did not find differences in the concentrations of triglycerides and blood cholesterol (Dawood et al., 2020, 2015).

The intestinal microbiota of fish is composed of microorganisms that live in association with their hosts, and include commensal, symbiotic, or pathogenic communities; they play important physiological functions in the overall health and immunity of the host that, in turn, provides a nutrient-rich environment for development and maintenance of these microorganisms (Butt and Volkoff, 2019; Eichmiller et al., 2016; Tarnecki et al., 2017). In the present work, the greater than 99.9% coverage of the samples indicates that the microbial communities were highly represented in the metagenomic analysis, thereby strengthening the reliability of the results.

Overall, the core microbiota identified in the present study corroborates that previously found in the Nile tilapia gut. Phyla such as Firmicutes, Fusobacteria, and Proteobacteria are widely represented in Nile tilapia (Fan et al., 2017; Kohl et al., 2014; Souza et al., 2020; Suphoronski et al., 2019; Zhang et al., 2016), as are members of the genus *Cetobacterium* (Fusobacteria) (Suphoronski et al., 2019; Zhang et al., 2016). Another genus abundantly identified in the three treatments, *Rombutsia* (Firmicutes), comprises species that can metabolize different carbohydrate sources through different metabolic pathways (Gerritsen et al., 2017; Wang et al., 2015), and has also been identified as one of the most abundant genera in tilapia (Souza et al., 2020). In the present study, we could not classify the members of the family Vibrionaceae (Proteobacteria), the abundance of which was significantly greater in the 0.1 than control group ($P < 0.05$). Members of this family comprise genera such as *Vibrio*, *Photobacterium*, *Salinivibrio*, *Enterovibrio*, *Grimontiae* e *Aliivibrio* (Gomez-Gil. et al., 2014), which may exhibit pathogenic, symbiotic, or probiotic characteristics, depending on the genus and/or strain of each species (Gomez-Gil. et al., 2014; Gomez-Gil et al., 2002; Silva et al., 2011). Bacteria of this family are commonly found in marine fish such as sea bream, sea bass, and flatfish (Grisez et al., 1997; Tapia-Paniagua et al., 2010), and some strains are considered pathogenic, whereas others are beneficial in preventing gut pathogen colonization (Gomez-Gil. et al., 2014; Grisez et al., 1997). Some genera have also been reported in freshwater species, including *Photobacterium* sp. (Zhang et al., 2016) and *Vibrio* spp., in both Nile tilapia (Pakingking-Jr et al., 2015) and Anjak fish (*Schizocypris altidorsalis*) (Ghanbari et al., 2019). The order Vibrionales was found to be abundantly present in the fecal microbiota of bighead carp (*Hypophthalmichthys nobilis*) (Eichmiller et al., 2016), while the Vibrionaceae family was abundantly identified in goldfish (*Carassius auratus*) (Silva et al., 2011). However, the failure to classify the genera of this family in the present study makes it difficult to infer the effects of β-glucan on this bacterial community; nevertheless, the increase in Vibrionaceae family members observed in this study was not likely to have been related to a greater abundance of pathogenic species, considering the other beneficial effects afforded by the inclusion of 0.1 mg L^{-1} β-glucan in water. This is corroborated by other studies that found that administration of this ingredient decreased mortality rates in olive flounder (*Paralichthys olivaceus*) infected with *V. harveyi* (Lee et al., 2018) and Taiwan abalone (*Haliotis diversicolor supertexta*) infected with a pathogenic strain of *V. alginolyticus* (Wu et al., 2016). Using qPCR, (Carballo et al., 2019) demonstrated that Vibrio DNA copy number was decreased in Senegalese sole (*Solea senegalensis*) administered β-glucan by oral intubation. This highlights that further studies are needed to clarify how β-glucan exerts its modulatory effects on members of the Vibrionaceae family in Nile tilapia, especially at the species level, given the complexity and multifunctionality of members of this family.

