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Suplementação alimentar com *Synechococcus elongatus* PCC 7942 em zebrafish (*Danio rerio*): impacto no desempenho zootécnico, transcriptoma cerebral, fisiologia hepato-intestinal e microbioma do trato digestório

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Tese apresentada ao Programa de Pós-graduação em Aquicultura, como parte dos requisitos para obtenção do Título de Doutor.

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Por

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LISTA DE ACRÔNIMOS, ABREVIAÇÕES E SÍMBOLOS

ASVs: amplicon sequence variants

ATP: Adenosine triphosphate

cat: catalase

cDNA: complementary DNA

CEUA: Ethics Committee on Animal Use of the Federal University of Rio Grande

CF: control group fed with commercial feed

CF+C: experimental group fed with commercial feed supplemented with Synechococcus elongatus PCC 7942

CONCEA: National Council for the Control of Animal Experimentation

CPM: counts per million

cyp19a1a: cytochrome P450 family 19 subfamily A

cyp1a: cytochrome P450 family 1

DNA: Deoxyribonucleic Acid

EF: Experimental Feed

FAO: Food and Agriculture Organization of the United Nations

FDA: Food and Drug Administration

FURG: Federal University of Rio Grande

gapdh: Glyceraldehyde-3-Phosphate Dehydrogenase

GCL: glutamate-cysteine ligase

gpx: glutathione peroxidase 1a

GPX: Glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

GSSG: oxidized glutathione

gst1a: glutathione S-transferase tandem duplicated alpha 1

gstπ: glutathione S-transferase pi

H_2O_2: hydrogen peroxide

ICB: Institute of Biological Sciences

K: condition factor

ldhbb: Lactate dehydrogenase Bb

micu3a: Mitochondrial Calcium Uptake Family Member 3a

myhc4: Myosin Heavy Chain 4

myhz2: Myosin Heavy Chain 2 Fast Muscle Specific

mylpfa: Myosin Light Chain a

myripa: Myosin VIIA and Rab Interacting Protein a

NGS: Next-Generation Sequencing

NRC: National Research Council

OD: optical density

OD_{750}: optical density at λ = 750 nm

pANL: endogen plasmid of *Synechococcus elongatus PCC7942*

pANS: *endogen plasmid of Synechococcus elongatus PCC7942*

PBS: Phosphate-buffered saline

PCC: Pasteur Culture Collection

PCoA: Principal Coordinate analysis

PCR: Polymerase chain reaction

pecam1a: Platelet and Endothelial Cell Adhesion Molecule 1a

PERMANOVA: Permutational multivariate analysis of variance

PHB: poly-3-hydroxybutyrate

pvalb1: Parvalbumin 1

pvalb2: Parvalbumin 2

pvalb4: Parvalbumin 4

qPCR: quantitative PCR

RNA: Ribonucleic acid, Ribonucleic Acid

ROS: reactive oxygen species

rpl13a: ribosomal protein L13 alpha

rRNA: ribosomal RNA

S: survival

sesn1: Sestrin 1

SGR: specific growth rate

SOD: superoxide dismutase

sod1: soluble superoxide dismutase 1

sod2: mitochondrial superoxide dismutase 2

tnni2b.2: Troponin I Type 2b

tnnt3b: Troponin T Type 3b

tsc1a: TSC Complex Subunit 1a

USA: United States of America

WG: weight gain

WHO: World Health Organization

SUMÁRIO

RESUMO

A busca por ingredientes alternativos e sustentáveis para a aquicultura tem levado ao uso de microrganismos fotossintetizantes, como as cianobactérias, devido ao seu alto valor nutricional e potencial biotecnológico. *Synechococcus elongatus* PCC 7942 destaca-se por sua rápida taxa de crescimento, capacidade de produzir compostos bioativos e facilidade de manipulação genética. No entanto, seus efeitos como suplemento alimentar para peixes ainda não foram amplamente investigados. Neste contexto, esta Tese avaliou os impactos da suplementação alimentar com *S. elongatus* PCC 7942 em zebrafish (*Danio rerio*), com foco no desempenho zootécnico, metabolismo cerebral, saúde hepato-intestinal e microbioma do trato digestório. Para isso, 120 zebrafish foram divididos em dois grupos: um controle, alimentado com ração comercial, e outro experimental, alimentado com a mesma ração suplementada com *S. elongatus* PCC 7942 durante 35 dias. Foram avaliados parâmetros zootécnicos, expressão gênica no cérebro e no fígado, histologia hepática, morfometria intestinal e composição do microbioma intestinal por sequenciamento de terceira geração. Os resultados indicaram que os peixes suplementados apresentaram um aumento significativo no consumo alimentar e no fator de condição, sugerindo uma possível melhoria na palatabilidade da ração. A análise transcriptômica revelou alterações na expressão gênica cerebral, com indução de genes envolvidos no metabolismo energético e resposta antioxidante, enquanto genes associados à neuroplasticidade foram reduzidos, sugerindo um ambiente mais estável para o metabolismo neural. No fígado, foi observada uma menor incidência de danos hepáticos nos peixes suplementados, indicando um possível efeito hepatoprotetor da cianobactéria. Além disso, a expressão reduzida de genes relacionados ao metabolismo de xenobióticos e enzimas antioxidantes sugere que a suplementação não induziu estresse oxidativo significativo. A análise

morfológica do intestino revelou que a suplementação não causou alterações estruturais relevantes, demonstrando boa tolerância ao suplemento. A composição do microbioma intestinal indicou estabilidade na diversidade microbiana geral, mas com mudanças específicas na abundância de certos gêneros. Foi observada uma redução do gênero *Pirellula* e a ausência de micoplasmas nos peixes suplementados, o que sugere um possível efeito benéfico sobre a microbiota intestinal, reduzindo a presença de potenciais patógenos oportunistas. Os achados desta pesquisa demonstram que *S. elongatus* PCC 7942 é uma fonte segura de suplementação alimentar para peixes e pode atuar como um modulador metabólico e microbiológico, sem causar efeitos adversos sobre a fisiologia hepato-intestinal. Seu potencial como veículo de compostos bioativos pode abrir novas perspectivas para aplicações na nutrição e saúde de peixes cultivados. Estudos futuros devem aprofundar a avaliação de sua digestibilidade, impacto no sistema imunológico e aplicações na engenharia genética para aprimoramento de seus efeitos probióticos e nutracêuticos na aquicultura.

Palavras-chave: cianobactéria; nutrição de peixes; modulação gênica; microbiota intestinal; saúde hepato-intestinal.

ABSTRACT

The search for alternative and sustainable ingredients in aquaculture has led to the use of photosynthetic microorganisms, such as cyanobacteria, due to their high nutritional value and biotechnological potential. *Synechococcus elongatus* PCC 7942 stands out for its rapid growth rate, ability to produce bioactive compounds, and ease of genetic manipulation. However, its effects as a dietary supplement for fish remain largely unexplored. In this context, this Thesis evaluated the impact of *S. elongatus* PCC 7942 supplementation in zebrafish (*Danio rerio*), focusing on zootechnical performance, brain metabolism, hepato-intestinal health, and gut microbiota composition. For this, 120 zebrafish were divided into two groups: a control group, fed with a commercial diet, and an experimental group, fed with the same diet supplemented with *S. elongatus* PCC 7942 for 35 days. Zootechnical parameters, gene expression in the brain and liver, liver histology, intestinal morphometry, and gut microbiota composition (using third-generation sequencing) were analyzed. The results showed a significant increase in feed intake and condition factor in the supplemented fish, suggesting improved palatability of the diet. Transcriptomic analysis revealed alterations in brain gene expression, with upregulation of genes involved in energy metabolism and antioxidant response, while genes associated with neuroplasticity were downregulated, suggesting a more stable neural metabolic environment. In the liver, a lower incidence of hepatic damage was observed in supplemented fish, indicating a potential hepatoprotective effect of the cyanobacterium. Furthermore, the reduced expression of genes related to xenobiotic metabolism and antioxidant enzymes suggests that supplementation did not induce significant oxidative stress. Morphological analysis of the intestine showed no major structural changes, demonstrating good tolerance to the supplement. Gut microbiota composition remained stable in terms of overall diversity, but specific shifts in bacterial

abundance were detected. A reduction in *Pirellula* abundance and the absence of mycoplasmas in the supplemented fish suggest a beneficial effect on the gut microbiome, potentially limiting the presence of opportunistic pathogenic microorganisms. The findings of this study demonstrate that *S. elongatus* PCC 7942 is a safe dietary supplement for fish and may act as a metabolic and microbiological modulator without causing adverse effects on hepato-intestinal physiology. Its potential as a delivery vehicle for bioactive compounds opens new perspectives for applications in fish nutrition and health. Future studies should further investigate its digestibility, effects on the immune system, and genetic engineering strategies to enhance its probiotic and nutraceutical benefits in aquaculture.

Keywords: cyanobacterium; fish nutrition; gene modulation; gut microbiota; hepato-intestinal health.

INTRODUÇÃO GERAL

O crescimento da população mundial requer maior disponibilidade de alimentos, e a aquicultura contribui cada vez mais para suprir essa demanda. Em 1961, o consumo anual *per capita* de alimentos de origem aquática era de 9.1 kg, atingindo 20.6 kg em 2021 (FAO, 2024). No entanto, os principais insumos utilizados nas rações aquícolas, como a farinha e óleo de peixe, apresentam limitações porque sua disponibilidade e qualidade estão em declínio (FAO, 2024). Para resolver isso, a pesquisa em nutrição aquícola e biotecnologia tem buscado ingredientes inovadores que possam substituir ou fazer sinergia com esses insumos como sementes oleaginosas, grãos, hidrolisados, subprodutos e microrganismos como bactérias, leveduras, fungos e algas.

As cianobactérias, também conhecidas como algas verde-azuis, são bactérias Gram-negativas que possuem ficobilissomos, carboxissomos e enzimas RuBisCO, que lhes permitem fixar CO_2 e realizar fotossíntese. Algumas também são capazes de fixar nitrogênio atmosférico, uma função particularmente importante em ambientes com baixa disponibilidade de nutrientes (Bustos-Díaz et al., 2019). As cianobactérias, foram responsáveis pela criação da atmosfera oxidante há milhões de anos e possuem distribuição cosmopolita devido à elevada plasticidade fenotípica diante das mudanças ambientais (Amin et al., 2024; Huang et al., 2021). Ecologicamente, são essenciais ao sustentar cadeias alimentares por meio da fotossíntese, promovendo a estabilidade ecológica que fortalece a resiliência dos ecossistemas (Singh et al, 2016). No entanto, sua proliferação excessiva representa uma preocupação global devido à produção de dermatotoxinas, neurotoxinas e hepatotoxinas, que podem exercer efeitos danosos sobre microrganismos e organismos superiores que entram em contato com elas e serem

transferidas ao longo das cadeias tróficas por meio da bioacumulação (Chorus & Welker, 2021).

Por outro lado, nem todas as cianobactérias produzem toxinas; algumas espécies sintetizam polímeros, polissacarídeos e metabólitos secundários com aplicações na indústria de biocombustíveis, farmacêutica, cosmética e de alimentos (Agarwal et al., 2022; Bouyahya et al., 2024; Castro et al., 2023). O uso de cianobactérias como alimento de organismos aquáticos já demonstrou efeitos benéficos sobre a digestão e absorção de nutrientes, o metabolismo lipídico, o sistema de defensa antioxidante e a imunidade inata em peixes (Coli et al., 2024; El-Salam et al., 2024; Faheem et al., 2022). O estudo realizado por Liang et al. (2015), suplementando a ração de peixes dourados (*Carassius auratus*) com *Microcystis aeruginosa*, demostrou que a baixa inclusão, 10-20 % na ração, promove o crescimento, enquanto doses altas (30 e 40 %,) o inibe e o peixe acumula microcistinas, gerando um risco para saúde do consumidor. No camarão *Penaeus vannamei*, a adição de 3 g/kg de *Haematococcus pluvialis* na ração aumentou significativamente o rendimento do camarão em escala piloto, melhorou o balanço de aminoácidos essenciais no músculo, aumentou a capacidade antioxidante no hepatopâncreas e favoreceu a presença de proteobactérias com potencial probiótico na microbiota intestinal (Huang et al., 2023). El-Salam et al. (2024) observaram que a ração suplementada com *Arthrospira platensis* (5 g/kg) evitou os efeitos deletérios do pesticida diazonin sobre o crescimento de tilápia melhorando, também, a capacidade antioxidante e diminuindo os níveis de colesterol e triglicerídeos.

Na indústria alimentar, as spirulinas do gênero *Arthrospira* se destacam pelo alto valor nutricional com 60 % de proteína e 12 % de lipídeos (Ahmad et al., 2023). É amplamente cultivado e comercializado devido à sua segurança para consumo humano e animal (FAO, 2024; Ahmad et al., 2023). Esse reconhecimento abre caminho para a

exploração de outras cianobactérias na formulação de rações aquícolas e na nutrição animal em geral.

Do ponto de vista biotecnológico, algumas cepas de *Synechococcus* demonstram robustez e adaptabilidade que as tornam ideais para várias aplicações industriais. A cepa UTEX 2973, uma variante de crescimento rápido de *Synechococcus elongatus*, tem aplicações na bioprodução de biocombustíveis e ácidos graxos ômega-3 (Sengupta et al., 2024; Wendt et al., 2022). A cepa PCC 7942 de água doce também é de rápido crescimento e naturalmente possui elevada competência para a integração de DNA exógeno, favorecendo os processos na engenharia genética. Inicialmente, esta cepa foi isolada na California State University (USA) e identificada como *Anacystis nidulans* R2, tendo sido depositada na Pasteur Culture Collection (PCC) no ano 1979 sob o registro 42, dando origem ao sufixo PCC 7942. Em 2001, a introdução do clado *Synechococcus* produziu uma realocação taxonômica e seu nome mudou para *Synechococcus elongatus* PCC 7942 (Golden, 2019). Esta cepa tem sido geneticamente manipulada para a produção aprimorada de bioplásticos (PHB), ácidos graxos poli-insaturados (ômega-3) e zeaxantina, um carotenoide antioxidante (Santos-Merino et al., 2018; Sarnaik et al., 2018; Takahashi et al., 1998). Assim, *S. elongatus* PCC 7942 surge não apenas como microrganismo biofábrica, mas também como um veículo para a entrega direta de compostos com alto perfil nutricional por meio da ingestão por organismos aquáticos como moluscos, crustáceos e peixes. Embora o potencial biotecnológico de *S. elongatus* PCC 7942 seja promissor, esta cianobactéria ainda não foi avaliada como suplemento alimentar em peixes e se desconhece seus efeitos sobre o metabolismo e sua segurança para consumo.

Zebrafish como modelo biológico

Nesta tese foi utilizado o peixe zebrafish (*Danio rerio*) para avaliar os efeitos do consumo de *S. elongatus*. O zebrafish é um peixe tropical da Ordem Cypriniformes, com proximidade filogenética a carpas e tilápias, validando seu uso como modelo translacional para aquicultura. Na fase adulta atinge, em média, 3 cm e a maturidade sexual a partir dos 3 meses de idade, produzindo novas gerações rapidamente (Wixon, 2000). O rápido desenvolvimento e o tamanho do peixe favorecem seu uso no laboratório, pois permite a obtenção de resultados rapidamente. Conseguindo responder as perguntas relacionadas aos efeitos da suplementação da cianobactéria na expressão de genes em tecidos específicos como o cérebro, ou como este microrganismo fotossintetizante utilizado como suplemento alimentar pode afetar o microbioma intestinal do hospedeiro. Além de utilizar o zebrafish como modelo translacional, esta Tese também utiliza técnicas modernas de sequenciamento de DNA.

Do DNA à piscicultura

Todo organismo possui a informação completa sobre sua composição, codificada em sequências de nucleotídeos que formam unidades funcionais denominadas genes. O conjunto total desses genes constitui o genoma cuja estrutura química é o DNA. Essa informação está presente em todas as células e é única para cada organismo, permanece inalterada ao longo da vida e é transmitida de geração em geração. O estudo da informação genética é realizado pelas ciências ômicas, que investigam o funcionamento e a organização dos seres vivos em nível molecular. O sufixo ômica refere-se à totalidade de algo. Por exemplo, a genômica estuda a totalidade dos genes, a transcriptômica a totalidade dos transcritos (RNAs mensageiros) e a metagenômica a totalidade dos genomas (geralmente microbianos).

Desde a descrição da estrutura do DNA por Watson e Crick em 1953, a decodificação das sequências de nucleotídeos tornou-se essencial para a compreensão dos mecanismos moleculares da vida. As técnicas de sequenciamento permitem identificar a ordem precisa dos nucleotídeos, analisar a expressão e função biológica dos genes, construir mapas genéticos e comparar sequências que ajudam a estabelecer relações funcionais e evolutivas. O método de Sanger, desenvolvido por Frederick Sanger e colaboradores em 1977, revolucionou a biologia molecular ao permitir o sequenciamento de fragmentos de DNA com alta precisão, baseando-se na terminação de cadeia mediada por dideoxinucleotídeos (Sanger et al., 1977). Pouco depois, o método químico de Maxam e Gilbert surgiu como alternativa, utilizando clivagens específicas de bases nitrogenadas, embora com menor popularidade devido à sua complexidade e uso de reagentes tóxicos (Maxam & Gilbert, 1980). Apoiada em automação surgiu a Shotgun de Sanger possibilitando sequenciar moléculas maiores (100-1000 nucleotídeos). Este método inclui etapas de fragmentação do DNA, clonagem com plasmídeos bacterianos, extração e purificação de DNA e uso de nucleotídeos marcados com fluoroforos que emitem uma luz durante a leitura. A bioinformática se tornou essencial para ordenar as regiões sobrepostas e reconstruir o DNA. Essa tecnologia foi fundamental para sequenciar o genoma humano (Collins et al., 1998).

Com o avanço da genômica, surgiu a necessidade de aumentar drasticamente o rendimento do sequenciamento, o que levou ao desenvolvimento das tecnologias de segunda geração, ou Next-Generation Sequencing (NGS). Entre as primeiras, destacou-se o pirosequenciamento da 454 Life Sciences, lançado em 2005, que utilizava emissão de luz para detectar a incorporação de nucleotídeos (Margulies et al., 2005). Pouco depois, a plataforma Illumina revolucionou o campo ao oferecer uma tecnologia

baseada em terminadores reversíveis, que combinava altíssimo rendimento, baixo custo por base e alta acurácia (Bentley et al., 2008). Essas tecnologias viabilizaram o sequenciamento em larga escala de genomas, transcriptomas e microbiomas, ainda que limitadas por leituras curtas e pela necessidade de amplificação por PCR.

Para superar essas limitações, emergiram as tecnologias de terceira geração, que permitem o sequenciamento de moléculas individuais de DNA ou RNA em tempo real, sem etapas de amplificação. Entre essas, destaca-se o sequenciamento por nanoporos da Oxford Nanopore Technologies. Essa tecnologia baseia-se na passagem de ácidos nucleicos por poros nanométricos imersos em uma membrana, onde cada base afeta de maneira distinta a corrente elétrica, permitindo a leitura direta da sequência (Clarke et al., 2009; Bayley, 2015). Suas principais vantagens incluem leituras ultralongas, portabilidade dos dispositivos, detecção de modificações epigenéticas e o sequenciamento direto de RNA, características que têm ampliado consideravelmente as possibilidades em estudos de genômica, transcriptômica e metagenômica.