Although the results of microbial diversity have not shown significant differences, it is worth highlighting an upward trend in the Simpson index ($P = 0.1$) in the 0.1 group in relation to the control group, followed by a greater Chao richness ($P <0.05$), and a greater sharing of taxa between groups 0.1 and 03 according to the Venn diagram. These results, in general, corroborate with other studies of fish that received β-glucan. The intestinal microbiota of carp fed diets supplemented with 10 g kg^{-1} β-glucan (Macrogard) for two weeks showed significant increases in Shannon–Wiener and Simpson index values, as well as a greater number of OTUs, compared to those of the microbiota of fish not fed the additive (Jung-Schroers et al., 2016). It is known that β-glucan, as a prebiotic, can stimulate the growth of certain bacteria because it is a “selectively fermentable” ingredient, allowing specific changes in the microbiota (Hamaker and Tuncil, 2014). In an experiment conducted by Miest et al. (2016), turbot larvae fed Macrogard-enriched rotifers presented an increase in microbiota richness 24 days after hatching. According to the authors, β-glucan contributed to faster maturation of the larval microbiota compared to that in fish fed rotifers without β-glucan. Moreover, Kuhlwein et al. (2013) identified a decrease in species richness in the intestinal microbiota of mirror carp fed a diet supplemented with 1% Macrogard for two weeks; however, by the fourth week of supplementation, there was no significant difference in microbial richness compared to the control group, concomitant with increased intestinal villi length and density in the fish fed diets supplemented with 1% Macrogard.

In the present study, the supply of β-glucan administration in water affected the composition and richness of the Nile tilapia intestinal microbiota. In this sense, the Chao richness value was increased in the 0.1 group and the composition of the microbial community was altered, as evidenced by the increase in Vibrionaceae family members in the same group. Although it is difficult to draw any firm conclusions as members of this family could not be identified to the species level, it is possible that an increase in richness and changes in microflora community composition may lead to the competitive exclusion of pathogenic bacteria through competition for nutrients or occupation of bacterial-binding receptors in the gut, as reported by (Jung-Schroers et al., 2016). Importantly, no studies to date have evaluated the effect of β-glucan on the intestinal microbiota of Nile tilapia. Therefore, our results may serve as a basis for future research on the modulatory effect of this ingredient on tilapia or other species. Technologies such as transcriptomics may provide information on the potential effects of β-glucan on bacterial gene expression, thereby helping to understand the functional alterations occurring in the intestinal microbiome of fish exposed to β-glucans.

5.1 Conclusion

In conclusion, our study indicated that β-glucan administered in water at a concentration of 0.1 mg L^{-1} minimized the effect of hypoxia-induced stress in Nile tilapia, as evidenced by the lower mortality, and modulating plasma glucose concentrations. Administration of β-glucan at this concentration also increased the Chao richness and changed the composition of the Nile tilapia intestinal bacterial community compared to that in the control group. By other hand, the 0.3 mg L^{-1} concentration presented a greater modulatory effect on hematological response and lymphocytes count compared to that of β-glucan administration at 0.1 mg L^{-1}. Biochemical parameters evaluated (plasma lactate, triglyceride, and cholesterol concentrations), as well as several immunological parameters (serum total protein and serum lysozyme concentrations), were not significantly affected by β-glucan administration in Nile tilapia water.

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8 CONSIDERAÇÕES FINAIS

Levando em consideração a crescente demanda de produtos naturais, economicamente viáveis, e não nocivos à natureza, os estudos realizados apresentaram resultados promissores para utilização da microalga *Schizochytrium* sp. e do β-glucana em cultivos de tilápias-do-Nilo.

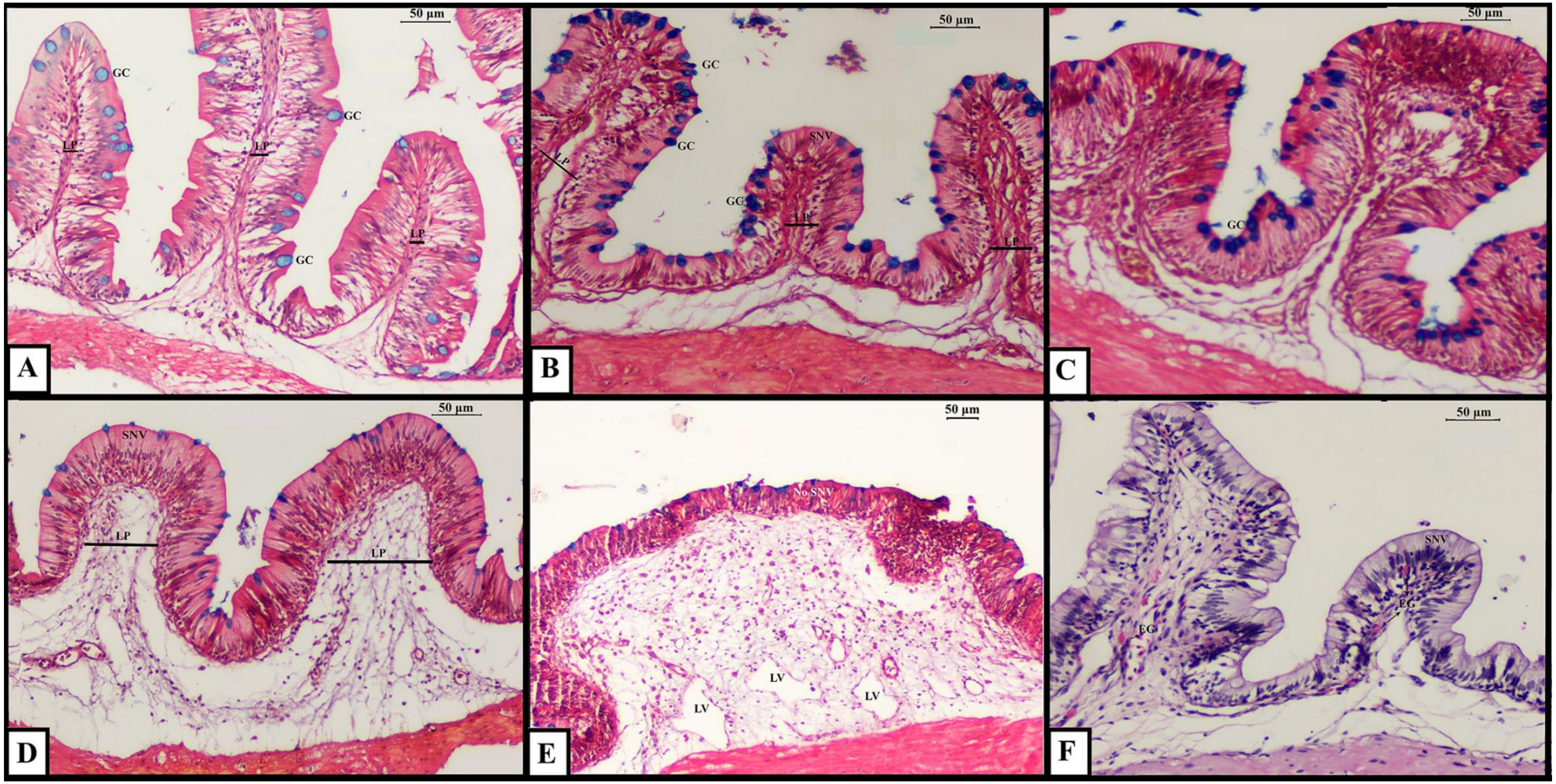
Em relação à inclusão da microalga, outras concentrações na dieta e diferentes tempos de administração podem ser avaliados em novos estudos, bem como em outras fases de cultivo de tilápias-do-Nilo. Respostas metabólicas frente a desafios com estresse (ambientais ou bacterianos, por exemplo) poderiam esclarecer se essa microalga possui alguma característica protetora frente a fatores estressantes. Para isso, outros indicadores de estresse, como cortisol e glicose, devem ser mensurados para averiguar tal característica. Além disso, pesquisas em espécies nativas com grande potencial produtivo, como tambaqui e pacu, também podem trazer resultados interessantes sobre os efeitos da inclusão dessa microalga na dieta.

Assim como no estudo da *Schizochytrium* sp, diferentes níveis de inclusão de β-glucana na água podem ser avaliados, como concentrações intermediárias às avaliadas pelo presente estudo (1,5 mg L^{-1} e 2,0 mg L^{-1}, por exemplo). Ademais, um maior período de avaliação, como 30 ou 42 dias, poderia trazer resultados diferentes quanto ao desempenho zootécnico dos animais. Também, propõe-se estudar outros tipos respostas frente a diferentes fatores estressantes, como salinidade e desafios bacterianos, incluindo novos métodos de análises de estresse, como mensuração de enzimas responsáveis pela proteção antioxidante e expressão de imunoglobinas, as quais já demonstraram ser afetadas pela inclusão desse aditivo via dietética.