Além do avanço na genômica estrutural, o sequenciamento por nanoporos tem ganhado destaque em aplicações transcriptômicas e metagenômicas, especialmente em abordagens como a metataxonomia baseada no gene 16S rRNA. A principal vantagem dessa tecnologia para análise de RNA é a capacidade de realizar sequenciamento direto de RNA mensageiro, sem necessidade de conversão para cDNA ou amplificação, permitindo a identificação de transcritos completos (full-length) e suas isoformas com maior fidelidade (Garalde et al., 2018). No contexto de tecidos complexos como o cérebro de peixes, isso possibilita o estudo detalhado de perfis de expressão gênica relacionados ao desenvolvimento neural, resposta a dietas funcionais, estresse ambiental e interação com microbiota.

Na metagenômica, o sequenciamento por nanoporos tem sido amplamente aplicado para a análise do gene 16S rRNA, permitindo a caracterização taxonômica precisa de comunidades bacterianas em diferentes nichos ambientais e organismos hospedeiros. Por conta da capacidade de leitura de fragmentos longos (por exemplo, das regiões V1 a V9 do 16S), a tecnologia da Oxford Nanopore possibilita uma resolução taxonômica até o nível de espécie, algo difícil de obter com as plataformas NGS convencionais, que normalmente analisam apenas regiões parciais do gene (Benítez-Páez et al.,2016). Esse enfoque metataxonômico tem aplicações diretas na aquicultura, como na avaliação da microbiota intestinal de peixes, da água de cultivo, do biofilme de tanques e da ração funcional, contribuindo para o monitoramento da saúde animal, detecção precoce de patógenos e desenvolvimento de estratégias probióticas (Hoseinifar et al., 2018; Rajeev et al., 2023).

Na aquicultura moderna, entender como dietas, suplementos ou mudanças ambientais afetam a expressão gênica e a composição microbiana é essencial para promover crescimento sustentável, melhora da conversão alimentar, resiliência ao estresse e redução no uso de antimicrobianos. O sequenciamento por nanoporos se apresenta como uma ferramenta poderosa, acessível e flexível para atender a essas demandas, sendo cada vez mais incorporado em pesquisas translacionais e programas de melhoramento zootécnico. Dessa forma, esta Tese visou avaliar os efeitos da cianobactéria de *S. elongatus* PCC 7942 como suplemento alimentar, utilizando o zebrafish (*Danio rerio*) como modelo translacional e técnicas de sequenciamento transcriptômico e metataxonômico, com a perspectiva de uso dessa cianobactéria como veículo de entrega de compostos bioativos e, também, como futura plataforma fotossintética de produção de moléculas recombinantes.

OBJETIVOS

Avaliar os efeitos do consumo de *S. elongatus* PCC 7942 sobre o desempenho zootécnico, transcrição de genes, morfometria hepato-intestinal e na comunidade microbiana do trato digestório do zebrafish (*Danio rerio*).

Objetivos específicos

- Medir parâmetros zootécnicos e analisar o transcriptoma cerebral de *D. rerio* alimentados por 35 dias com *S. elongatus* PCC 7942.
- Analisar morfometria de intestino, histopatologia de fígado e expressão de genes hepáticos envolvidos no metabolismo xenobiótico e defesa antioxidante de peixes alimentados com *S. elongatus* PCC 7942.
- Mapear as mudanças na comunidade microbiana do trato digestório de *D. rerio* alimentado com *S. elongatus* PCC 7942 por 35 dias com sequenciamento de terceira geração.

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CAPÍTULO I. Effect of dietary supplementation with the cyanobacterium *Synechococcus elongatus* PCC 7942 on the brain transcriptome of zebrafish (*Danio rerio*)

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Effect of dietary supplementation with the cyanobacterium *Synechococcus elongatus* PCC 7942 on the brain transcriptome of zebrafish (*Danio rerio*)

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Author's contributions

The conceptualization of the study was carried out by M.L.E.R., B.F.N. and L.F.M. Formal analysis was conducted by B.F.N. and L.F.M. Funding for the project was acquired by L.F.M. The investigation was undertaken by M.L.E.R., B.F.N. and L.F.M., while the methodology was developed by A.I.H., R.S.A., B.X.F., I.S.F., T.S., I.S.A.A., L.O.S., B.R.M, M.L.E.R. and B.F.N. Project administration was handled by L.F.M., and resources were provided by L.F.M. and L.O.S. Supervision was conducted by L.F.M., with validation performed by B.F.N, L.O.S., and L.F.M. The original draft was written by M.L.E.R. and L.F.M., and the manuscript was reviewed and edited by all authors.

Abstract

Dietary supplementation with the cyanobacterium *Synechococcus elongatus* PCC 7942 was evaluated for its effects on the zootechnical parameters and brain transcriptome of zebrafish (*Danio rerio*). After 35 days of experimentation, fish fed the supplemented diet showed higher daily feed intake and condition factor, while other parameters, such as weight gain and specific growth rate, did not differ significantly from the control group. Transcriptome analysis revealed differential expression of 15 genes in the brain, grouped into five main functions: energy metabolism, calcium homeostasis, neuroplasticity, oxidative stress response, and metabolic regulation. Genes related to energy metabolism, such as *ldhbb* and *gapdh*, were overexpressed, indicating increased glycolytic activity to meet elevated metabolic demand. The gene *tsc1a*, associated with the regulation of the mTORC1 pathway, was overexpressed, suggesting a compensatory mechanism to promote autophagy and metabolic homeostasis. Genes related to calcium homeostasis, such as *micu3a* and parvalbumins, were also induced, reflecting neural adaptations to metabolic changes. On the other hand, the repression of *myripa* and *pecam1a*, linked to neuroplasticity, may be associated with structural adjustments in the brain to maintain functional stability. The results suggest an integration between metabolic and neural pathways, indicating that *S. elongatus* PCC 7942 exerts positive systemic effects by modulating brain metabolism and promoting neuroprotective mechanisms. This study highlights the potential of the cyanobacterium as a functional dietary supplement for aquaculture, paving the way for future research on the interaction between dietary supplementation and metabolic regulation in aquatic organisms.

Keywords: palatability, feed intake, condition factor, brain metabolism, neuroprotection.

1. Introduction

The increasing global population requires greater food availability, and aquaculture is increasingly contributing to meeting this demand. In 1961, the annual per capita consumption of aquatic-origin food was 9.1 kg, reaching 20.6 kg in 2021. However, the main inputs in aquaculture feeds, such as fish meal and fish oil, present disadvantages because their availability and quality are declining (FAO, 2024). To address this, research focused on aquaculture nutrition is seeking innovative and sustainable ingredients that can replace or synergize with these inputs, such as oilseeds, grains, hydrolysates, by-products, and microorganisms like bacteria, yeasts, fungi, and algae.

Blue-green algae, or cyanobacteria, are highly nutritious. For example, the proximate composition of spirulina (*Arthrospira platensis*) is 60 % of protein and 12% of lipids (Ahmad et al. 2023). The biomass of cyanobacteria has been used in the diets of farmed aquatic organisms. Supplements/additives are defined as substances added in minimal quantities to feed formulations that influence the physical or chemical properties of the diets, which may or may not affect animal performance or product quality (FAO, 1987; NRC, 2011). While additives do not have a direct nutritional effect, supplements complement the diet. Liang et al. (2015) supplemented the diet of goldfish (*Carassius auratus*) with *Microcystis aeruginosa* and observed that low inclusion levels of 10% and 20% in the diet promoted growth, whereas higher doses (30% and 40%) inhibited growth and led to the accumulation of microcystins in the fish, posing a risk to consumer health. The addition of 3 $g.kg^{-1}$ of *Haematococcus pluvialis* to the diet of *Penaeus vannamei* significantly enhanced shrimp performance on a pilot scale, with increased feed intake, feed conversion ratio, and survival rate. This supplementation also improved the balance of essential amino acids in the muscle, boosted antioxidant

capacity in the hepatopancreas, and altered the intestinal microbiota composition, favoring the presence of Proteobacteria with probiotic potential (Huang et al., 2023). El-Salam et al. (2024) demonstrated that tilapia exposed to the organophosphate pesticide diazinon and fed a diet supplemented with *Arthrospira platensis* (5 $g.kg^{-1}$) did not experience altered growth. However, the antioxidant capacity of the tilapia improved, along with reductions in cholesterol and triglyceride levels.

Among cyanobacteria, species from *Arthrospira* (commonly known as spirulina) stands out due to its worldwide commercialization for human consumption, with approval from the Food and Drug Administration (FDA, USA) (Ahmad et al., 2023; FAO, 2024). However, species of *Synechococcus* have also shown significant potential for biotechnological applications. *Synechococcus elongatus* is a fast-growing, freshwater photoautotroph that does not produce toxins and naturally absorbs and integrates external DNA into its genome. Additionally, several strains of this species have sequenced genomes available in international databases, making it a promising platform for genetic engineering (Wendt et al., 2022).

Previous studies have modified the *S. elongatus* PCC 7942 strain to produce poly-3-hydroxybutyrate (PHB) and ethanol for bioplastic and biofuel production (Deng and Coleman, 1999; Takahashi et al., 1998). In the nutritional context, Santos-Merino et al. (2018) developed *S. elongatus* PCC 7942 mutants capable of synthesizing α-linolenic acid, a member of the omega-3 fatty acid group, and Azevedo et al. (2019) employed this strain as a biofactory for recombinant β-glucosidase, an enzyme with applications in second-generation ethanol production. Although the biotechnological potential of *S. elongatus* PCC 7942 is evident, this cyanobacterium has not yet been evaluated as a dietary supplement.

In parallel with nutritional advancements, aquaculture research integrates molecular tools to evaluate the physiological impacts of feed ingredients. Transcriptomic analysis, particularly in the brain tissue, provide valuable insight into gene expression changes associated to feeding, metabolism and stress response (Ahi et al., 2019; Shang et al., 2022; Shi et al. 2025). The zebrafish (*Danio rerio*) is an established model in neuroscience (Kalueff et al. 2014) and has been adopted as translational model in aquaculture research due to its phylogenetic proximity to commercially important species.

In the present study, *S. elongatus* PCC7942 was evaluated as a dietary supplement in the feed of *Danio rerio*. The proximate composition and fatty acid profile of the cyanobacterium were determined. After a 35-day experimental period, zootechnical performance and brain transcriptome were evaluated, comparing fish fed with commercial feed to those fed with commercial feed supplemented with *S. elongatus* PCC 7942.

2. Material and methods

2.1. Cyanobacterium

The strain *Synechococcus elongatus* PCC 7942 (ThermoFisher Scientific, Brazil) was cultivated in 250 mL Erlenmeyer flasks containing 125 mL of BG-11 medium (Rippka et al., 1979) with an initial optical density (OD) of 0.05, measured using a BioMate 3 spectrophotometer (ThermoFisher Scientific, Brazil) at λ = 750 nm, with BG-11 medium used as the blank. The cultivation was carried out at 35°C under constant light (50 µM photons $m^{-2}.s^{-1}$). Weekly maintenance was performed by adding

BG-11 medium to replenish volumes lost due to evaporation or consumption during feed preparation.

2.2. Feed|

The commercial feed Discus Gran D-50 Plus (Tropical, Brazil) was used as the base for the experiments. This feed was chosen because it meets the protein and lipid requirements of zebrafish (Fernandes et al., 2016; O'Brine et al., 2015). Additionally, it is available in Brazil, where there is no commercial feed specifically formulated for this specie. According to the manufacturer, this feed contains 50% protein derived from fish and fish by-products, plant protein extracts, plant by-products (including red pepper extract at 3,000 $mg.kg^{-1}$), mollusks and crustaceans, cereals, algae (*Arthrospira platensis* min. 1.5%), yeasts, oils, and fats, as well as mineral substances (including zeolite 1%). Additives (per kg): vitamin A 31,000 IU, vitamin D3 1,950 IU, vitamin E 110 mg, vitamin C 550 mg, beta-carotene 140 mg. Minimum concentrations: iron: 40.5 $mg.kg^{-1}$, zinc: 11.2 $mg.kg^{-1}$, manganese: 8.4 $mg.kg^{-1}$, copper: 2.0 $mg.kg^{-1}$, iodine: 0.24 $mg.kg^{-1}$, selenium: 0.24 $mg.kg^{-1}$, molybdenum: 0.05 $mg.kg^{-1}$, astaxanthin: 120 $mg.kg^{-1}$. Nutritional composition: crude protein: 50%, crude fat: 7.5%, crude fiber: 3%, moisture: 8%.

The commercial feed was ground and sieved to obtain pellet sizes between 100 and 500 µm. One portion was reserved for feeding the control group fish, while another was separated to add 10 mL of *S. elongatus* culture (OD_{750} = 1) per gram of dry feed. To determine the amount of experimental feed required, the following calculations were performed:

- Total fish biomass for the treatment: average initial fish weight (126 mg) × number of fish per tank (12) × number of tanks (5) = 7,560 mg.

- Estimated feed consumption at 3% of biomass**:** 7,560 × 0.03 = 226.8 mg of feed per day.

- Feed for one week: 226.8 mg × 7 days = 1,587 mg. A total of 2 g of feed was prepared to feed 60 fish for one week.

- *S. elongatus* culture aliquot (OD_{750} = 1) to be added: 10 ml × 2 = 20 mL.

The optical density of the culture was initially measured with BG-11 medium as the blank control. The aliquot to be added to the feed was separated, and if the OD_{750} exceeded 1, BG-11 medium was added to dilute it. The remaining aliquot volume was discarded. The Erlenmeyer flask volume was adjusted back to 125 mL with sterile BG-11 medium to allow cultivation to continue and was returned to incubation conditions.

The *S. elongatus* culture aliquot with OD_{750} = 1 was transferred to a 50 mL Falcon tube and centrifuged at 7,000 rpm for 10 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 2 mL of sterile Phosphate-buffered saline (PBS) to wash and eliminate potential bacterial contamination. It was then centrifuged again at 7,000 rpm for 10 minutes, and the supernatant was discarded. The cell pellet was resuspended in 10 mL of sterile PBS. The feed was weighed and distributed in a Petri dish, and the cyanobacteria were added. The feed was completely submerged to ensure thorough impregnation with the cyanobacteria. The Petri dish containing the moist feed, uncovered, was placed in an incubator at 28°C for 48 hours to dry. The dried feed was ground and sieved to achieve pellet sizes between 100 and 500 µm. Finally, the feed was stored at 4°C until use.

2.3. Zebrafish (*Danio rerio*)

A total of 120 AB-line zebrafish (*Danio rerio*), male and female aged between 10 and 12 weeks, were used in the experiment. The fish were supplied by the

Transgenic Fish Facility of the Institute of Biological Sciences (ICB) at the Federal University of Rio Grande (FURG). They were acclimated for two weeks under a photoperiod of 12 hours of light and 12 hours of darkness. The fish were distributed among six aquaria in a 300-liter recirculation system equipped with a heater with a thermostat set to 28°C, an ozonator, UV radiation sterilization, and an external biochemical canister filter (OceanTech, model CF-1200). Water quality (pH and conductivity) was monitored using an AKSO multiparameter probe (model AK88) and commercial Labcon Test kits to measure ammonia and nitrites. Water quality parameters were maintained within optimal ranges for the species as described by Lawrence (2007), Kutter et al. (2023) and Longkumer et al. (2024): temperature 27.1 ± 0.03 °C, dissolved oxygen 7.1 ± 0.1 $mg.L^{-1}$, pH 6.7 ± 0.09, ammonia 0.001 ± 0.0001 $mg.L^{-1}$, nitrite 0.042 ± 0.01 $mg.L^{-1}$, and conductivity 1,011.9 ± 70.5 µS. During acclimation, the fish were fed *ad libitum* three times a day. During the first and second feedings, commercial Discus Gran D-50 Plus feed (Tropical, Brazil) was provided, while *Artemia* sp. nauplii (BioArtemia, Brazil) were offered during the third feeding.

2.4. Experiment

The zebrafish experiment was conducted in accordance with the provisions of Law No. 11.794 of October 8, 2008, Decree No. 6.899 of July 15, 2009, and the regulations issued by the National Council for the Control of Animal Experimentation (CONCEA, Brazil). It was approved by the Ethics Committee on Animal Use of the Federal University of Rio Grande (CEUA-FURG), as certified by CEUA-FURG 23116.003565/2023-41.

The experiment consisted of two treatments: **CF** (control group fed with commercial feed) and **CF+C** (experimental group fed with commercial feed

supplemented with *Synechococcus elongatus* PCC 7942). Each group was assigned a recirculation system and 60 fish, with an initial average weight of 125.4 ± 5 mg for the CF treatment and 126.2 ± 3 mg for the CF+C treatment. The fish were randomly distributed into five aquariums (10 L), with 12 fish per tank. For 35 days, the fish were fed *ad libitum* three times a day. The feed was weighed at the beginning and end of each day, and the amount consumed was divided by the number of fish per tank to determine daily feed intake (Molinari et al., 2024). To evaluate zootechnical performance, biometrics were performed under fasting conditions for 24 hours at the beginning and end of the experiment. Prior to handling, the fish were anesthetized with tricaine 168 $mg.L^{-1}$ (ethyl 3-aminobenzoate methanesulfonate, Sigma, Brazil), measured (mm) with a caliper, and weighed (mg) using an electronic balance. During the final biometric session, all fish were euthanized by overdose of tricaine (500 $mg.L^{-1}$). Three male fish were randomly captured from each of four aquariums per treatment for brain collection. The brains were frozen in liquid nitrogen and stored in an ultrafreezer at -70 °C until use in molecular analyses.

2.5. Zootechnical parameters

The zootechnical parameters considered were weight gain (WG), specific growth rate (SGR), condition factor (K), and survival (S). The calculations were performed as follows:

- WG (mg) = final weight (mg) – initial weight (mg)

- SGR (%/day) = [(ln final weight – ln initial weight) / days of experiment] × 100

- K (%) = (final weight / final total length^3) × 100

- S (%) = (number of fish at the end of the experiment / number of fish at the beginning of the experiment) × 100

2.6. Total RNA extraction

For total RNA isolation, the fish brains from each treatment were randomly distributed into groups of three, forming a total of four pools per treatment. The tissues were homogenized in 1 mL of Trizol (Invitrogen, Brazil). Subsequently, each sample was treated with DNase I (Invitrogen, Brazil) to remove genomic DNA and purified using the PureLink RNA Mini Kit (Ambion, Brazil). The purified RNA was eluted in 50 µL of ultrapure water (Invitrogen, Brazil) and quantified using a Qubit fluorometer (Invitrogen, Brazil) and a NanoDrop One spectrophotometer (ThermoFisher Scientific, Brazil). RNA quality and integrity were assessed by electrophoresis on a 1% agarose gel. The purified RNA was used for cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil), following the manufacturer's protocols. The extracted RNA was utilized for transcriptome library preparation.

2.7. Brain transcriptome

Direct cDNA sequencing was performed using the Ligation Sequencing Kit V14 (SQK-LSK114, Oxford Nanopore Technologies), following the manufacturer's protocol. A total of 1 µg of total RNA extracted from brains was used as input for subsequent reactions.

cDNA synthesis began with reverse transcription using the Maxima H Minus Reverse Transcriptase enzyme (ThermoFisher, Brazil) and the VN Primer (5'-phos/ACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTVN-3'), which targets polyadenylated RNAs. Following the synthesis of the first cDNA strand, the reaction for double-stranded cDNA synthesis was initiated using the Strand-Switching Primer (5'-TTTCTGTTGGTGCTGATATTGCTmGmGmG-3') in conjunction with the

PR2 Primer (5'-Phos/TTTCTGTTGGTGCTGATATTGC-3'). Residual RNA was degraded using a specific enzymatic mixture (RNase Cocktail, ThermoFisher Scientific, Brazil), eliminating any interference during library preparation.

The next step involved end repair of the double-stranded cDNA, carried out using the NEBNext Ultra II End Repair/dA-Tailing module (New England Biolabs, Brazil), which adds adenine tails to the 3' ends, making the molecules compatible with adapter ligation. Sequencing adapters provided in the kit were then ligated to the cDNA using the NEBNext Quick Ligation Module (New England Biolabs, Brazil). Subsequently, samples were purified using AMPure XP magnetic beads (Beckman Coulter, Brazil) to remove contaminants and unwanted fragment sizes. The final concentration of cDNA was measured using a Qubit fluorometer (Invitrogen, Brazil).

Prepared libraries were loaded onto R10.4.1 flow cells (FLO-MIN114) that had been pre-equilibrated with Flow Cell Priming Mix, according to the manufacturer's instructions. Before loading, library concentrations were adjusted to ensure optimal pore occupancy (> 95%). Sequencing was conducted on the PromethION 2 device, using MinKNOW software for both data acquisition and basecalling, converting electrical signals into nucleotide sequences in FASTQ format. The sequencing data were processed for bioinformatics analysis, including the identification of full-length transcripts, splicing variants, and other features of interest. The Epi2ME-Labs platform (Oxford Nanopore Technologies) was employed for aligning the data to the zebrafish genome and transcriptome reference sequences available in GenBank under accession number GCF_000002035.6, as well as for functional analyses using the Wf-transcriptomes workflow. The sequencing data were processed for bioinformatics analysis, including the identification of full-length transcripts, splicing variants, and other features of interest. The Epi2ME-Labs platform (Oxford Nanopore Technologies)

was employed for aligning the data to the zebrafish genome and transcriptome reference sequences available in GenBank under accession number GCF_000002035.6, as well as for functional analyses using the Wf-transcriptomes workflow. The EPI2ME-Labs platform utilizes a suite of powerful software tools in its wf-transcriptomes workflow, each playing a crucial role in ensuring accurate and reliable transcriptome analysis. Pysam (version 0.21.0) is used for handling alignment files (SAM/BAM), enabling the extraction of essential data for downstream analyses. Aplanat (version 0.6.20) automates large-scale analyses, streamlining the execution of the pipeline. Pandas (version 1.3.5) is a robust library for managing tabular data, essential for organizing gene counts and statistical results. Scikit-learn (version 1.0.2) provides support for advanced statistical analyses and machine learning, such as clustering of expressed genes. Fastcat (version 0.10.2) assists in analyzing the integrity and quality of sequencing reads. Minimap2 (version 2.24-r1122) aligns long reads to the reference genome, while Samtools (version 1.17) and Bedtools (version 2.30.0) handle and analyze alignment files, including filtering and operations on specific genomic regions. Pychopper (version 2.7.10) identifies and classifies full-length molecules, enabling more precise transcript analyses. Gffread (version 0.12.7) is used to manipulate genomic annotations, and SeqKit (version 2.2.0) supports preprocessing and cleaning of FASTQ data. Finally, StringTie (version 2.1.1) performs transcript assembly and quantification, enabling the identification of alternative isoforms and differentially expressed genes. Together, these tools provide a robust and efficient pipeline for transcriptome analysis.

2.8. Proximate composition and fatty acid profile of the cyanobacterium

The strain *S. elongatus* PCC 7942 was cultured in triplicate using 2 L Erlenmeyer flasks containing 1.2 L of BG-11 medium. The flasks were equipped with

737 glass pipettes and sealed with cotton and gauze to allow gas exchange. The cultures
738 started with an optical density (OD_{750}) of 0.05 and were maintained in an incubator at
739 35°C under constant illumination (50 µmol photons m^{-2} s^{-1}) and continuous aeration
740 supplied by an air pump connected to tubing. To minimize contamination, 0.22 µm
741 syringe filters were attached to the tubing before the pipettes. Optical density was
742 monitored every four days, and evaporated volumes were replaced with BG-11 medium.
743 After 22 days, the cultures were harvested at an OD_{750} of 1, and the biomass was
744 concentrated by centrifugation (11,000 rpm, 3 minutes, room temperature) in 50 mL
745 Falcon tubes. The supernatant was discarded, and the process was repeated until the
746 entire volume from each Erlenmeyer flask was concentrated, which were subsequently
747 stored at -70°C for 12 hours and lyophilized in a SOLAB lyophilizer (SL-404) for 16
748 hours. The dry biomass was weighed, concentrated into a single tube per replicate,
749 sealed with Parafilm, and stored in an ultrafreezer until use.

750 For protein and carbohydrate analyses, 10 mg of dry biomass was resuspended
751 in 20 mL of distilled water and sonicated (QSONICA Q55, 20 kHz, 40% amplitude) for
752 10 minutes, resulting in the sonicated cyanobacterial extract. The soluble protein
753 content was determined using the method of Lowry et al. (1951) with 500 µL of the
754 extract, while carbohydrates were quantified using the method of Dubois et al. (1956)
755 with 1 mL of the same extract. Total lipid determination was performed using the
756 method of Marsh and Weinstein (1966), with 10 mg of dry biomass and glass beads for
757 cell lysis. Ash content was quantified by incinerating 80 mg of dry biomass in a muffle
758 furnace at 550°C.

759 Lipid extraction for fatty acid identification was conducted using the method of
760 Bligh and Dyer (1959) with 350 mg of dry biomass. The extracted lipids were analyzed

by gas chromatography at the Integrated Analysis Center (CIA) of the Federal University of Rio Grande (FURG).

2.9. Statistical analyses

Prior to statistical analysis, the data were transformed into a linear model and subjected to the Shapiro-Wilk test to assess normality and the Bartlett test to evaluate homoscedasticity. Data with normal distribution and homogeneous variances were analyzed using one-way ANOVA, followed by Tukey's post hoc test. For non-normally distributed data, the Kruskal-Wallis test was applied, followed by the Wilcoxon test. Differences were considered statistically significant at $p < 0.05$. Results were expressed as means ± standard deviation. All statistical analyses were performed using R software (version 2024).

3. Results

3.1. Zootechnical parameters

The survival of the fish was not affected by the addition of *S. elongatus* PCC 7942 to the diet. Similarly, total length, weight gain, and specific growth rate showed no significant differences. However, the condition factor and daily feed intake were higher in fish fed with the diet supplemented with S. elongatus PCC 7942 (Table 1).

Table 1 Zootechnical parameters of *Danio rerio* fed for 35 days with commercial feed (CF) and commercial feed supplemented with the cyanobacterium *Synechococcus elongatus* PCC 7942 (CF+C). Data represent the mean of five replicates ± standard deviation. The different superscript letters indicate statistical differences.

Parameters	CF	CF+C
Survival (%)	100^{a}	98.3 ± 3.7^{a}
Initial weight (mg)	$125,4 \pm 5^{a}$	$126,2 \pm 3^{a}$
Final weight (mg)	167 ± 6^{a}	175 ± 8^{a}
Initial total length (mm)	23.5 ± 0.3^{a}	23.5 ± 0.1^{a}
Final total length (mm)	25.3 ± 0.3^{a}	25.1 ± 0.4^{a}
Weight gain (mg)	41.6 ± 4.2^{a}	48.8 ± 8.9^{a}
Specific growth rate (%)	0.80 ± 0.08^{a}	0.93 ± 0.15^{a}
Condition factor (K)	0.99 ± 0.03^{a}	1.05 ± 0.03^{b}
Daily feed intake (mg feed/fish/day)	3.4 ± 0.17^{a}	3.75 ± 0.20^{b}

3.2. Brain transcriptome

Among the zootechnical parameters, the increase in feed intake stood out. Because of this, a transcriptome analysis of the brain was conducted to verify whether feed supplementation with the cyanobacterium was inducing any appetite-related genes. However, the results showed the differential expression of unexpected genes. The initial differential expression analysis identified 96 differentially expressed genes. However, further analysis revealed that the vast majority of these genes were classified as differentially expressed due to high transcript counts in only one of the four samples for

each treatment. Thus, a stricter criterion was applied, requiring at least two samples to show high transcript counts for a gene to be considered differentially expressed. After this refinement, only 15 differentially expressed genes were identified between the treatments (CF and CF+C), of which 13 were upregulated and two were downregulated in the brains of fish fed the cyanobacteria-supplemented diet. Table 2 shows the 15 differentially expressed genes between treatments, their abundance measured in counts per million (CPM), and the level of induction of each gene relative to the control treatment (LogFC). The differentially expressed genes were: Lactate Dehydrogenase Bb (*ldhbb*), Mitochondrial Calcium Uptake Family Member 3a (*micu3a*), Parvalbumin 1 (*pvalb1*), Sestrin 1 (*sesn1*), TSC Complex Subunit 1a (*tsc1a*), Parvalbumin 2 (*pvalb2*), Myosin Heavy Chain 2 Fast Muscle Specific (*myhz2*), Troponin I Type 2b (*tnni2b.2*), Myosin Heavy Chain 4 (*myhc4*), Glyceraldehyde-3-Phosphate Dehydrogenase (*gapdh*), Troponin T Type 3b (*tnnt3b*), Myosin Light Chain a (*mylpfa*), Parvalbumin 4 (*pvalb4*), Myosin VIIA and Rab Interacting Protein a (*myripa*), Platelet and Endothelial Cell Adhesion Molecule 1a (*pecam1a*).

Table 2. Gene expression levels in the brain of zebrafish *(Danio rerio)* fed with commercial feed (CF) and commercial feed supplemented with *Synechococcus elongatus* PCC 7942 (CF+C). Values represent transcript counts per million (CPM) for four biological replicates of each treatment. The LogFC reflects the difference in expression between treatments, with positive and negative values indicating higher or lower expression in the CF+C group compared to the CF (control) group, respectively.

Genes	**CF**				**CF+C**				**LogFC**
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	
ldhbb	0,00	0,00	0,00	0,00	14,05	0,00	200,21	28,02	6,8

micu3a	0,00	0,00	0,00	0,00	31,62	75,87	9,10	0,00	5,59
pvalb1	0,00	14,90	10,26	0,00	42,16	105,38	81,90	0,00	3,54
sesn1	0,00	0,00	20,52	0,00	56,21	88,52	54,60	28,02	3,17
tsc1a	9,37	14,90	0,00	11,37	66,75	63,23	59,15	46,70	3,12
pvalb2	0,00	119,18	5,13	5,69	66,75	290,85	204,76	4,67	3,07
myhz2	9,37	0,00	30,78	0,00	14,05	139,10	150,16	9,34	2,94
tnni2b.2	9,37	44,69	15,39	0,00	31,62	118,03	136,51	0,00	2,71
myhc4	93,70	432,04	169,27	90,98	221,33	1635,52	1342,33	9,34	2,57
gapdh	131,18	104,28	92,33	0,00	7,03	181,26	778,10	23,35	2,22
mylpfa	46,85	44,69	46,16	11,37	28,11	185,47	213,86	23,35	2,02
pvalb4	65,59	134,08	82,07	5,69	31,62	316,14	295,77	28,02	1,76
ttn.1	178,02	104,28	82,07	17,06	45,67	333,00	455,03	42,03	1,73
myripa	56,22	148,98	35,91	28,43	0,00	0,00	0,00	14,01	-3,71
pecam1a	74,96	44,69	25,65	0,00	0,00	0,00	0,00	0,00	-5,44

Table 3 shows the 15 differentially expressed genes grouped into five basic functions that may affect brain neurons. The identified functions are energy metabolism (2 genes), calcium homeostasis (4 genes), neuroplasticity (2 genes), oxidative stress response (1 gene), and metabolic regulation (6 genes).

Table 3. Functional classification of differentially expressed genes in the brain of zebrafish *(Danio rerio*) fed with commercial feed (CF) and feed supplemented with *Synechococcus elongatus* PCC 7942 (CF+C). Green indicates upregulated genes (higher expression in the CF+C group compared to the CF group), and red indicates downregulated genes (lower expression in the CF+C group compared to the CF group).

Genes	Functions				
	Energy metabolism	Calcium homeostasis	Neuroplasticity	Oxidative stress response	Metabolic regulation
ldhbb					
gapdh					
micu3a					
pvalb1					
pvalb2					
pvalb4					
myripa					
pecam1a					
sesn1					
tsc1a					
myhz2					
myhc4					
mylpfa					
tnni2b.2					
tnnt3b					

3.3. Proximal composition and fatty acid profile of the cyanobacterium

The proximal composition of the cyanobacterium, determined per 100 mg of dry biomass, resulted in 45.19 ± 1.73 mg of protein, 21.34 ± 0.39 mg of lipids, 10.08 ± 0.06 mg of carbohydrates, and 8.01 ± 0.48 mg of ash. The fatty acid profile is shown in Table 4, with the predominant fatty acids being palmitic acid (C16:0), palmitoleic acid (C16:1), linoleic acid (C18:2), stearic acid (C18:0), and elaidic acid (C18:1). These five fatty acids account for 93.53% of the lipid fraction of the dry biomass.

Table 4. Fatty acid profile of the dry biomass of the cyanobacterium *Synechococcus elongatus* PCC 7942.

Fatty acid	Formula	Relative content (%)
Palmitic acid	C16:0	39.96
Palmitoleic acid	C16:1Δ^{9}	20.8
Linoleic acid	C18:2$\Delta^{9,12}$	12.25
Stearic acid	C18:0	11.26
Elaidic acid	C18:1Δ^{9} *trans*	9.26
Oleic acid	C18:1Δ^{9} *cis*	3.52
Cis-10-Heptadecenoic acid	C17:1Δ^{10}	1.78
Myristoleic acid	C14:1Δ^{9}	0.82
Myristic acid	C14:0	0.35

4. Discussion

The increased daily feed intake in the supplemented fish is particularly interesting, as this observation contrasts with the common perception that cyanobacteria

often exhibit low palatability due to the content of secondary metabolites such as malyngamide, malyngolide, ypaoamide, and lyngbioic acid, reported as defense mechanisms against herbivores (Capper et al., 2016; Nagle and Paul, 1999; Thacker et al., 1997). Conversely, the cyanobacteria spirulina *Arthrospira platensis* are recognized for their nutritional and sensory properties attributed to the presence of bioactive compounds such as carotenoids, phycocyanin's, and essential amino acids. These compounds are known to enhance intestinal health by promoting nutrient absorption and stimulating appetite (Youssef et al., 2023).

Its relevant note that the commercial feed used in the present study already included spirulina in its formulation. Therefore, the supplementation with *S. elongatus* may have produced a synergistic effect, further enhancing feed intake in zebrafish. The proximate composition of *S. elongatus* PCC 7942 supporting this, revealing a high protein content (45.19%), complemented by lipids (21.34%), carbohydrates (10.08%), and ash (8.01%). This protein-rich profile is consistent with other cyanobacteria, such as spirulina, which is widely recognized for its nutritional value and biotechnological potential.

Cyanobacterial proteins are known to provide a well-balanced amino acid profile, which can enhance the dietary value of aquafeeds (Galafat et al., 2022). Furthermore, the lipid fraction, stand out by essential fatty acids like palmitic acid (C16:0) and linoleic acid (C18:2), offers additional benefits for cellular metabolism and energy production (O'Brine et al., 2015). The presence of significant carbohydrate levels further highlights the potential of *S. elongatus* as a functional feed additive, as carbohydrates can serve as an energy source, particularly during periods of increased metabolic demand (Polakof et al., 2012). These nutritional attributes are also reflected in the significantly higher condition factor of the supplemented fish, reinforcing the

positive impact of adding *S. elongatus* to the diet, indicating an improved weight-to-length relationship and related to a better health and body composition. Abdel-Tawwab et al. (2008) reported similar results when supplementing tilapia diets with yeast, suggesting that functional ingredients can improve body composition independently of absolute weight gain.

On the other hand, the absence of significant differences in parameters such as weight gain and SGR may be associated with the short duration of the experiment (35 days). Lawrence (2007) highlighted that zebrafish have limited growth under laboratory conditions, and changes in these parameters may require longer experimental periods to be detected. These results suggest that *S. elongatus* PCC 7942 is a promising functional supplement capable of enhancing the beneficial effects of commercial diets.

Brain transcriptomic analysis of zebrafish indicated that dietary supplementation with *S. elongatus* triggers substantial alterations in metabolic functions and adaptative process of fish. Regarding genes associated with energy metabolism, differential expression analysis revealed a significant induction of the *ldhbb* (Lactate Dehydrogenase Bb) and *gapdh* (Glyceraldehyde-3-Phosphate Dehydrogenase) genes. LDH Bb is a crucial enzyme in the glycolytic pathway, catalyzing the conversion of pyruvate to lactate, which is essential for energy production, particularly under hypoxic conditions or increased energy demand. Although few specific studies address the role of LDH Bb in the fish brain, data from other models, such as rodents, provide important insights into its metabolic relevance. Park et al. (2022) report experimental evidence that LDH B deficiency in knockout mouse models for the *ldhb* gene can lead to mitochondrial dysfunction, oxidative stress, and neurodegeneration. These findings suggest that adequate expression of this enzyme is essential for metabolic homeostasis in tissues with high energy demands, such as the brain. Thus, the increased expression

of *ldhbb* in zebrafish supplemented with the cyanobacterium may indicate a neuroprotective effect on the brain and a metabolic adaptation to meet the increased energy demands associated with greater feed intake and neural activity.

Still regarding cerebral energy metabolism, GAPDH plays a central role in glycolysis, facilitating the conversion of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate. In zebrafish, *gapdh* was frequently used as a reference gene in gene expression analyses due to its previously assumed stable expression across various tissues and experimental conditions, which is no longer considered accurate. Rassier et al. (2020) report that this gene is one of the most variable across different zebrafish tissues. According to Nicholls et al. (2012), this common enzyme has uncommon functions. These authors highlight its role as a multifunctional protein involved in processes such as transcription regulation, RNA stability, and apoptosis, especially in response to oxidative stress. Additionally, GAPDH also performs extraglycolytic roles, such as mRNA regulation through binding to AU-rich elements (AREs), which can influence the stability and translation of specific transcripts under cellular stress conditions. This function is particularly relevant in the brain, where rapid changes in signaling pathways and gene expression are critical to maintaining homeostasis. It is challenging to hypothesize the consequences of increased *gapdh* expression, as observed in the brains of zebrafish whose diet was supplemented with *S. elongatus* PCC 7942. It is possible that this increased expression represents a neuroprotective effect, particularly regarding oxidative stress, without disregarding the potential impact on other functions as those highlighted by Nicholls et al. (2012).

In the context of supplementation with *S. elongatus* PCC 7942, the calcium metabolism-related genes in the zebrafish brain that showed differential expression were *micu3a* (Mitochondrial Calcium Uptake Family Member 3a), *pvalb1*, *pvalb2*, and

pvalb4 (Parvalbumins). These genes play critical roles in regulating intracellular calcium fluxes, an essential element for cellular processes such as signaling, neurotransmitter release, and energy homeostasis. The MICU3 protein works in conjunction with other members of the MICU family to regulate calcium levels in mitochondria, ensuring cellular homeostasis and protection against calcium overload, which can cause mitochondrial dysfunction and apoptosis. Studies in mammalian models have shown that alterations in the expression of MICU family genes can directly impact mitochondrial function in neurons, particularly under conditions of metabolic stress (Patron et al., 2014). In the present study, the increased expression of *micu3a* in supplemented zebrafish may reflect a metabolic adaptation to optimize mitochondrial calcium uptake in response to dietary-induced changes. This adaptation might be associated with the need for greater energy efficiency to meet the brain's demands under conditions of higher feed intake and neural activity.