ANEXOS

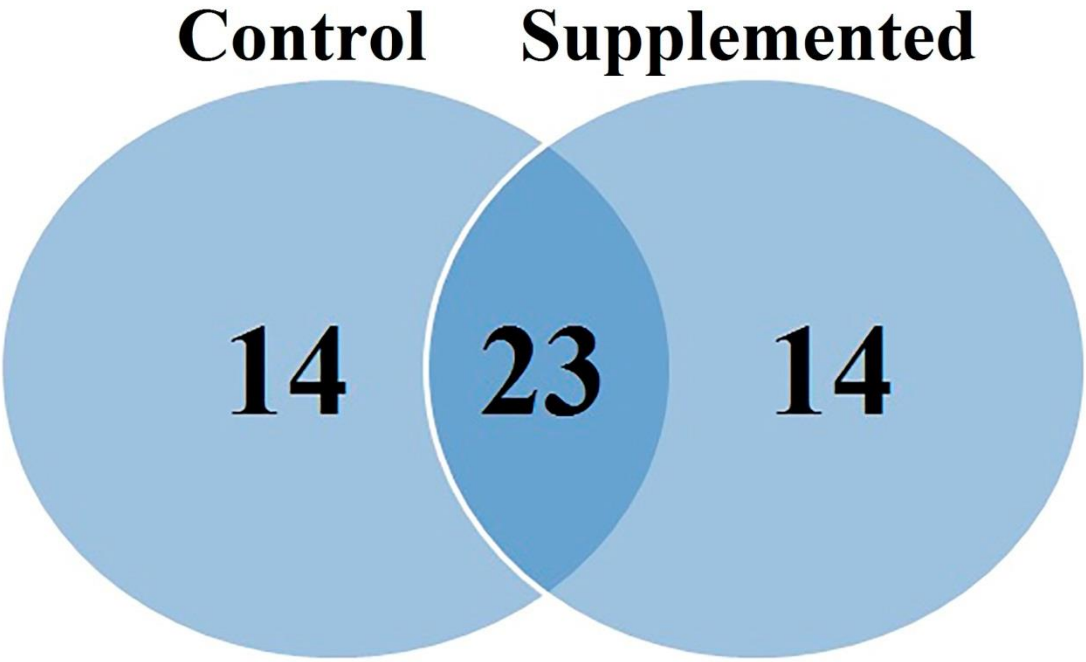
ANEXO A

S1 Fig. Intestinal morphology of Nile tilapia (*Oreochromis niloticus*). GC, goblet cells; LP; lamina propria; EG, eosinophilic granulocytes; SNV, supranuclear vacuoles; LV, lymphatic vessel. (A) Epithelium and whole villi, with no signs of flattening; fine and complete LP; and GC in small amounts. (B) Increase in CG; LP with slight thickening; diffuse reduction of SNV. (C) GC increased. (D) Shrinkage of villi; LP with increased size; SNV present and aligned. (E) 'Crumbling' (disruption) of villi; LV present; absence of SNV. (F) Increased presence of EG in LP; SNV present and aligned. (A, B, C, D, E: Alcian blue staining; F: H&E staining). Scale bar = 50 µm.



ANEXO B

S2 Fig. Venn diagram showing the intersection between the bacterial diversity in the gut microbiota of the control (CON) and supplemented (SUP) diet groups.



ANEXO C

S1 Table. Relative abundances (at order and genus level) of the 15 most abundant gut bacteria of Nile tilapia fed with a diet containing 1.2% *Schizochytrium* sp. meal (SUP) or a control diet (CON).

Order			Genus			
	CON (%)	SUP (%)		CON (%)	SUP (%)	Total of reads
Clostridiales	44.35	71.87	Romboutsia	44.28	71.76	128238
Fusobacteriales	52.6	17.86	Cetobacterium	51.83	14.1	117108
Rhizobiales	0.92	3.64	Fusobacteriaceae_unclassified	0.69	3.76	3254
Pseudomonadales	0.25	3.54	Pseudomonas	0.25	3.51	2195
Erysipelotrichales	0.55	0	Methylocystis	0.3	2.31	1731
Enterobacteriales	0.27	0.13	Turicibacter	0.55	0	1170
Caldilineales	0.28	0	Rhizobiales_unclassified	0.24	0.59	789
Burkholderiales	0.04	1.05	Caldilineaceae_unclassified	0.28	0	599
Bacteroidales	0.22	0	Escherichia/Shigella	0.23	0.09	541
Actinomycetales	0.17	0.17	Prevotella	0.22	0	465
Gamma	0.11	0.36	Gamma	0.11	0.36	396

proteobacteria_unclassified			proteobacteria_unclassified			
Bacteria_unclassified	0.04	0.46	Hyphomicrobium	0.15	0	320
Legionellales	0.08	0.28	Hyphomicrobiaceae_unclassified	0.1	0.19	311
Lactobacillales	0	0.33	Bacteria_unclassified	0.04	0.46	304
Rhodospirillales	0.06	0.04	Ralstonia	0.04	0.48	304