Parvalbumins are calcium-binding proteins that act as intracellular buffers, controlling the levels of free calcium in the cytosol. These genes play an essential role in calcium regulation in excitable tissues such as muscles and neurons, where rapid changes in calcium fluxes are necessary for cellular signaling. The overexpression of *pvalb1*, *pvalb2*, and *pvalb4* may indicate an increased need for calcium buffering in the brains of supplemented zebrafish. Some studies have already demonstrated that elevated parvalbumin levels are associated with protection against excitotoxicity induced by calcium overload, conferring a neuroprotective role (Van Den Bosch et al., 2002; Chandrasekar et al., 2019). Thus, the increased expression of these genes may represent a response to enhanced neural excitability resulting from metabolic changes induced by *S. elongatus* PCC 7942 supplementation, which appears to induce adaptations in

calcium metabolism-related genes, suggesting a functional adjustment in the zebrafish brain to cope with changes in metabolic and neural demands.

Among the neuroplasticity-related genes analyzed in this study, two showed reduced expression in the brains of zebrafish supplemented with *S. elongatus* PCC 7942: *myripa* (Myosin VIIA and Rab Interacting Protein a) and *pecam1a* (Platelet and Endothelial Cell Adhesion Molecule 1a). Both genes play significant roles in cellular dynamics and cell interaction, processes that are fundamental to neural plasticity. The *myripa* gene encodes a protein that interacts with myosin VIIA, playing critical roles in intracellular transport and cytoskeletal dynamics. El-Amraoui et al. (2002) associated dysfunctions in myosin VIIA and its interacting proteins with alterations in cellular architecture, particularly in neural and sensory tissues. The repression of *myripa* in supplemented fish may indicate a reduction in intracellular transport activity associated with the supplementation. This decrease could result from adaptations in cellular metabolism or neural dynamics induced by the bioactive compounds present in *S. elongatus*. However, the specific function of this gene in zebrafish neuroplasticity remains poorly understood, highlighting the need for further studies.

The *pecam1a* gene is primarily known for its function in endothelial cells, where it regulates cell adhesion and migration during angiogenesis. In neural contexts, PECAM1 is involved in processes of cellular remodeling and maintenance of the blood-brain barrier (Wimmer et al., 2019). The reduced expression of *pecam1a* in supplemented zebrafish may reflect an alteration in the structural support of cell-cell interactions in the brain. One hypothesis is that this repression is associated with a reduction in cellular remodeling due to stabilized metabolic conditions or a lower need for structural plasticity in response to supplementation. The repression of *myripa* and *pecam1a* in supplemented fish suggests adjustments in the structural and functional

dynamics of brain cells. These results may indicate a state of reduced neural plasticity, possibly associated with a more stable and less demanding metabolic environment.

In the context of oxidative stress, only the *sesn1* gene was significantly induced in the brains of zebrafish supplemented with *S. elongatus* PCC 7942. This gene, which encodes the protein sestrin 1 (SESN1), plays a central role in the cellular response to oxidative stress and in maintaining redox homeostasis. As highlighted by Chen et al. (2022), SESN1 regulates the production of reactive oxygen species (ROS), promoting cell survival and protecting against oxidative damage. SESN1 is widely recognized for its ability to inhibit the mTORC1 pathway and activate AMPK, thereby promoting autophagy (Chen et al., 2019). Autophagy is an essential cellular process for the degradation and recycling of damaged components, contributing to the elimination of misfolded proteins, dysfunctional organelles, and accumulated cellular waste, especially in tissues with high metabolic demand, such as the brain. This mechanism not only preserves cellular integrity but is also critical for adaptation to metabolic and oxidative stress conditions. The overexpression of *sesn1* observed in the supplemented zebrafish may be interpreted as an adaptive response to the metabolic changes induced by the diet enriched with *S. elongatus*. Bioactive compounds present in the cyanobacterium, such as antioxidant pigments and essential fatty acids, may be promoting a cellular environment requiring greater antioxidant and autophagic regulation to maintain homeostasis. This increase in *sesn1* expression may also indicate a neuroprotective effect, particularly in response to alterations in metabolic and oxidative pathways.

In the group of genes related to metabolic regulation, significant differential expression was observed for *tsc1a*, *myhz2*, *myhc4*, *mylpfa*, *tnni2b.2*, and *tnnt3b*. Although these genes are traditionally associated with muscle functions, they have relevant metabolic implications, especially in neural tissues. The *tsc1a* gene plays a

critical role in regulating the mTORC1 pathway, which controls processes such as cell growth, lipid metabolism, and protein synthesis. The overexpression of *tsc1a* in zebrafish supplemented with *S. elongatus* suggests a negative regulation of the mTORC1 pathway, promoting autophagy and preventing the accumulation of damaged proteins, particularly in response to increased food intake and metabolic demands (Dibble and Cantley, 2015). This regulation can be interpreted as a protective mechanism to maintain cellular homeostasis.

The *myhz2*, *myhc4*, and *mylpfa* genes encode components of the muscle contractile machinery, but their metabolic functions extend beyond muscle tissue. The presence and overexpression of these genes in the brain may indicate a role in maintaining cytoskeletal architecture and regulating local energy dynamics. Hodge et al. (2000) demonstrated that myosins are involved in the intracellular transport of organelles and vesicles, critical processes for neuronal function and responses to metabolic changes. The increased expression of these genes could reflect functional adaptations induced by dietary supplementation, such as the need for greater efficiency in intracellular transport and energy delivery to specific brain regions. Similarly, *tnni2b.2* and *tnnt3b*, generally associated with muscle contraction regulation, may play metabolic roles in the brain. These proteins are involved in modulating intracellular calcium, which is essential for neural functions such as neurotransmitter release and synaptic plasticity (Gomes et al., 2002). The overexpression of these genes may be linked to neuronal metabolic adaptations in response to the increased metabolic demand associated with *S. elongatus* supplementation. These findings highlight that genes traditionally associated with the musculoskeletal system may have significant metabolic roles in the brain, particularly in the context of dietary supplementation.

This study demonstrated that dietary supplementation with the cyanobacterium *S. elongatus* PCC 7942 induced significant changes in the zootechnical parameters and brain metabolism of zebrafish, as evidenced by transcriptome analysis and functional outcomes. The increase in daily feed intake and condition factor suggests that the supplementation modulated diet palatability and metabolic efficiency, likely due to the biochemical composition of *S. elongatus* and its bioactive compounds. At the molecular level, the differential expression of 15 genes grouped into five main functions (energy metabolism, calcium homeostasis, neuroplasticity, oxidative stress response, and metabolic regulation) indicated a complex metabolic and neural adaptation. In energy metabolism, genes such as *ldhbb* and *gapdh* were prominently overexpressed, pointing to an intensification of glycolytic pathways, while the *tsc1a* gene suggested negative regulation of the mTORC1 pathway, promoting metabolic homeostasis and autophagy. Additionally, the induction of genes related to calcium homeostasis (*micu3a* and parvalbumins) indicated adjustments to meet ionic demands under altered metabolic conditions.

The integration of transcriptome results suggests that the observed metabolic effects are interconnected. For instance, the increased expression of *tsc1a*, *ldhbb*, and *gapdh* aligns with the need for metabolic adaptation to higher food intake. Simultaneously, the regulation of genes related to oxidative stress (*sesn1*) and calcium metabolism may act synergistically to preserve neuronal functionality in an environment of increased energy demand. On the other hand, the repression of neuroplasticity-related genes (*myripa* and *pecam1a*) could reflect a compensatory structural adjustment to maintain the brain's functional stability. These findings highlight the potential of *S. elongatus* PCC 7942 as a functional supplement capable of

modulating central metabolic pathways and promoting systemic positive effects in zebrafish, a relevant translational model for aquaculture.

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Declarations

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Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CAPÍTULO II. Hepatic protective effects and oxidative stress modulation via gene expression in zebrafish (*Danio rerio*) fed with *Synechococcus elongatus* PCC 7942 as a functional feed additive

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Hepatic protective effects and oxidative stress modulation via gene expression in zebrafish (*Danio rerio*) fed with *Synechococcus elongatus* PCC 7942 as a functional feed additive

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Abstract

The inclusion of cyanobacteria in aquafeeds is a sustainable alternative to traditional fishmeal. This study evaluated the effects of *Synechococcus elongatus* PCC 7942 supplementation on intestinal morphology, liver histopathology, and antioxidant gene expression in zebrafish (*Danio rerio*). Fish were fed a commercial diet (CF) or the same diet supplemented with *S. elongatus* (EF) for 35 days. Liver histopathology revealed that 62% of fish in the CF group exhibited generalized liver alteration, while fish in the EF group showed a lower frequency of generalized alteration (31%) and a higher frequency of multifocal lesions (46%), suggesting improved hepatic homeostasis. Intestinal morphometry showed no significant changes in villus length between groups. Gene expression analysis demonstrated a significant downregulation of xenobiotic metabolism genes (*cyp1a*, *gst*), antioxidant defense genes (*sod1*, *sod2*, *cat*), and steroid metabolism (*cyp19a1a*) in fish fed *S. elongatus*, except for *gpx*, which remained unchanged. The reduction in antioxidant gene expression, along with improved liver histology, suggests a lower oxidative stress in the EF group, likely due to synergistic effects of *S. elongatus* in mitigating oxidative damage. These findings indicate that *S. elongatus* supplementation does not impair intestinal morphology or liver function but supports hepatic homeostasis by reducing oxidative stress and modulating liver histopathology. This highlights its potential as a functional feed additive in aquaculture.

Keywords: Cyanobacteria, gut, histopathology, antioxidant defense, xenobiotic metabolism, liver health.

Introduction

Cyanobacteria, or blue-green algae, are Gram-negative, photosyntetic bacteria found in a wide range of aquatic an terrestrial environment. They possess specialized structures such as phycobilisomes, carboxysomes, and rubisco enzymes, enabling them to fix CO_2 and thrive in oligotrophic ecosystems (Whitton and Potts, 2002). Due their metabolic plasticity and ecological versatility, cyanobacteria play a central role in aquatic food webs as primary producers (Oren, 2014). However, the excessive proliferation of some species can lead to the production of cyanotoxins, including hepatotoxins, neurotoxins, and dermatotoxins, which may bioaccumulate in aquatic organisms and pose risks to environmental and human health (Chorus & Welker, 2021).

Importantly, not all cyanobacteria strains produce toxins. Several species have gained attention for their ability to synthesize bioactive metabolites, pigments ans macromolecules of industrial relevance (Agarwal et al., 2022; Bouyahya et al., 2024; Castro et al., 2023). Non toxigenic cyanobacteria such as *Arthrospira platensis* (commonly known as spirulina) are the most widely used in aquaculture due to their proven safety and well-documented benefits in fish nutrition. Spirulina supplementation has been shown to enhance antioxidant defenses, immune function, and growth performance in several fish species (Coli et al., 2024; El-Salam et al., 2024; Rosas et al., 2019). Recent protocols have demonstrated the feasibility of transforming spirulina strains (Tabakh et al. 2023), but issues such as low transformation efficiency, genomic instability, and complex cellular architecture continue to limit its utility as a biotechnological chassis. In contrast, Synechococcus elongatus (another non-toxigenic cyanobacteria) exhibits natural competence for DNA uptake, a well-annotated genome, and compatibility with standard genetic tools, allowing for stable and targeted modifications (Taton et al., 2020). *S. elongatus* PCC7942 has been engineered to

produce polyhydroxybutyrate (PHB), long-chain polyunsaturated fatty acids such omega-3, and high-value carotenoid such as zeaxanthin (Santos-Merino et al., 2018; Sarnaik et al., 2018; Takahashi et al., 1998). These developments have reinforced the role of S. elongatus as a photosynthetic microbial platform with promising applications in food, feed and environmental biotechnology. In aquaculture, where sustainable alternatives to fishmeal and fish oil are increasingly sought (FAO, 2024), *S. elongatus* emerges as a potential source of bioactive supplementation.

Despite its promising features, the safety and physiological impacts of dietary inclusion of S. elongatus PCC7942 in aquafeeds have not been comprehensively evaluated. It is critical to determine whether the ingestion of this cyanobacterium affects the morphology or function of the intestinal tract or liver – organs central to nutrient absorption, metabolism and detoxification. Therefore, this study aimed to investigate the physiological and molecular responses of zebrafish (*Danio rerio*) to dietary supplementation with Synechococcus elongatus PCC7942, focusing on intestinal structure, liver and the expression of genes related to detoxification and antioxidant defense. These endpoints were selected because they reflect key aspects of nutritional impact: intestinal architecture is directly related to absorption efficiency and gut health; the liver is the primary organ involved in metabolism and xenobiotic detoxification; and antioxidant gene expression serves as a molecular marker of oxidative stress modulation, which is often influenced by bioactive dietary components. Zebrafish were selected as the experimental model due to their well-characterized physiology, high genetic homology with farmed fish species, and stablished utility in nutritional and toxicological research (Lawrence, 2002; Hill et al., 2005).

Material and methods

Cultivation of *Synechococcus elongatus* PCC 7942 and Feed Preparation

The strain *Synechococcus elongatus* PCC 7942 (ThermoFisher Scientific, Brazil) was cultured in BG-11 medium (Rippka et al., 1979) in an incubator at 34 °C under continuous illumination (50 µmol photons m^{-2} s^{-1}). Culture maintenance was performed weekly by supplementing with fresh BG-11 medium to compensate for volume loss due to evaporation or sampling for feed preparation.

The control feed (CF) consisted of the commercial diet Discus Gran D-50 Plus (Tropical, Brazil). According to the manufacturer, this feed contains 50% protein derived from fish and fish byproducts, plant protein extracts, plant-based byproducts (including red pepper extract, 3,000 mg/kg), mollusks and crustaceans, cereals, algae (*Arthrospira platensis*, minimum of 1.5%), yeast, oils and fats, and mineral substances (including zeolite, 1%). The feed formulation includes the following additives (per kg): vitamin A (31,000 IU), vitamin D_3 (1,950 IU), vitamin E (110 mg), vitamin C (550 mg), and beta-carotene (140 mg). Minimum concentrations of trace elements include iron (40.5 mg/kg), zinc (11.2 mg/kg), manganese (8.4 mg/kg), copper (2.0 mg/kg), iodine (0.24 mg/kg), selenium (0.24 mg/kg), molybdenum (0.05 mg/kg). The guaranteed composition includes crude protein (50%), crude fat (7.5%), crude fiber (3%), and moisture (8%). This commercial feed was selected because it is widely adopted in zebrafish nutritional studies and provides a balanced nutritional profile suitable for maintenance and growth (Fernandes et al., 2016). Although the diet contains functional compounds such as carotenoids and astaxanthin, which may influence physiological responses, its standardized composition allows for reproducibility and comparative interpretation of supplementation effects.

The experimental feed (EF) was prepared by supplementing the commercial feed (CF) with 10 mL of *S. elongatus* PCC 7942 culture (OD_{750} = 1) per gram of dry feed.

The feed was fully immersed in the cyanobacterial culture and subsequently dried in an oven at 28 °C for 48 hours. Both the control and experimental feeds were ground and sieved to obtain pellets ranging in size from 100 to 500 µm. Finally, the feeds were stored at 4 °C until use.

Zebrafish feeding experiment

The experimental design was submitted to the Ethics Committee on Animal Use of the Federal University of Rio Grande (CEUA-FURG) and approved under certificate CEUA-FURG 23116.003565/2023-41. The feeding trial lasted 35 days and involved *Danio rerio* (AB strain) with an initial mean weight of 125.8 ± 3 mg and a total length of 23.5 ± 0.3 mm. Two experimental groups were established: a control group and an experimental group, each consisting of 60 fish. The fish were randomly distributed into five aquaria (12 fish/aquarium with a volume of 10 L each) per group, maintained in a recirculating system. The control group was fed the CF diet, while the experimental group received the EF diet. Fish were fed *ad libitum* three times daily. The environmental conditions were maintained according to species-specific recommendations (Kütter et al., 2023), with the following parameters: temperature of 27.1 ± 0.03 °C, dissolved oxygen of 7.1 ± 0.1 mg/L, pH of 6.7 ± 0.09, ammonia of 0.001 ± 0.0001 mg/L, nitrite of 0.042 ± 0.01 mg/L, and conductivity of 1,011.9 ± 70.5 µS.

At the end of the experiment, all fish underwent a 24 h fasting period. After that, all fish were euthanized by overdose of Tricaine MS-222 (Sigma-Aldrich, Brazil; cat. A-5040) prior to fixation. Before sample collection, biometric measurements of body weight and total length were recorded to assess growth performance.

Histological analyses

To evaluate the condition of organs involved in nutrient degradation and metabolism in response to *S. elongatus* ingestion, four whole fish per aquarium were collected. To ensure optimal fixation, a ventral transverse incision was made near the anal pore to allow the free penetration of 4% paraformaldehyde, in which the specimens were immersed for 12 hours. Subsequently, fish were rinsed in running water, transferred to 70% ethanol, and the organs of the ventral cavity, including the intestine and liver, were dissected together.

The organs were processed in an automated LEICA ASP 200S tissue processor for dehydration, clearing, and embedding in paraplast (Sigma-Aldrich, Brazil; P3808). Histological sections of 5 µm were obtained using a Leica RM 2255 microtome, stained with hematoxylin and eosin, and mounted with slide synthetic mounting medio DPX (Sigma-Aldrich, Brazil; O6522). The stained sections were examined under a light microscope and photographed using an Olympus DP72 camera. Images were processed using ImageJ software (Rasband, 1997). Histological analyses were conducted in a double-blind study, with fish and organs randomly labeled and examined. Subsequently, data were categorized into control and experimental groups for statistical analysis.

Intestinal morphometry

To determine whether cyanobacteria ingestion induces structural changes in the intestine that could affect nutrient absorption, intestinal villi morphometry was performed. Measurements were taken from 15 villi per fish in the rostral to mid-intestine section (S1 to S5), following the section identification criteria described by Wang et al. (2010). The intestinal villus length (**c**) was calculated using the following

formula: **c = a – b**; where “a” represents the length from the serosa to the epithelium, and “b” represents the length from the serosa to the submucosa (Figure 1A).

Liver histopathology

Liver histopathological evaluation was conducted through the analysis of histological images. Each sample was classified based on observed cellular morphology into one of three categories: normal, multifocal damage, and generalized damage. Classification was performed according to the criteria established by Triana-García et al. (2013). Briefly, normal hepatocytes exhibit a well-defined cell membrane, central nucleus, homogeneous cytoplasm, and well-organized sinusoids. Multifocal alteration is characterized by alterations in isolated tissue regions interspersed with normal areas. In contrast, generalized alteration involves widespread morphological alterations throughout the tissue, with few or no regions containing normal hepatocytes. Key morphological indicators of hepatocyte damage include the loss of cell membrane integrity, cell fusion, nuclear displacement to the periphery, karyolysis, and the presence of intracellular vacuoles. After individual classification of liver samples in a double-blind study, samples were identified by group, and the categorized data were converted into frequencies for statistical analysis.

Gene expression

To assess the metabolic response in the liver, the expression of genes involved in different physiological processes was analyzed: I) xenobiotic metabolism: cytochrome P450 family 1 (*cyp1a*), glutathione S-transferase pi (*gstπ*), glutathione S-transferase tandem duplicated alpha 1 (*gst1a*); II) antioxidant defense system: soluble superoxide dismutase 1 (*sod1*), mitochondrial superoxide dismutase 2 (*sod2*), catalase (*cat*),

glutathione peroxidase 1a (*gpx*), glutamate-cysteine ligase (*gcl*); III) steroid metabolism: cytochrome P450 family 19 subfamily A (*cyp19a1a*).

Following euthanasia, four fish per aquarium (20 in total per treatment) were dissected for liver collection. The tissues were individually stored in cryotubes and immediately frozen in liquid nitrogen, then kept at -80 °C in an ultrafreezer until molecular analysis. Frozen liver samples were individually homogenized in 1 mL of Trizol (Invitrogen, Brazil) for total RNA extraction. Following homogenization in 1mL of Trizol, samples were centrifuged at 12,000 x g for 10 min at4°C to remove debris, and the supernatant mas used for RNA extraction. The samples were treated with DNase I (Invitrogen, Brazil) and quantified using a Qubit fluorometer (Invitrogen, Brazil). RNA quality and integrity were verified by 1% agarose gel electrophoresis. RNA was extracted individually from 20 fish per treatment group and subsequently pooled in randomized pairs (two samples per pool) prior to analysis, resulting in n= 10 biological replicates per treatment group. This pooling strategy was adopted to account for individual variability while maintaining statistical power. RNA samples were then used for cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil), following the manufacturer's protocols.

Gene expression quantification was performed by real-time quantitative PCR (qPCR) using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Brazil) in 96-well plates. Reactions were carried out in duplicate, using cDNA as a template, specific primers (Table 1), and PowerUp SYBR Green Master Mix (Applied Biosystems, Brazil), with a final reaction volume of 15 µL per well. The thermal cycling conditions were: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min, with final steps at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s.

The ΔCt for each sample was calculated by subtracting the geometric mean Ct of the reference genes from Ct of the target gene. The ΔΔCt value was obtained by subtracting the average ΔCt of the control group (CF) from each individual ΔCt. Relative gene expression was then calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All reactions showing abnormal melting curves or amplification inconsistencies were excludes from the analysis. The reference genes used for expression normalization were *rpl13a* (ribosomal protein L13 alpha) and *eef1α* (eukaryotic elongation factor 1 alpha), tested using geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002).

Table 1 Analyzed genes, primer sequences used for qPCR, and their respective efficiencies. F: forward; R: reverse.

Gene	**Sequence (5´-3´)**	**Efficiency (%)**	**GenBank accession number**
cat	F: aacaacacccccatcttctttat R: atgtgtgtctgggtaggagaaaa	103	BC051626
cyp1a	F:cgcttgcatggccttgtc R: gcggtatgtttgaaagcacaaa	100	NM131879
cyp19a1a	F:cgcttgcatggccttgtc R: gcggtatgtttgaaagcacaaa	107	NM131879
gcl	F: aggcctgagtatggcagcta R: gtggtccgattcgttctcat	116	BC068331
gpx	F: gaagaaatcctgcagtctctgaa	109	BC083461

	R: gaaccttctgctgtacctcttga		
gst pi	F: cagttgcctaaatttgaagatgg R: agcttccagaagatgaacatcag	123	BI979167
gst1a	F: cgcaggaaaatacaacctctatg R: agcttccagaagatgaacatcag	106	BC060914
sod1	F: caccgtctatttcaatcaagagg R: agaatgttggcctgacaaagtta	114	BC055516
sod2	F: tctccctgacctcacatatgact R: tggcagctgatatcttctctttc	105	BC060895
rpl13a	F: tctggaggactgtaagaggtatgc R: agacgcacaatcttgagagcag	99	NM212784
eef1α	F: caaaattggaggtattggaactgtac R: tcaacagacttgacctcagtggtt	99	NM131263

Statistical analyses

Growth was assessed using the Shapiro-Wilk test for normality, the Bartlett test for homoscedasticity, one-way analysis of variance (ANOVA), and the Tukey post-hoc test. The villi morphometry data were analyzed using the Kruskal-Wallis test. Liver histopathology was analyzed using the chi-square test (χ^2) and standardized residual analysis to identify differences between observed and expected values. These tests were performed using the R software (2024). Gene expression was analyzed using the Shapiro-Wilk test for normality and the Bartlett test for homoscedasticity. The gene expression data (mean ± standard error) were analyzed using an unpaired Student's t-test or Mann-Whitney U test when assumptions of normality or homoscedasticity were not met, with a significance level of $p < 0.05$, using GraphPad Prism 9.0 software.

Results

Growth

The final weight of the fish in the control group was 167 ± 6 mg, with a final total length of 25.3 ± 0.3 mm. In the group fed with the *S. elongatus*-supplemented diet, the final weight was 175 ± 8 mg and the total length was 25.1 ± 0.4 mm. No statistically significant differences were observed between the groups ($p > 0.05$).

Intestinal morphometry

The intestinal villus length in the control diet (CF) group ranged from 76.78 to 121.03 µm, with a median and standard deviation of 99.03 ± 12.49 µm. In the EF group, values ranged from 74.66 to 115.05 µm, with a median and standard deviation of 91.22 ± 13.20 µm. The data distribution is shown in Figure 1B. Statistically, *S. elongatus* supplementation had no significant effect ($p > 0.05$) on the morphology of intestinal villi in fish.

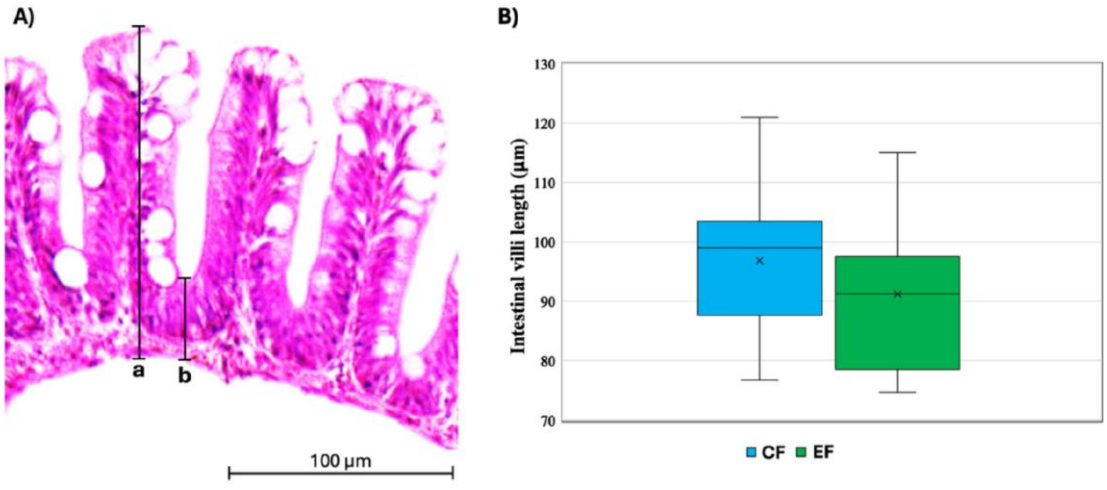

Figure 1. Intestinal villi morphology in the rostral to mid-intestine section of zebrafish (*Danio rerio*). (A) Measurement of villus length obtained by subtracting "a" from "b", where "a" is the length from the serosal layer to the epithelium, and "b" is the length

from the serosal layer to the submucosa. (B) Distribution of villus length data by group (CF: commercial feed; EF: experimental feed), showing the range between the minimum (⊥) and maximum (┬) values, the mean (X), and the median (—).

Liver histopathology

Fish in the CF group exhibited a higher frequency of generalized liver alteration (62%), characterized as hepatic steatosis, evidenced by the presence of intracellular vacuoles that deformed and compromised cell membrane integrity. Each fish was assigned a single categorical classification: normal (Figure 2A), multifocal (Figure 2B), or generalized alteration (Figure 2C), based on the most representative pattern observed across all histological sections. In cases where more than one pattern was present, the predominant or most extensive alterations was used for classification. Consequently, the reported percentages reflect the proportion of individuals in each group exhibiting each type of liver condition.

Fish fed the cyanobacteria-supplemented diet (EF) exhibited livers with normal morphology, multifocal alteration, and generalized alteration, with multifocal alteration being the most frequent condition (46 %). Statistical analyses revealed significant differences between groups in the frequency of multifocal alteration ($p < 0.05$), which was predominant in the EF group but absent in fish fed the commercial diet (Figure 3).

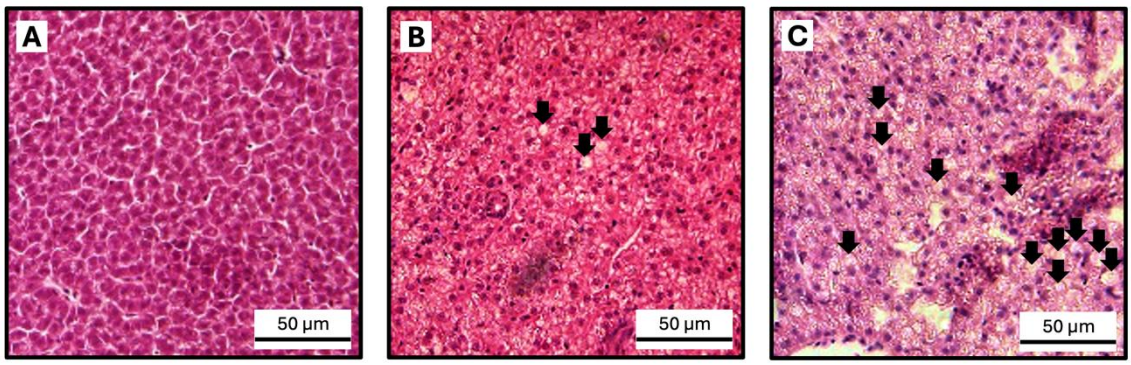

Figure 2. Histological assessment of *Danio rerio* livers stained with hematoxylin and eosin. (A) Liver with normal morphology, showing hepatocytes with a well defined cell membrane, central nucleus, and homogeneous cytoplasm. (B) Liver with multifocal alteration, characterized by localized morphological alterations in isolated areas of the tissue interspersed with normal regions. Intracytoplasmic vacuoles are observed, altering hepatocyte morphology; (C) Liver with generalized alteration, showing morphological alterations distributed throughout the tissue. Arrows indicate the presence of intracytoplasmic vacuoles.

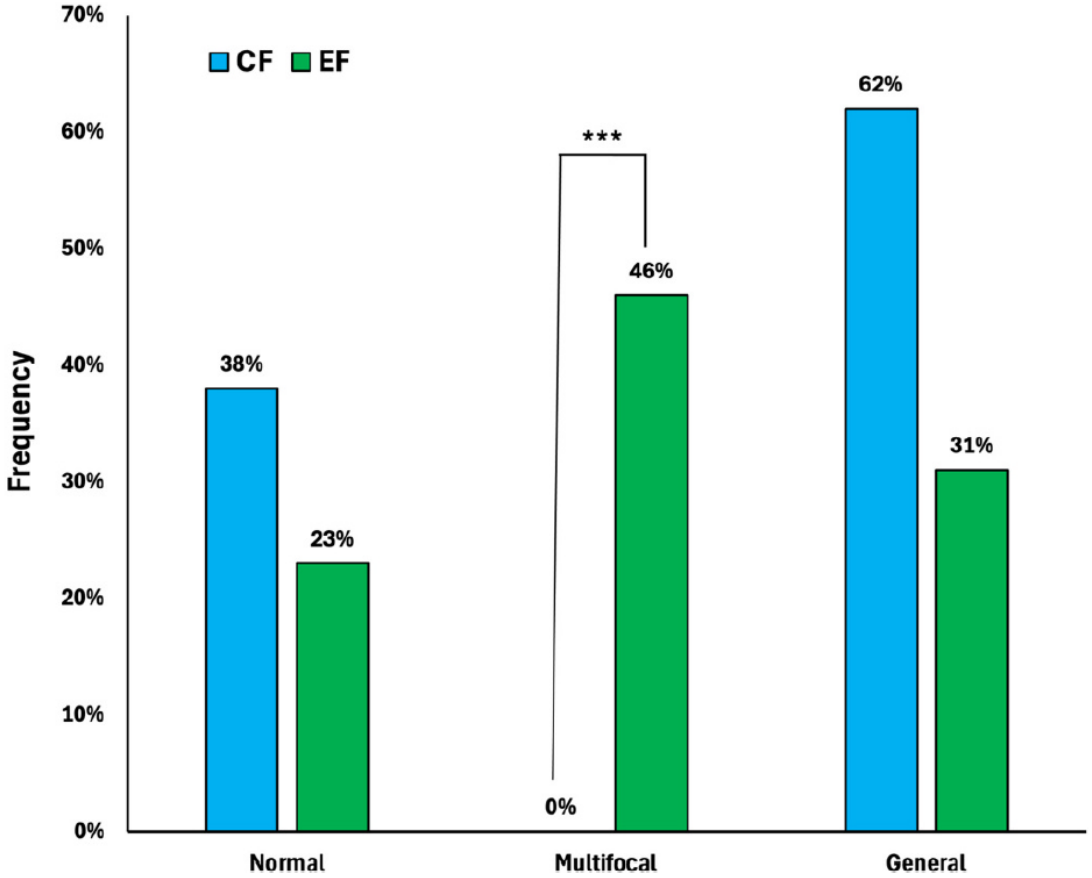

Figure 3. Frequency distribution of liver histopathological patterns in zebrafish (*Danio rerio*) fed a commercial diet (CF) or a diet supplemented with *Synechococcus elongatus* PCC 7942 (EF). Liver conditions were categorized as normal, multifocal alteration, or generalized alteration. Each fish was assigned a single classification based on the predominant histological pattern observed. A significantly higher frequency of multifocal alteration was observed in the EF group compared to the CF group (*** $p < 0.001$).

Gene Expression

Gene expression analysis revealed a consistent reduction in the hepatic expression of genes related to detoxification, antioxidant defense and steroid metabolism in zebrafish fed the *S. elongatus*-supplemented diet (Figure 4). The genes *cyp1a*, *gst1a* and *gst pi*, which are involved in xenobiotic metabolism, showed

reductions of 80%, 85% and 89%, respectively. Genes involved in antioxidant defense also showed substantial downregulation with *sod1*, *sod2*, *cat* and *gcl* expression reduced by 84%, 82%, 83% and 76% respectively. The gene *gpx* showed 92% reduction, although this difference was not statistically significant, likely due to high intra-group variability. Additionally, *cyp19a1a*, a gene associated with estrogen synthesis, was downregulated by 90 %.

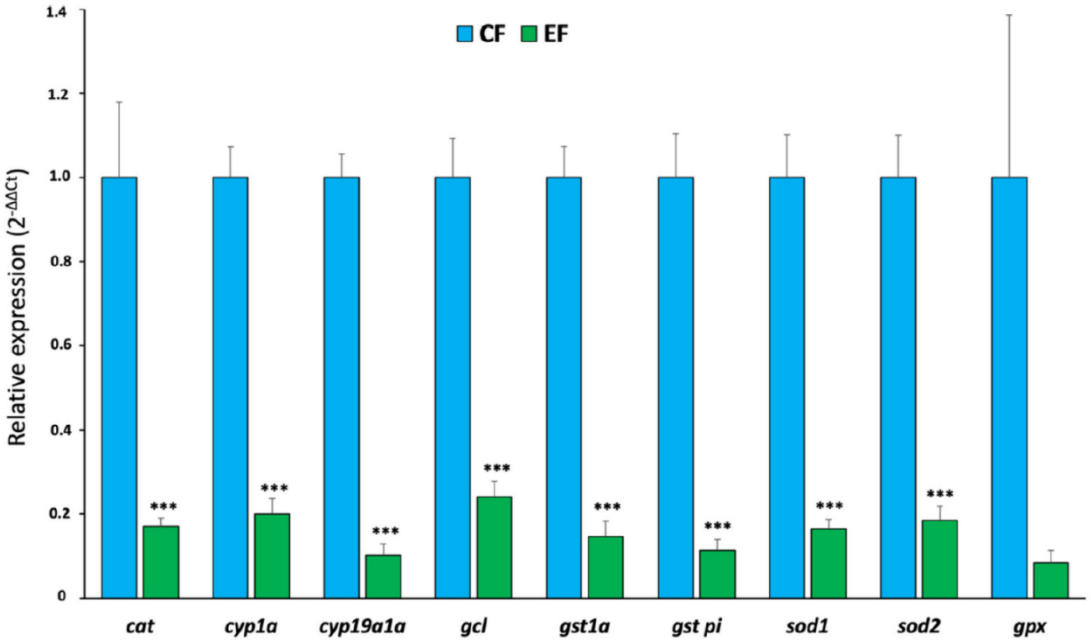

Figure 4 Relative expression of detoxification, antioxidant, and steroid metabolism genes in the liver of zebrafish (*Danio rerio*) fed a commercial diet (CF) or a commercial diet supplemented with *Synechococcus elongatus* PCC 7942 (EF) for 35 days. Gene expression was quantified by qPCR using the 2-ΔΔCt method, with normalization to the geometric mean of two reference genes (*eef1α* and *rpl13a*). Bars represent the mean ± standard error (n = 10 per group). Statistical comparisons between groups were performed using Student's t-test or Mann–Whitney U test when assumptions of normality or homoscedasticity were not met. Asterisks (***) indicate statistically significant differences between groups ($p < 0.001$).

Discussion

This study evaluated the effects of dietary supplementation with *S. elongatus* PCC7942 on zebrafish intestinal morphology, liver histology, and the expression of genes involved in detoxification and antioxidant defense. Overall, the results indicate that the inclusion of *S. elongatus* in the diet was well tolerated. No significant changes were observed in intestinal villus length, suggesting preserved absorptive function. In the liver, a shift in the histopathological profile was observed, with a reduction in the frequency of generalized alteration and a higher incidence of multifocal alteration in fish receiving *S. elongatus*, potentially indicating milder hepatic alteration. At the molecular level, the expression of detoxification-related genes and most antioxidant enzymes was downregulated, which may reflect reduced oxidative challenge in response to the supplemented diet. It is important to note, however, that gene expression analysis do not always reflect functional enzyme activity, as post-transcriptional, translational, and post-translational mechanisms may decouple mRNA levels from physiological function (Greenbaum et al., 2003). Therefore, further studies are necessary to assess whether the observed transcriptional changes translate into functional metabolic alterations.

Intestinal villus length did not differ significantly between groups, indicating that dietary supplementation with *S. elongatus* did not affect the absorptive surface or induce structural remodeling of the intestinal mucosa. This results suggest that the cyanobacterium was well tolerated by the intestinal tract and did not elicit trophic or inflammatory responses. Previous studies have shown that intestinal villus morphology is highly responsive to dietary composition, particularly to protein content, trace elements and functional additives (Özel et al., 2018; Anwar et al., 2024). For instance, diets supplemented with spirulina or *Chlorella vulgaris* have been associated with

increased villus height and improved nutrient absorption in fish species such as tilapia and carp (Youssef et al., 2023; El-Mashtoly et al., 2024). The absence of such changes in the present study may reflect the balanced nutritional profile of the diets tested, as well as the lack of a stimulatory effect of the *S. elongatus* on intestinal epithelial turnover. Additionally, no signs of tissue alteration or inflammatory were observed in the intestinal histology of either group, further confirming the safety of *S. elongatus* for the gastrointestinal tract of zebrafish.

Liver histopathology revealed contrasting profiles between dietary groups. In the control group (CF), generalized hepatic alteration was predominant and characterized by diffuse steatosis, with widespread hepatocellular vacuolization and compromised membrane integrity. In contrast, fish in the EF group exhibited a reduced frequency of generalized alterations and higher proportion of multifocal alterations, with localized lesions interspersed with normal parenchyma. Although multifocal alteration may still indicate a pathological condition, this pattern is generally considered less severe an may reflect a milder or recovering hepatic status (Wolf and Wolfe, 2005). These findings suggest that supplementation with S. elongatus may have attenuated the progression of liver injury associated with the control diet. Notably, no signs of necrosis, inflammation, or fibrosis were detected in either group, indicating that the hepatic alterations observed are likely reversible and non-degenerative. The observed differences may be linked to antioxidant or lipid-modulating compounds naturally present in *S. elongatus*, which have been reported in other cyanobacterial species used as functional feed additives (Rosas et al., 2019; Faheem et al., 2022).

The observed downregulation of *cyp1a*, *gst1a* and *gst pi* in the EF group suggest that the xenobiotic metabolism pathway was not activated in response to dietary supplementation with S. elongatus. As the gene expression analysis was conducted in

the liver, this finding indicates that no significant hepatic stimulus for detoxification was present. One possible explanation is that the components of S. elongatus absorbed after digestion were not recognized as reactive or harmful by the organism. Alternatively, the supplement may content compounds capable of binding, neutralizing or modulating potential xenobiotic activity. Previous studies have reported that cyanobacteria such as *S. elongatus* produce a variety or bioactive substances, including phycobiliproteins, carotenoids and phenolic compounds, which may contribute to redox balance and interfere with phase I and II metabolic responses (Faheem et al., 2022; Ahmad et al., 2023). These mechanisms may underlie the observed reduction in detoxification gene expression and the absence of hepatocellular alteration in the EF group.

The expression of most antioxidant genes was significantly reduced in fish fed the *S. elongatus*-supplemented diet, suggesting a diminished requirement for enzymatic antioxidant defense. This finding is consistent with the absence of histological liver alteration in this group and may reflect lower oxidative stress compared to fish maintained on the control diet. Although *gpx* expression was not significantly different between groups, mean values followed the same downward trend observed for other antioxidant-related genes. However, due to high intragroup variability, this result should be interpreted with caution. Several cyanobacteria species are known to contain non-enzymatic antioxidants such as carotenoids, phycobiliproteins and phenolic compounds, which may contribute to the mitigation of oxidative stress (Ahmad et al., 2023; Faheem et al., 2022). Therefore, the observed reduction in gene expression may indicate a shift in redox balance driven by dietary antioxidants, potentially reducing the need for endogenous enzymatic responses.

The expression of *cyp19a1a*, which encodes aromatase A and catalyzes the conversion of androgens into estrogens, was significantly reduced in fish fed *S.*

elongatus compared to the control group. This finding suggests that *S. elongatus* may exert a modulatory effect on estrogen biosynthesis pathways. While no direct evidence currently links *S. elongatus* to aromatase inhibition, other cyanobacteria such as *Microcystis aeruginosa* have been show to alter *cyp19a1a* expression in fish, possibly through bioactive compounds that interfere with endocrine signaling (Zhang et al., 2025). The observed reduction may therefore result from metabolites produces by s. elongatus, including phycobiliproteins or phenolic compounds. Although reproductive parameters were not assessed in this study, these results indicate potential endocrine-modulating effects that warrant further investigation of gonadal development, hormone levels and reproductive performance.

These potential endocrine effects should be considered alongside the digestive physiology on zebrafish. The feeding activity of Danio rerio begins with food recognition involving multiple sensory stimuli (Licitra et al., 2024), and following ingestion, digestion is initiated through the combined action of digestive enzymes and intestinal contractions (Farrell et al., 2011). Notably the absence of a stomach in this species means bile-released trypsin acts directly in the intestine, potentially influencing the bioavailability of S. elongatus compounds that may affect endocrine pathways. This unique digestive physiology may therefore play a role in both the nutrient utilization and any bioactive effects of the cyanobacterial supplement.

The intestine is composed of four layers: mucosa, consisting of epithelial tissue; submucosa, made up supportive connective tissue, organized into fold and the lamina propria; muscular layer; and serosal layer. The connective tissue folds have a finger-like structure and gradually decrease in size along the rostrocaudal axis. Each of these folds is called villus (plural: *villi*), whose primary function is to increase the surface area for nutrient absorption. Morphologically, the zebrafish intestine is dived into three sections:

rostral bulb, mid-intestine and caudal region (Wallace et al., 2005). The zebrafish intestine exhibits functions analogous to those of the mammalian digestive tract, with the rostral bulb and mid-intestine performing roles similar to those of the small intestine, serving as the primary site for food digestion and nutrient absorption including fatty acids, organic acids, vitamins, glucose, carbohydrates and ions, and contribuiting to key metabolic and homeostatic functions (Wang et al., 2010). Therefore, this region is functionally comparable to the large intestine, where amino acid metabolism and water retention take place (Wang et al., 2010).

The intestine is a highly plastic surface, and variations in villus length are common among organisms of the same species when exposed to different diets. Özel et al. (2018) observed an increase in intestinal villus length in black trout (*Salmo labrax*) associated with a higher protein content in the diet. Similarly, Anwar et al. (2024) investigated different selenium levels in the diet of *Danio rerio* and found that the increase in villus length and width, as well as in intestinal muscle layer thickness, was dependent on selenium inclusion levels. Additionally, the inclusion of microorganisms in the diet can modify intestinal morphology, enhancing food digestion and nutrient absorption when villus length increases. Youssef et al. (2023) supplemented the diet of tilapia with *Spirulina* and observed an increase in villus length and width, which improved nutrient absorption, as evidenced by higher serum albumin levels and enhanced zootechnical performance. Similarly, El-Mashtoly et al. (2024) supplemented the diet of common carp (*Cyprinus carpio*) with *Chlorella vulgaris* and *Saccharomyces cerevisiae*, reporting an increase in villus size, higher serum protein content, improved zootechnical performance, and increased activity of innate immune and antioxidant system enzymes. In the present study, intestinal villus length showed no significant differences, which aligns with the homogeneous growth observed among fish in both

groups. These findings suggest that the incorporation of *S. elongatus* into the diet did not affect intestinal morphology or absorptive function. Additionally, no intestinal tissue damage or structural impairment was observed, indicating that *S. elongatus* supplementation was well tolerated by the digestive tract of the fish.

After digestion in the intestine, hydrolyzed nutrients are absorbed and transported through the bloodstream to the liver, a key organ in lipid metabolism (Nagaraj et al., 2012; Nelson & Cox, 2014). Hepatocytes convert nutrients into energy through the uptake of circulating lipids, lipogenesis, and fatty acid oxidation, while also synthesizing precursors that are exported to other tissues, such as very low-density lipoproteins (VLDL) (Nelson & Cox, 2014). When there is an imbalance in lipid homeostasis between uptake and export, excessive lipid accumulation can occur, leading to hepatic steatosis (Ipsen et al., 2018). This condition was observed in fish from both experimental groups, which suggests that the commercial feed used in this study may not be suitable for zebrafish. In fact, its protein content (50%) is slightly above the recommended level for zebrafish (37.5%) reported by Fernandes et al. (2016), while its lipid content (8%) aligns with the level suggested by O'Brine et al. (2015). The main difference may lie in the carbohydrate content, which is not specified in the commercial feed. Xi et al. (2023) state that diets with carbohydrate levels exceeding 30% can lead to lipid accumulation in zebrafish. Thus, the importance of developing species-specific diets becomes evident.

Other factors like exposure to xenobiotics can modulate the expression of obesogenic genes (Ibor et al., 2019). Xenobiotics are exogenous compounds, meaning substances that do not naturally belong to the fish's organism. The liver is the primary organ responsible for detoxifying these compounds through xenobiotic metabolism. This process begins when the xenobiotic is ingested or comes into contact with cells,

initiating its elimination, which occurs via two pathways depending on the compound's polarity: if hydrophilic, it can be directly filtered by the kidneys and excreted in urine; if apolar, it is transported through the bloodstream to the liver for metabolism. In the liver, phase I, or biotransformation, takes place, mediated by enzymes such as cytochrome P450 (*CYP1A*). This step converts the xenobiotic into a more water-soluble product, generating substrates for the subsequent phase. Phase II, known as conjugation, involves the binding of Phase I products with glutathione S-transferase (*GST*) and reduced glutathione (*GSH*), resulting in a neutralized and soluble metabolite that can be excreted in urine or feces (Stanley, 2017). Although *S. elongatus* does not produce toxins, unlike other cyanobacteria (Chorus & Welker, 2021; Williams et al., 2020), its inclusion in the diet classifies it as a xenobiotic. Therefore, it is essential to ensure that none of the compounds present in this strain induce toxic effects in fish, ensuring its safety for consumption.

In this study, the expression of *cyp1a* and *gst* genes was reduced in fish fed *S. elongatus*, suggesting two possible interpretations: detoxification metabolism is saturated – in this scenario, membrane transporters responsible for GSH uptake into hepatocytes would be coupled to a xenobiotic compound, blocking GSH entry. As a result, GST would have oxidized all available GSH, neutralizing the xenobiotic but failing to complete detoxification. This blockade could trigger oxidative stress-induced cellular damage, leading to excessive reactive oxygen species (ROS) production and causing adverse effects such as genotoxicity, lipid peroxidation, membrane denaturation, and apoptosis. However, this hypothesis would imply significant hepatic tissue damage and an overall decline in fish health (Cazenave et al., 2006; Jiao et al., 2020). Another hypothesis is: xenobiotic metabolism was not activated, indicating that the tested strain was not recognized by the organism as a toxic substance.

Histopathological analysis showed an improvement in liver health in fish fed *S. elongatus*, evidenced by a reduced frequency of generalized liver alteration and an increased occurrence of multifocal damage, suggesting a possible hepatic recovery process. Thus, based on histological evidence, the second hypothesis appears to be the most plausible.

The antioxidant defense system consists of mechanisms involved in the removal and neutralization of reactive oxygen species (ROS), such as the superoxide and hydroxyl radicals, which can induce oxidative stress (Halliwell and Gutteridge, 2015). Oxidative stress occurs when there is an imbalance between free radical production and the organism's capacity to neutralize them (Sies, 2015). The antioxidant defense system functions through enzymatic and non-enzymatic pathways (Lushchak, 2014). Among the primary enzymatic mechanisms, superoxide dismutase (SOD) converts the superoxide radical ($^{\cdot}O_2^-$) into hydrogen peroxide (H_2O_2) (McCord & Fridovich, 1969). Catalase (CAT) then degrades hydrogen peroxide, producing water (H_2O) and molecular oxygen (O_2) (Chevion,1988). Glutathione peroxidase (GPX), in conjunction with reduced glutathione (GSH), also degrades hydrogen peroxide, yielding water and oxidized glutathione (GSSG) (Brigelius-Flohé & Maiorino, 2013), which can be regenerated by glutathione reductase (GR) (Meister and Anderson, 1983). GSH is a tripeptide composed of glutamate, cysteine, and glycine, whose synthesis involves the action of two enzymes: glutamate-cysteine ligase (GCL), which requires ATP, and glycine synthase (GS), which is also ATP-dependent (Nelson & Cox, 2014).

Since the commercial diet used in this study already contains spirulina, it is not surprising that the expression of antioxidant enzymes is upregulated, as spirulina is well-documented to contain both enzymatic and non-enzymatic antioxidants (Ahmad et al., 2023; Coli et al., 2024; Faheem et al., 2022; Rosas et al., 2019). However,

supplementation with *S. elongatus* significantly reduced the expression of most antioxidant enzymes, except for *gpx*. However, the lack of a significant difference for this important gene may be explained by high variance among the samples, as the means were considerably different between the analyzed groups and followed the same expression pattern as the other related genes. This finding suggests that hepatic lipids are being metabolized within hepatocyte peroxisomes, leading to hydrogen peroxide production and the subsequent stimulation of *gpx* expression. This mechanism may generate a substrate-enzyme feedback loop, while non-enzymatic antioxidant compounds may contribute to lipid peroxidation neutralization, reducing the need for increased expression of other antioxidant enzymes.

It appears that the combination of *S. elongatus* and spirulina exerts a synergistic effect on lipid metabolism, mitigating liver alteration through the action of GPX and non-enzymatic antioxidant compounds. This observation aligns with the decreased incidence of hepatic steatosis in fish. Therefore, the reduction in antioxidant enzyme expression in fish supplemented with *S. elongatus* may be associated with decreased oxidative stress and, consequently, lower ROS production. With fewer lipids requiring breakdown, hepatic homeostasis improves, resulting in reduced ROS generation and a diminished stimulus for the expression of other antioxidant enzymes. Recent studies have investigated the association between cyanobacterial compounds and steroid metabolism. Exposure of *Daphnia magna* to *Microcystis aeruginosa* has been reported to alter lipid production, promoting reproduction through its influence on reproductive hormones (Zhang et al., 2025). One way to assess this influence is through the expression of the aromatase *CYP19a1a*, an enzyme that catalyzes the conversion of androgens into estrogen and plays a crucial role in female fish development and sexual differentiation (Uno et al., 2012).

In this study, the commercial diet containing spirulina stimulated *CYP19a1a* expression. Given the role of this enzyme in estrogen biosynthesis, this finding suggests a potential effect on female gonadal development and progeny survival, as proposed by Shaw et al. (2023). This effect may also be linked to the improved synthesis and deposition of vitellogenin in *Danio rerio* oocytes fed a spirulina-supplemented diet, as described by Coli et al. (2024). Although this aspect was not deeply explored in the present study, it represents a promising avenue for future research.

Conclusions

The results of this study suggest that supplementing the commercial diet with *S. elongatus* in *Danio rerio* did not negatively impact intestinal morphology or absorptive function. Furthermore, no signs of toxicity associated with the consumption of this cyanobacterium were observed. The reduction in the expression of most antioxidant enzymes, suggests a possible synergistic effect between spirulina and *S. elongatus*, contributing to oxidative stress reduction through non-enzymatic antioxidant compounds. The improvement in histopathological parameters indicates that *S. elongatus* may serve as a safe dietary supplement for fish without detectable adverse effects.

Declaration of Competing Interest

The authors declare no conflict of interest.

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CRediT authorship contribution statement

Mirna Reyes: Methodology, Investigation, Conceptualization, Validation, Writing - review and editing. **Andrea Hernandez:** Methodology. **Raíza Azevedo**: Methodology. **Beatriz Figueiredo**: Methodology. **Isaac Flores**: Methodology. **Arthur Cardoso**: Methodology. **Tony Silveira:** Methodology. **Iuri Anni:** Methodology. **Antonio Sergio Varela Junior**: Methodology, Resources, Validation. **Bruna Nornberg:** Methodology, Supervision, Conceptualization, Writing – review and editing. **Luis Fernando Marins:** Conceptualization, Investigation, Validation, Project administration, Resources, Supervision, Writing - review and editing.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CAPÍTULO III. Impact of the cyanobacterium *Synechococcus elongatus* PCC 7942 as a dietary supplement on the intestinal microbiota of zebrafish (*Danio rerio*)

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Impact of the cyanobacterium *Synechococcus elongatus* PCC 7942 as a dietary supplement on the intestinal microbiota of zebrafish (*Danio rerio*)

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Author's contributions

The conceptualization of the study was carried out by M.L.E.R., L.F.M. and B.F.S.N. Formal analysis was conducted by R.S.A. and B.F.S.N. Funding for the project was acquired by L.F.M. The investigation was undertaken by M.L.E.R., L.F.M. and B.F.S.N., while the methodology was developed by R.S.A. and B.F.S.N. Project administration was handled by L.F.M., and resources were provided by L.F.M. Supervision was conducted by L.F.M., with validation performed by M.L.E.R., L.F.M. and B.F.S.N. The original draft was written by L.F.M. and B.F.S.N., and the manuscript was reviewed and edited by all authors.

Abstract

This study investigated the effects of dietary supplementation with *Synechococcus elongatus* PCC 7942 on the intestinal microbiota of zebrafish (*Danio rerio*). Using metataxonomic analysis based on 16S rRNA gene sequencing, the impact of supplementation on microbial composition and diversity was assessed. Results revealed that the intestinal microbiota remained generally stable, with no significant changes in alpha (Shannon and Simpson) or beta (Bray-Curtis) diversity indices, suggesting that supplementation did not compromise overall microbial health. Despite this general stability, a significant reduction in the relative abundance of the genus *Pirellula* was observed in the treated group. This genus, belonging to the Planctomycetota phylum, is associated with the degradation of complex organic compounds, and its decline may indicate specific interactions between *S. elongatus* metabolites and the basal microbiota. Additionally, micoplasmas were exclusively detected in control samples, suggesting that supplementation may have created an intestinal environment less conducive to colonization by these opportunistic microorganisms. Another relevant factor was the dietary context, as the feed used included *Arthrospira platensis*, known to promote microbial stability. This likely contributed to the resilience of the microbiota, mitigating the effects of new interventions. The absence of dysbiosis and the selective metabolic impact of *S. elongatus* highlight its potential as a safe and functional dietary supplement in aquaculture.

Keywords: cyanobacteria; dietary supplementation; gut microbiome; metataxonomic analysis.

1. Introduction

Cyanobacteria are essential components of aquatic ecosystems, playing a critical role in carbon dioxide (CO_2) fixation and oxygen production, thus significantly contributing to global carbon and oxygen cycles (Singh et al., 2016). Through photosynthesis, these microorganisms sustain aquatic food chains, supporting biodiversity across multiple trophic levels and promoting ecological stability. Beyond their ecological importance, some cyanobacteria are recognized for their ability to produce a wide range of bioactive compounds with antibacterial, antiviral, and anticancer properties, positioning them as promising sources for the development of novel pharmaceuticals (Nowruzi et al., 2018). Moreover, due to their high growth rate and photosynthetic efficiency, they are considered ideal candidates for the sustainable production of biofuels such as biodiesel, contributing to renewable energy alternatives (Bhandari & Sharma, 2006). In agriculture, cyanobacteria act as biofertilizers, enhancing soil fertility through biological nitrogen fixation and promoting plant growth (Roeselers et al., 2008). Additionally, they play a crucial role in bioremediation by removing metallic contaminants from the environment (Mota et al., 2016).

Another significant attribute of cyanobacteria is their nutritional profile, which includes high-quality biomolecules such as proteins, carbohydrates, and essential fatty acids. According to Passos et al. (2023), who conducted a comprehensive systematic analysis of the composition of these molecules across various cyanobacteria, some species are rich in polyunsaturated fatty acids like alpha-linolenic acid (ALA) and other omega-3 precursors, known for their cardiovascular and immunological health benefits in humans and animals. The presence of these essential compounds positions certain species as promising dietary supplements, particularly in contexts such as animal production, where nutritional quality is a key parameter. In addition to fatty acids, these

photosynthetic microorganisms contain high levels of proteins, providing essential amino acids to aquatic organisms, thereby promoting growth and overall health. Combined with carbohydrates, these proteins also act as prebiotic agents capable of influencing the host's intestinal microbiota, offering benefits for animal production systems aimed at optimizing intestinal health.

From a biotechnological perspective, certain strains of *Synechococcus* demonstrate robustness and adaptability, making them ideal for various industrial applications. The UTEX 2973 strain, a fast-growing variant of *Synechococcus elongatus*, is a notable example. This strain can grow under high-light conditions and elevated CO_2 concentrations, making it advantageous for biofuel production and the generation of high-value compounds such as omega-3 fatty acids. Due to its efficiency in converting CO_2 into biomass and its ease of genetic modification, UTEX 2973 is widely used in bioproduction processes that require high productivity (Sengupta et al., 2024).

Synechococcus elongatus PCC 7942 has gained attention in biotechnology due to its well-characterized genome, which facilitates genetic engineering and transformation. Unlike many microalgae, this strain contains two endogenous plasmids (pANL and pANS), allowing the insertion of genes encoding bioactive compounds, enzymes, and metabolites of interest. This characteristic positions *S. elongatus* PCC 7942 as an excellent candidate for use as a living biofactory, producing functional molecules that could enhance fish health and nutrition. Moreover, unlike toxin-producing cyanobacteria, *S. elongatus* PCC 7942 has not been associated with the production of harmful secondary metabolites, supporting its safety as a feed supplement. Its compact genome, approximately 2.7 megabases (Mb), includes two endogenous plasmids (pANL and pANS), which facilitate genetic transformation and

modification for the production of bioactive compounds. This genomic organization enables the insertion of specific genes and the exploration of metabolic pathways for the production of biofuels (such as ethanol and isoprene), essential fatty acids, and other value-added metabolites like 3-hydroxypropionate and propanediol. The genetic transformability of *S. elongatus* PCC 7942 was a milestone that paved the way for its use in genetic engineering, making it an efficient and sustainable biofactory (Jaiswal et al., 2020; Sengupta et al., 2024). Given its genetic plasticity and ability to produce bioactive molecules, *S. elongatus* PCC 7942 emerges as a promising candidate for aquaculture applications.

Despite the significant biotechnological potential of *Synechococcus*, the use of these cyanobacteria in aquatic systems raises concerns about toxin production by certain species, especially under environmental stress conditions. According to the review by Jakubowska & Szeląg-Wasielewska (2015), some *Synechococcus* strains can produce toxins such as microcystins and nodularins, which may cause significant neurodegenerative and ecological impacts, particularly under eutrophication and high-temperature conditions. These toxins can bioaccumulate in the food chain, posing risks to aquatic organisms and humans. Furthermore, *Synechococcus* can produce compounds like geosmin and 2-methylisoborneol, which alter the taste and odor of drinking water. Toxic blooms associated with *Synechococcus* have also been reported in tropical environments, highlighting the importance of monitoring and controlling the use of these cyanobacteria in natural systems or as dietary supplements.

In this study, the objective was to investigate the effects of using *Synechococcus elongatus* PCC 7942 as a dietary supplement on the intestinal microbiota of *Danio rerio* (zebrafish). The central hypothesis is that supplementation with *S. elongatus* may induce changes in the intestinal microbiota. To evaluate this hypothesis, an analysis of

the 16S ribosomal gene from the fish intestinal DNA was performed, using third-generation sequencing to map changes in the microbial community. This study aims to provide a comprehensive assessment of the risks and benefits associated with the use of *Synechococcus* in aquaculture, contributing to safe and sustainable fish farming practices.

2. Material and methods

2.1 Production and maintenance of zebrafish

Zebrafish (*Danio rerio*) were used as the experimental model. All protocols followed the ethical guidelines established by Brazilian regulations, including Law No. 11.794/2008, Decree No. 6.899/2009, and the National Council for the Control of Animal Experimentation (CONCEA, Brazil). A total of 120 zebrafish from the AB strain, aged four months, were used in the experiment. The entire process of reproduction, larviculture, and maintenance to obtain the animals was carried out at the Transgenic Fish Facility of the Federal University of Rio Grande (FURG, Brazil). During growth and maintenance, fish were fed *ad libitum* twice a day with Discus Gran D-50 Plus commercial feed (Tropical, Brazil). According to the manufacturer, this feed contains 50% protein from fish and fish by-products, along with plant protein extracts, plant-based by-products (including 3,000 mg/kg of red pepper extract), mollusks, crustaceans, cereals, and algae (*Arthrospira platensis*, minimum 1.5%). Additionally, it includes yeast, oils, fats, and minerals such as zeolite (1%). Additives (per kg): vitamin A 31,000 IU, vitamin D3 1,950 IU, vitamin E 110 mg, vitamin C 550 mg, beta-carotene 140 mg. Minimum concentrations: iron 40.5 mg/kg, zinc 11.2 mg/kg, manganese 8.4 mg/kg, copper 2.0 mg/kg, iodine 0.24 mg/kg, selenium 0.24 mg/kg, molybdenum 0.05

mg/kg, astaxanthin 120 mg/kg, crude protein 50%, crude fat 7.5%, crude fiber 3%, moisture 8%. Until reaching the desired age, the fish were kept in closed recirculating water systems at an average temperature of 27 °C with a photoperiod of 12 hours of light and 12 hours of darkness, and the water quality parameters were maintained as follows: pH = 6.7, dissolved oxygen = 7 mg/L, conductivity = 1,011 µS, salinity = 0.16 ppm, NH_3 and NO_2 = 0 mg/L.

2.2 Experimental design

The experimental design was approved by the Ethics Committee on Animal Use of the Federal University of Rio Grande (CEUA-FURG) under certificate CEUA-FURG 23116.003565/2023-41. Two treatments were established, each consisting of five replicates (12 fish per replicate). The fish were randomly distributed into two independent recirculating water systems, with each system containing 12 aquariums. In the control group, the fish were fed the previously described commercial Discus Gran D-50 Plus feed (Tropical, Brazil). In the treated group, the fish were fed the same commercial feed supplemented with *S. elongatus* PCC 7942.

For feed supplementation, cyanobacteria were cultured in BG-11 medium at 34 °C without agitation under constant fluorescent light (50 µmol/m²/s) until reaching OD_{750} = 1 (~10^8 cells/mL). The culture (10 mL) was then centrifuged at 1,500 × g (4 °C, 10 min), and the supernatant was discarded. The pellet was washed twice with 3 mL of phosphate-buffered saline (PBS) and resuspended in 5 mL of PBS to obtain a final concentration of 2 × 10^8 cells/mL. Although the chosen concentration was based on prior studies using cyanobacterial supplements in aquaculture, future research should investigate different supplementation levels to determine optimal dosing strategies. This solution was mixed with 5 g of feed, homogenized, and dried at 28 °C for 24 hours. The

dried feed was sieved to obtain granules between 100 and 500 µm and stored at 4 °C for a maximum of 15 days.

In all treatments, diets were administered three times a day *ad libitum*. The initial mean weight of the fish was 125 ± 5 mg for the control group and 126 ± 3 mg for the treated group, with no significant differences between treatments. At the end of the 35-day experiment, the fish were euthanized with a lethal dose of anesthetic (MS-222, 400 mg/L). Following euthanasia, fish were weighed and intestinal samples were collected for metataxonomic analyses. The dissected intestinal tissues were immediately stored in liquid nitrogen and subsequently maintained at -80 °C.

2.3 Metataxonomic analysis

2.3.1 DNA extraction and polymerase chain reaction (PCR)

To assess the impact of *S. elongatus* supplementation on the zebrafish intestinal microbiota, metataxonomic analysis was performed. Intestines were collected from four randomly selected fish per experimental group. Genomic DNA (gDNA) was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, Brazil). The concentration was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Brazil), purity was assessed with a NanoDrop 1000 spectrophotometer, and integrity was verified by 0.8% agarose gel electrophoresis.

The metataxonomic analyses were conducted individually for each sample. The full-length 16S rRNA gene (V1–V9 regions) was amplified from the gDNA of each sample via PCR using universal primers 16S-27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16S-1492R (5′-TACCTTGTTACGACTT-3′), targeting a region of approximately 1,500 bp. The PCRs were performed in a 25 µL reaction volume using

Platinum Taq DNA Polymerase (Invitrogen, Brazil), comprising 2.5 μL of 10X PCR buffer, 0.5 μL of 10 mM dNTPs, 0.75 μL of 50 mM $MgCl_2$, 0.5 μL of gDNA, and 0.2 μM of each primer, as per the manufacturer's instructions. The reactions were run on a Proflex thermocycler (Applied Technology, Brazil) using the following cycling conditions: initial denaturation at 94 °C for 2 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds, extension at 72 °C for 1 minute and 30 seconds, and a final extension at 72 °C for 5 minutes. A no-template control was included to ensure no amplification from contamination.

The PCR products were then purified using the PureLink PCR Purification Kit (Invitrogen, Brazil), quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Brazil), analyzed for purity using the NanoDrop 1000 spectrophotometer, and checked for integrity by 1% agarose gel electrophoresis. Amplicons from different samples were normalized to 200 ng of DNA per sample for sequencing.

2.3.2 Library preparation and sequencing

The Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technologies, Brazil) was used to prepare the amplicon library for loading onto the PromethION 2 (Oxford Nanopore Technologies, Brazil), following the manufacturer's protocol. DNA samples consisted of 1.5 μg of DNA with barcodes in a volume of 11.5 μL and 1 μL of control DNA (lambda DNA, used as a positive control for sequencing). DNA was processed for end repair and adenine tailing (dA-tailing) using the NEBNext Ultra II End Repair / dA-tailing Module (New England Biolabs, Brazil) and incubated at 20 °C for 5 minutes, followed by 65 °C for 5 minutes.

During the barcode ligation step, 0.75 μL of each prepared DNA sample was added to a mixture containing 5 μL of Blunt/TA ligase master mix (New England

Biolabs) and 1.25 μL of each barcode. The reaction was incubated at room temperature for 20 minutes. Adapter ligation was performed in a reaction containing 5 μL of Native Adapter (NA), 10 μL of NEBNext Quick Ligation Reaction Buffer (5X), and 5 μL of Quick T4 DNA Ligase (New England Biolabs, Brazil). The reaction was incubated for 20 minutes at room temperature and subsequently purified with AMPure XP Beads (AXP) (Beckman Coulter, Brazil).

For sequencing, 12 μL of the DNA library was mixed with Library Loading Beads (LIB) (25.5 μL) and Sequencing Buffer (SB) (35.5 μL). The run was performed using a PromethION 2 flow cell (FLO-PRO114M, R10.4.1). After verifying the quality of the flow cell, it was prepared with a mixture containing 1,170 μL of Flow Cell Flush (FCF), 5 μL of Bovine Serum Albumin (BSA) at 50 mg/mL, and 30 μL of Flow Cell Tether (FCT). Immediately after preparation, the library was gradually loaded through the inlet port. Once loaded, a standard 5-hour sequencing protocol was initiated using MinKNOW software (Oxford Nanopore Technologies).

The generated pod5 files were processed into FASTQ format via base-calling in super accuracy mode, demultiplexed, and trimmed using the Dorado software (v0.8.3 basecaller model “dna_r10.4.1_e8.2_400bps_sup@v4.3.0”).

2.3.3 Data analyses

The FASTQ files generated were used as input for a custom 16S analysis pipeline in the EPI2ME software (v1.3.0) (Ewels et al., 2020), employing several bioinformatics tools. These included Pysam (v0.21.0) for SAM/BAM file manipulation, Pandas (v2.0.3) for data processing and analysis, Fastcat (v0.15.1) for FASTA/FASTQ read manipulation, Samtools (v1.18) for alignment file processing and indexing, Taxonkit (v0.15.1) for taxonomic data handling, and Kraken2 (v2.1.3) for taxonomic

classification of reads. The wf-16S sequencing workflow was configured to include sequences with a minimum quality score of ≥10, read lengths between 1,400 and 1,600 bp, and utilized the SILVA_138_1 database (Quast et al., 2013) for taxonomic analysis. Taxonomic classification was performed with a minimum abundance threshold of 1 and a confidence threshold of 0.2 for Kraken2.

The data obtained from the SILVA 138.1 database (available in the supplementary material S1) were analyzed and visualized using R environment packages, including Vegan and Ggplot2. Alpha diversity and richness were calculated based on observed amplicon sequence variants (ASVs). Richness was determined using the "specnumber" function, and rarefied richness was calculated with the "rarefy" function. Simpson and Shannon diversity indices were computed using the "diversity" function from the Vegan package.

Beta diversity was calculated using the Bray-Curtis dissimilarity matrix to evaluate compositional dissimilarities among samples, implemented with the "vegdist" function in the Vegan package. Permutational multivariate analysis of variance (PERMANOVA) was conducted using the "Adonis" function in R to test for differences in beta dissimilarities ($P < 0.05$) between groups. Finally, the Kruskal-Wallis test was used to identify microbial taxa with differential abundances ($P < 0.05$) between treatments at the genus level.

3. Results

During the entire experiment, no mortality was observed in either of the two groups analyzed. The final weight of the fish in the control group was 167 ± 6 mg, while for the group supplemented with *S. elongatus* was 175 ± 8 mg, with no statistical difference ($p > 0.05$).

3.1 Sequencing yield

After sequencing the V1–V9 region of the 16S rRNA gene, a total of 1,749,839 reads were obtained from eight libraries (Table 1). Following the cleaning of raw data, 1,730,412 reads remained for statistical analysis, representing 98.8% of the total reads. The number of unclassified/unmapped sequences in the SILVA 138.1 database was 19,427, accounting for 1.12% of the total raw reads. The average read quality was 15.3. Regarding amplicon sequence variants (ASVs), 26 ASVs were identified in each group (control and treated).

Table. 1. Yield of nanopore sequencing in terms of number of reads obtained, classification against the SILVA 138.1 database, and average quality of reads obtained.

Treatment	Reads	Unclassified	Average quality
control 1	257,438	1,429	15.3
control 2	119,768	6,657	15.1
control 3	236,713	2,863	15.4
control 4	255,405	686	15.3
treated 1	248,427	2,359	15.2
treated 2	284,033	1,440	15.4
treated 3	162,059	1,898	15.3
treated 4	166,569	2,095	15.3

3.2 Composition and diversity of the microbiota associated with the intestine

The abundance of phyla present in the control and treated groups are shown in Figure 1A. There was no difference in the abundance of phyla between treatments, with the following phyla being found: Bacteroidota (control: 34.3% and treated: 39.6%), Bdellovibrionota (control: 0.2% and treated: 0%), Firmicutes (control: 30.1% and treated: 4.5%), Fusobacteriota (control: 0% and treated: 2.6%), Planctomycetota (control: 8.8% and treated: 2.9%) and Proteobacteria (control: 24.6% and treated: 49.4%). Twenty-five bacterial genera with average relative abundance > 1% were identified in all libraries (Figure 1B).

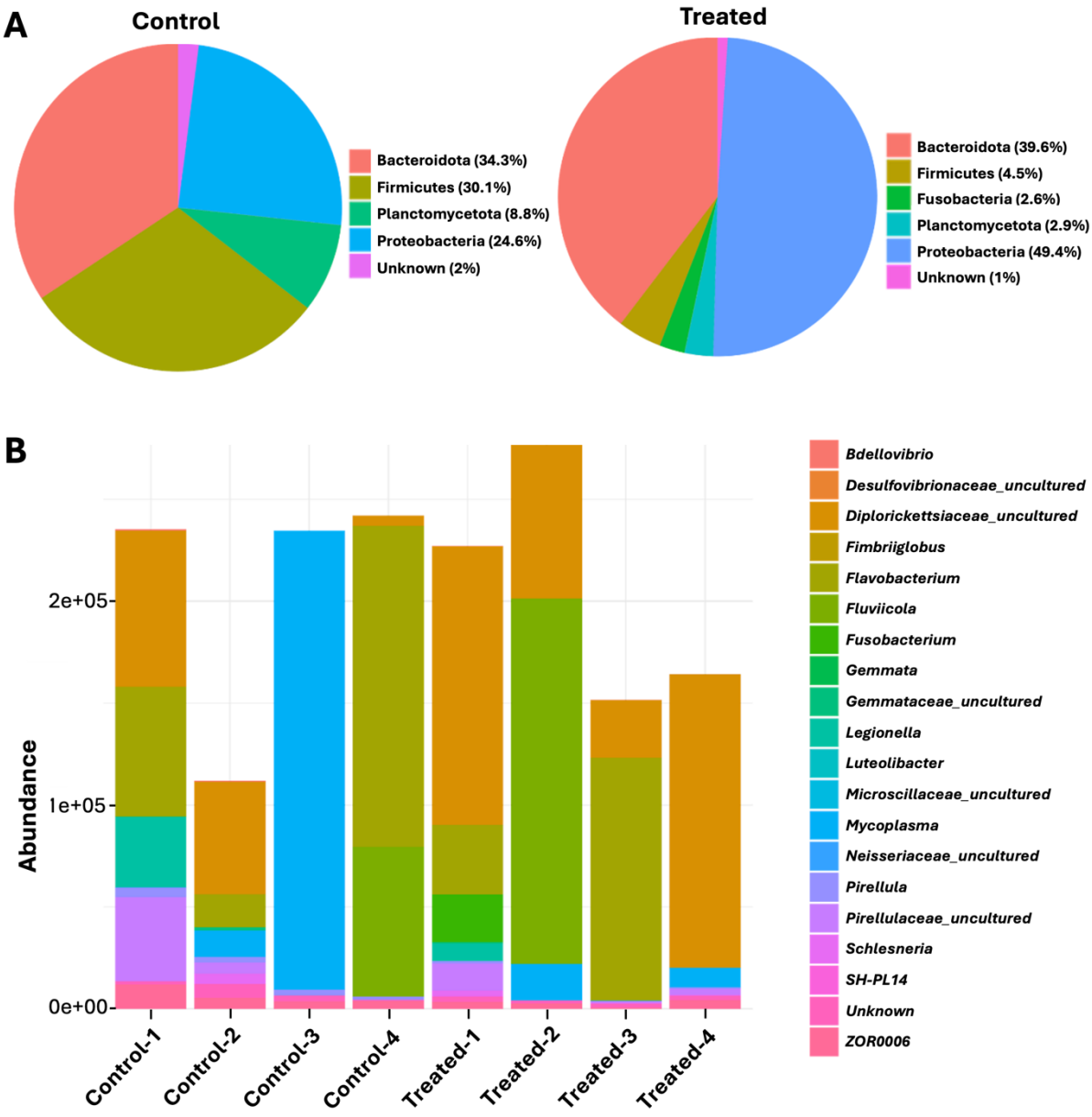

Figure 1. Diversity profile. A) Phylum profile of the intestinal microbiota of zebrafish (*Danio rerio*) fed with commercial feed (control), and fed with commercial feed supplemented with *S. elongatus* (treated). B) Genera profile of the intestinal microbiota of zebrafish (*Danio rerio*) from the control and treated groups.

A more detailed analysis comparing the control and treated groups for each of the 25 genera present in the samples showed that there was a significant variation between treatments in the abundance of only the genus *Pirellula* (Figure 2). This genus

showed a significant reduction ($P < 0.05$) in abundance in the group treated with *S. elongatus* when compared to the control.

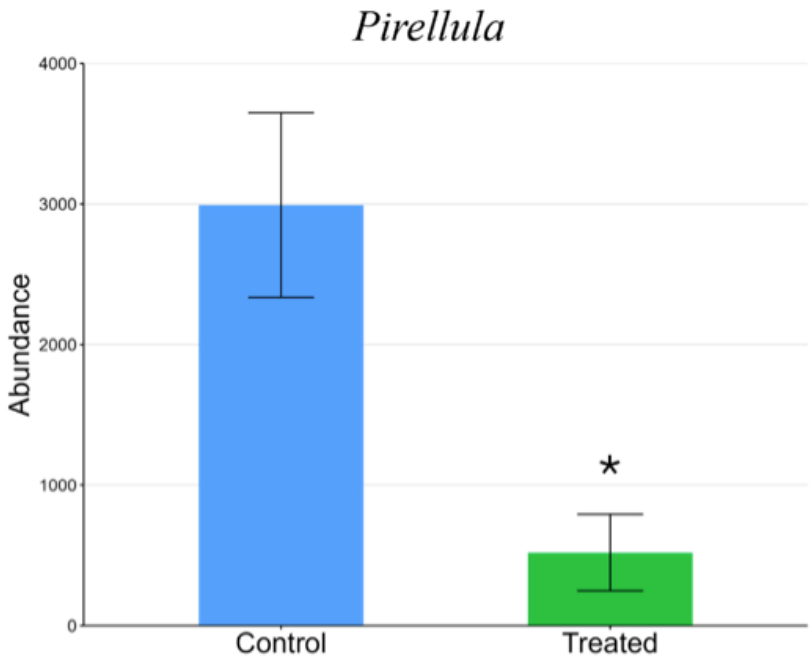

Figure 2. Average abundance of the genus *Pirellula* in the intestine of zebrafish (*Danio rerio*) fed commercial feed supplemented with *S. elongatus* (treated) compared to zebrafish fed non-supplemented commercial feed (control). Data are expressed as mean ± SD. Statistically significant difference between groups was calculated by the Kruskal-Wallis test. The asterisk indicates significant difference with $P < 0.05$.

The alpha diversity of the microbial community present in the zebrafish intestine of the different groups tested was evaluated using the Shannon and Simpson indices. The Shannon and Simpson indices indicated that there are no differences in the diversity of the intestinal microbiota between the groups studied (Fig. 3A and B).

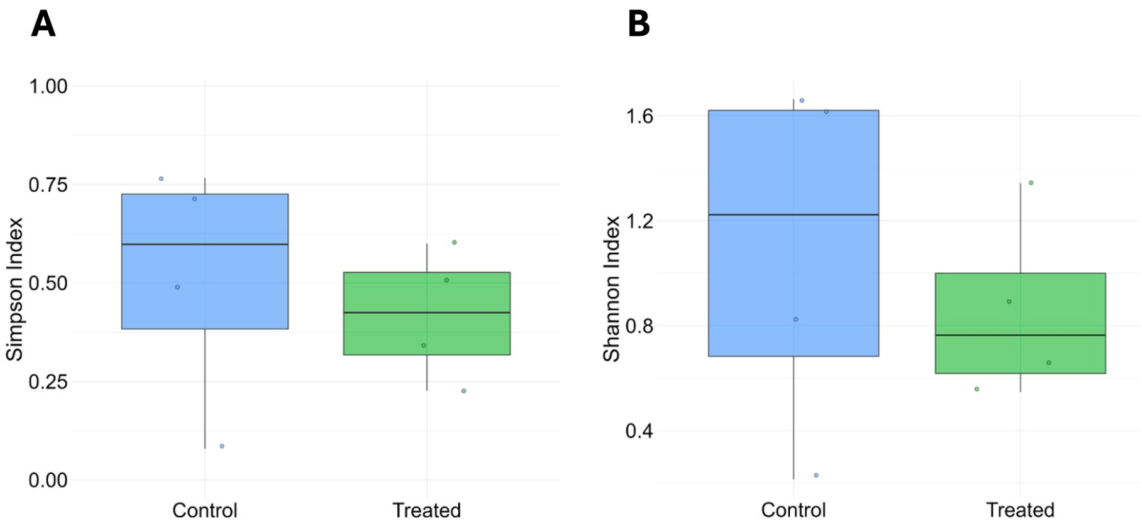

Figure 3. Alpha diversity analyses. A) Simpson index and, B) Shannon index of the gut microbiota of the control (not supplemented with *S. elongatus*) and treated (supplemented with *S. elongatus*) groups. Data are presented in boxplots with individual scatter plots. No significant differences were observed in these indices between groups ($P > 0.05$). Blue colors represent the control group and green colors represent the treated group.

To assess beta diversity, Bray-Curtis dissimilarity in a principal multidimensional scaling ordination (PCoA) was used. Microbiota composition was visualized between treatments based on the first two principal axes (Figure 4). A permutational multivariate analysis (PERMANOVA) was also performed to test for differences in beta distances (dissimilarities) between groups. No significant differences in beta diversity were observed between treatments ($P > 0.05$).

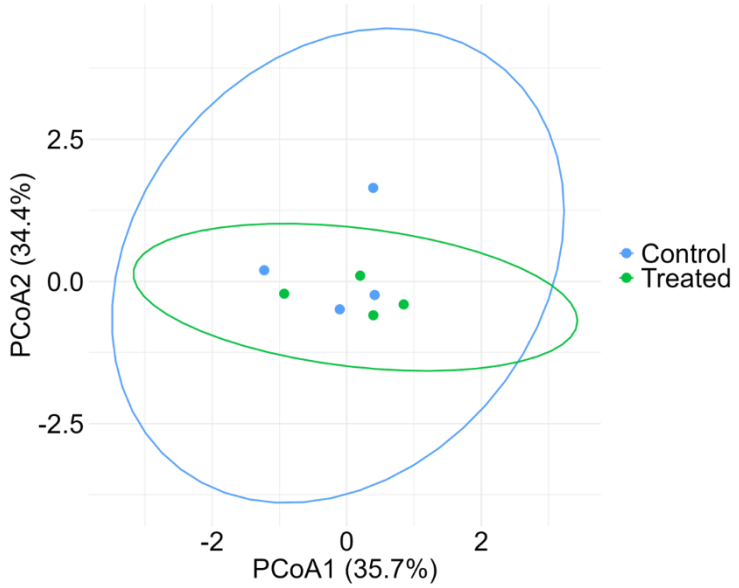

Figure 4. Beta diversity analysis. Principal Coordinates Analysis (PCoA) for each sample from the group fed commercial feed supplemented with *S. elongatus* (treated) compared to zebrafish fed non-supplemented commercial feed (control), using Bray-Curtis dissimilarity.

4. Discussion

The results of this study provide insights into the effects of dietary supplementation with *S. elongatus* PCC 7942 on the gut microbiota of zebrafish (*Danio rerio*). The stability in alpha (Shannon and Simpson) and beta (Bray-Curtis) diversity indices suggests that this cyanobacterium is a safe supplement, without causing dysbiosis or substantial changes in microbial composition. This stability is a desirable trait in aquaculture, where dietary interventions can often destabilize the microbiota, compromising the health of the organisms.

Despite the overall stability, the significant reduction in the relative abundance of *Pirellula* in the treated group suggests that *S. elongatus* PCC 7942 may metabolically impact specific genera of the gut microbiota. *Pirellula*, belonging to the phylum

Planctomycetota, is known for its ability to degrade complex organic compounds and is often associated with environments rich in organic matter (Glöckner et al., 2003). The decrease in *Pirellula* in the treated group may indicate that specific metabolites or components of *S. elongatus* interfere with microbial niches that favor *Pirellula*. This finding raises questions about the metabolic and ecological interactions between cyanobacteria and the basal gut microbiota, suggesting that supplementation with *S. elongatus* may have selective effects on certain members of the gut microbial community.

The presence of mycoplasmas in the control group, but not in the treated group, highlights a relevant aspect. Although mycoplasmas are often considered opportunistic pathogens, their absence in the group supplemented with *S. elongatus* may reflect a less permissive intestinal environment for colonization by these microorganisms. This could be explained by the production of bioactive compounds or by the modulation of immunological factors by the host in response to the supplement. Studies indicate that cyanobacteria, such as *S. elongatus*, produce bioactive compounds with antimicrobial activity, including volatile fatty acids that inhibit the growth of pathogenic bacteria (do Amaral et al., 2020). Furthermore, nutrition plays a crucial role in fish health, directly influencing the immune system and resistance to pathogens. An adequate diet can strengthen the natural defenses of fish, making them less susceptible to infections by mycoplasmas and other opportunistic pathogens (Martin & Król, 2017). Therefore, supplementation with *S. elongatus* may create an intestinal environment that is unfavorable to mycoplasma colonization, either by the direct action of antimicrobial compounds or by improving the host's immune response. Although the absence of *Mycoplasma* in the supplemented group suggests a less favorable environment for its colonization, the underlying mechanism remains unclear. Some microalgae, such as

Schizochytrium sp., *A. platensis*, and *Dunaliella salina*, have been shown to downregulate the expression of pro-inflammatory cytokines (IL6, IL8, and IL1β), contributing to an improved intestinal immune state (Ma et al., 2022). Future research should include serum IgM and lysozyme activity assays to investigate potential immunomodulatory effects of *S. elongatus* PCC 7942.

Another point to consider is the dietary context of the zebrafish used in the study. The inclusion of *Arthrospira platensis* in commercial feed may have contributed to the resilience of the microbiota, minimizing the effects of new interventions. The presence of *A. platensis* in the commercial feed may have influenced microbiota stability, potentially mitigating the effects of *S. elongatus* supplementation. Previous studies demonstrate that the intestinal microbiota of zebrafish is highly adaptable, but tends to stabilize in consistent diets free of antimicrobial or toxic compounds. For example, the total replacement of fishmeal by *A. platensis* in diets for African catfish (*Clarias gariepinus*) did not significantly affect the structure of the intestinal microbial community, indicating an adaptation of the microbiota to the diet provided (Rosenau et al., 2021). Furthermore, dietary supplementation with a combination of *A. platensis* and *Nannochloropsis gaditana* in juvenile European sea bass (*Dicentrarchus labrax*) resulted in significant increases in body weight without adverse changes in the composition of the intestinal microbiota (Peralta-Sánchez et al., 2024). Therefore, supplementation with *S. elongatus* may be an effective and safe strategy, especially in diets already optimized for intestinal health.

From a biotechnological perspective, the results highlight the potential of *S. elongatus* as a dietary supplement in aquaculture. In addition to being nutritionally rich and safe, its minimal impact on the overall microbiota may facilitate its adoption in farming systems without the risk of adverse effects on fish health. However, further

investigation into specific metabolic interactions is needed and the effects under stress conditions, such as changes in water quality, high population density and presence of pathogens, are explored. Additionally, the low plasticity of the microbiota observed suggests that *S. elongatus* can be incorporated into diets formulated for other fish species, which expands its commercial potential. Future studies should include functional analyses of the microbiota, with emphasis on the production of bioactive metabolites and intestinal gene expression, to better understand the mechanisms underlying host-microbiota interactions.

This study reinforces the potential of *S. elongatus* PCC 7942 as a promising dietary supplement for zebrafish and other fish species in aquaculture systems. Its ability to preserve microbial stability, together with specific metabolic interactions that can favor intestinal health, makes it a viable and safe choice to optimize zootechnical performance. Future studies should deepen the investigations to explore the long-term effects and validate its application on a large scale.

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Declarations

Ethical Approval: The experimental design was submitted to the Ethics Committee on Animal Use of the Federal University of Rio Grande (CEUA-FURG) and approved under certificate CEUA-FURG 23116.003565/2023-41.

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Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests: The authors declare no competing interests.

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DISCUSSÃO GERAL

Os peixes alimentados com ração suplementada com *Synechococcus elongatus* PCC 7942 apresentaram aumento no consumo alimentar diário. Considerando que a ração comercial utilizada já contém spirulina, reconhecida por suas propriedades nutricionais e sensoriais (Youssef et al., 2023), a adição de *S. elongatus* pode ter potencializado a palatabilidade da ração para o zebrafish. A biomassa dessa cianobactéria é rica em proteínas (aproximadamente 45 %) e ácidos graxos essenciais, como ácido palmítico (C16:0) e linoleico (C18:2$\Delta^{9,12}$) que podem ter contribuído para o aumento do fator de condição, refletindo melhora na saúde e a composição corporal dos peixes suplementados. Abdel-Tawwab et al. (2008) sugerem que ingredientes funcionais podem melhorar a composição corporal de maneira independente do ganho de peso. Nesta tese, não foram observadas diferenças significativas no ganho de peso e na taxa de crescimento especifica, o que poderia estar relacionado à curta duração do experimento (35 dias). Lawrence (2007) destacou que o zebrafish apresenta crescimento limitado em condições laboratoriais, sendo necessário um período experimental mais longo para detectar variações nesses parâmetros.

A análise do transcriptoma cerebral de zebrafish revelou 15 genes diferencialmente expressos, agrupados em cinco categorias: metabolismo energético, homeostase de cálcio, neuroplasticidade, resposta ao estresse oxidativo e regulação metabólica. Com exceção dos ligados à neuroplasticidade, todos os genes estavam superexpressos nos peixes suplementados com *S. elongatus*. Genes que codificam enzimas como Lactato Desidrogenase Bb (*ldhbb*) e Gliceraldeído-3-fosfato Desidrogenase (*gapdh*) indicam maior conversão de glucose a lactato, sugerindo aumento da demanda energética (Nicholls et al., 2012; Park et al., 2022). Genes relacionados ao metabolismo do cálcio (*micu3a*, *pvalb1*, *pvalb2* e *pvalb4*) são expressos

para atuar ante mudanças rápidas necessárias para a sinalização celular e proteger os neurônios contra a excitotoxicidade induzida por sobrecargas do íon, conferindo um papel neuroprotetor (Chandrasekar et al., 2019; Van Den Bosch et al., 2002). O gene *sesn1* codifica uma proteína que protege contra danos oxidativos e promove autofagia (Chen et al., 2019). Da mesma forma, os genes *tsc1a*, *myhz2*, *myhc4*, *mylpfa*, *tnni2b.2* e *tnnt3b* tem implicações na regulação metabólica, promovem atividades de autofagia evitando o acúmulo de proteínas danificadas, aumentam a eficiência no transporte intracelular, o fornecimento de energia, e modulação do cálcio intracelular para liberação de neurotransmissores (Dibble & Cantley, 2015; Gomes et al., 2002; Hodge & Cope, 2000). Em conjunto, estes genes tem implicações no metabolismo energético que promovem a sinapses e a manutenção do equilíbrio interno.

Em contraste, os genes relacionados à neuroplasticidade (*myripa* e *pecam1a)*, apresentaram expressão reduzida, o que poderia indicar um estado de menor plasticidade neural, possivelmente associado a um ambiente metabólico mais estável e menos demandante pela ingesta de *S. elongatus* (Wimmer et al., 2019), sugerindo que a participação dos genes superexpressos é exitosa. Estes achados ilustram o impacto significativo do consumo de *S. elongatus* PCC 7942 e a complexidade de adaptações que ocorrem no cérebro do zebrafish.

No fígado, a histologia revelou esteatoses hepática em peixes de ambos grupos, sugerindo que a ração comercial utilizada pode não ser adequada para o zebrafish por um excesso de carboidratos na formulação (Ibor et al., 2019; Xi et al., 2023). Embora, os peixes alimentados com *S. elongatus* apresentaram melhoras no estado de saúde do fígado, evidenciadas pela redução na frequência de fígados com alterações generalizadas (esteatoses) e pelo aumento da ocorrência de alterações multifocais, as quais são consideras menos graves e podem refletir um fígado com dano leve e/ou em

processo de recuperação (Wolf & Wolfe, 2005). Estas diferenças podem estar relacionadas com compostos antioxidantes e moduladores lipídicos naturalmente presentes em *S. elongatus*, os quais foram reportados em outras cianobactérias (Rosas et al., 2019; Faheem et al., 2022).

Para garantir que nenhum dos compostos presentes em *S. elongatus* PCC 7942 tem efeitos tóxicos para os peixes foi analisada a expressão dos genes hepáticos *cyp1a* e *gst* envolvidos no metabolismo xenobiótico. Observou-se redução na expressão desses genes nos peixes alimentados com a ração suplementada, sugerindo que o metabolismo xenobiótico não foi ativado, indicando que a cepa testada não foi reconhecida metabolicamente como substância tóxica, similar ao reportado por Williams et al. (2020).

A suplementação com *S. elongatus* reduziu significativamente a expressão de enzimas hepáticas antioxidantes, coincidindo com a menor incidência de esteatose e por tanto, menor estresse oxidativo. As cianobactérias podem produzir antioxidantes não enzimáticos como carotenoides, ficobiliproteinas e compostos fenólicos que podem combater as espécies reativas de oxigênio (ROS) mitigando o estresse oxidativo (Ahmad et al., 2023; Faheem et al., 2022). Assim, a redução na expressão genica destas enzimas pode refletir um equilíbrio redox por conta de antioxidantes dietéticos, reduzindo a necessidade da resposta enzimática endógena.

A expressão do gene *CYP19a,* responsável pela aromatase A que catalisa a conversão de androgênios a estrogênios, foi significativamente menor em peixes alimentados com *S. elongatus*. É possível que *S. elongatus* exerça um efeito modulador na biossíntese de estrogênio como reportado para outras cianobactérias que interferem no desenvolvimento gonadal das fêmeas, a sobrevivência da progênie, e na síntese e depósito de vitelogenina nos ovócitos (Coli et al., 2024; Shaw et al., 2023). Embora este

aspecto não tenha sido explorado em profundidade no presente estudo, ele representa um tema promissor para futuras investigações.

A histologia do intestino não apresentou diferenças significativas entre os peixes de ambos grupos, sugerindo que a incorporação de *S. elongatus* na ração não impactou a morfologia intestinal nem na função absortiva. Além disso, não foram observadas lesões no tecido intestinal indicando que a suplementação com *S. elongatus* foi bem tolerada pelo trato digestório dos peixes. Da mesma forma, o estudo da comunidade microbiana intestinal indicou que a suplementação com *S. elongatus* teve um impacto limitado, os índices de diversidade alfa e beta se mantiveram sem alterações significativas o que reflete estabilidade no microbioma. Isso é um indicador positivo associado a um microbioma saudável e resiliente (Lozupone et al., 2012). No entanto, a abordagem de análise individual do microbioma de cada peixe por tratamento permitiu observar a redução na abundância relativa de *Pirellula* e ausência de micoplasmas no grupo alimentado com *S. elongatus*. Assim, a presença destes no peixe do grupo controle destaca a importância de monitorar microrganismos oportunistas em estudos de microbiota intestinal.

CONCLUSÕES E PERSPECTIVAS

A inclusão de *S. elongatus* PCC 7942 na ração do zebrafish teve efeitos no consumo alimentar e relação peso-comprimento dos peixes, possivelmente por uma melhora na palatabilidade. Enquanto ao metabolismo as diferenças entre grupos foram a nível neurofisiológico e hepático, mostrando melhoras na neuroplasticidade, metabolismo energético e lipídico, e atividade antioxidante no grupo com a ração suplementada com *S. elongatus* PCC 7942; a nível histológico se observou a possibilidade de indução a processos de recuperação hepática. No correspondente ao sistema digestivo, as diferenças foram nulas entre grupos, mantendo a integridade da morfologia intestinal e estabilidade no microbioma. Assim, a informação obtida indica que a inclusão de *S. elongatus* PCC 7942 na dieta do zebrafish por 35 dias, pode trazer benefícios fisiológicos e metabólicos sem comprometer a saúde do peixe. Estudos futuros deverão aprofundar outras faces como sistema imune e reprodutivo.

Com as evidências obtidas, pode-se concluir que a cepa *S. elongatus* PCC 7942 apresenta-se como um veículo de entrega seguro para consumo, não tóxico, que pode ser utilizado para entrega de compostos nutricionais aos peixes com benefícios extras como palatabilidade, melhora no metabolismo lipídico e na resposta antioxidante. No futuro é recomendável realizar testes de digestibilidade de *S. elongatus* PCC 7942 para verificar que a parede celular seja quebrada durante a digestão permitindo que o composto produzido seja liberado e devidamente entregado.

A partir dos benefícios observados no zebrafish com a suplementação de *Synechococcus elongatus* PCC 7942, abrem-se novas possibilidades para a manipulação genética dessa cianobactéria visando a otimização de seus efeitos probióticos e nutricionais. *S. elongatus* PCC 7942 é um organismo modelo bem estabelecido para engenharia metabólica, sendo suscetível a transformações genéticas estáveis por meio

de recombinação homóloga, o que permite a inserção de genes de interesse com alta precisão. Assim, estratégias como a expressão de enzimas digestivas recombinantes, a produção de metabólitos bioativos ou até mesmo a modulação da composição de sua parede celular para aumentar a biodisponibilidade de seus compostos podem ser exploradas. Essas abordagens poderiam potencializar seu efeito na microbiota intestinal dos peixes e ampliar seu uso como suplemento funcional na aquicultura.

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