UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

SUPLEMENTAÇÃO DE FITASE E MIO-INOSITOL: EFEITOS NO DESEMPENHO PRODUTIVO E SAÚDE DE TILÁPIA-DO-NILO SUBMETIDA A DESAFIO POR INFECÇÃO BACTERIANA

EDGAR JUNIO DAMASCENO RODRIGUES

Tese apresentada ao Programa de Pós-graduação em Zootecnia como parte das exigências para obtenção do título de Doutor em Zootecnia

Botucatu – SP

2024

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Orientador: Profª. Ass. Dra. Margarida Maria Barros

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1. Aditivos alimentares. 2. Antioxidantes. 3. Adjuvantes imunológicos. 4. Alimentos funcionais. 5. Tilápia-do-Nilo.

Palavras-chave: Aditivo alimentar; Capacidade antioxidante; Imunoestimulante; Nutrição funcional; *Oreochromis niloticus*.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Botucatu

ATESTADO DE APROVAÇÃO - DEFESA

Atestamos que **EDGAR JÚNIO DAMASCENO RODRIGUES**, RA nº: ZNP200026, RG nº MG-17.165.381, expedido pela PC/MG, defendeu, no dia 29/07/2024, a tese intitulada **SUPLEMENTAÇÃO DE FITASE E MIO-INOSITOL: EFEITOS NO DESEMPENHO PRODUTIVO E SAÚDE DE TILÁPIA-DO-NILO SUBMETIDA A DESAFIO POR INFECÇÃO BACTERIANA**, junto ao Programa de Pós Graduação em Zootecnia, Curso de Doutorado, tendo sido 'APROVADO'.

Atestamos ainda que a obtenção do título dependerá de homologação pelo Órgão Colegiado competente.

Botucatu, 29 de julho de 2024

Cláudia Cristina Mereci
Assistente Administrativo
Seção de Pós-Graduação / FMVZ

BIOGRAFIA DO AUTOR

Edgar Junio Damasceno Rodrigues, formiguense e mineiro, nascido em 15 de setembro de 1991, filho de Lúcia Maria de Brito e Edgar Rodrigues de Sousa Faria, de criação simples. Iniciou seu bacharelado em Zootecnia na Universidade Federal de Lavras (UFLA) em julho de 2010. Sua carreira acadêmica começou como estudante de iniciação científica no setor de aquacultura da UFLA dando seguimento nesta área de pesquisa até a conclusão de seu bacharelado. No ano de 2013, participou do programa de graduação sanduíche "Ciência Sem Fronteiras", na The University of Queensland em Brisbane QLD, Austrália. Regressou ao Brasil em 2015 para finalizar sua graduação, e no ano de 2018 inicia seu mestrado em nutrição e produção animal na Universidade Estadual Paulista (UNESP) – Botucatu, finalizado em 2020.

DEDICATÓRIA

Aos meus pais Lucia e Edgar pelo dom da vida, incentivo e amor.

Ao meu irmão Euler Rodrigues, por todo apoio, amor fraternal e companheirismo.

Às minhas filhas, Helena e Sofia, por me inspirarem a ser um homem melhor.

Dedico

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Agradeço ao criador, Olorum, pelo dom da vida, por conceder saúde e força para seguir nessa jornada

Aos meus pais, em especial à minha mãe, pela firmeza, dedicação, amor e anos de luta e trabalho árduo para minha criação e estudos.

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Ao meu padrinho Mozar José de Brito, que me incentivou desde o ensino médio a ingressar em uma universidade, me deu todo o suporte necessário para a conclusão de minha graduação, sempre disposto a auxiliar e compartilhar os mais diversos ensinamentos e que me apresentou ao mundo acadêmico.

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Ao meu coorientador, Dr. Pedro Luiz Pucci Figueiredo de Carvalho que sempre me motivou, acreditou em meu potencial, pela amizade e companheirismo. Seu apoio foi fundamental para a conclusão desta etapa.

O apoio, ensinamentos e amizade de todos, amigos e professores, durante meu doutorado e os levarei para a vida toda. Todos vocês foram de suma importância para a conclusão desta etapa de minha carreira, a todos minha gratidão.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Código de Financiamento 001.

Try to be the person that your dog thinks you are.

Unkown

RESUMO GERAL

Este estudo objetivou avaliar os efeitos da suplementação *in vitro* de mio-inositol (MI) no *burst respiratório* e proliferação de leucócitos isolados de tilápia do Nilo. Adicionalmente, foram avaliados os efeitos da suplementação dietética de MI e fitase no perfil hematológico, desempenho produtivo, parâmetros dos sistemas imune e antioxidante de tilápias-do-Nilo submetidas à desafio imunológico por infecção bacteriana. A pesquisa foi dividida em três fases distintas: ensaio *in vitro*, desempenho produtivo e desafio imunológico. Para a primeira fase, foram utilizados 60 juvenis com aproximadamente 60 gramas mantidos por 30 dias em 30 aquários (38L). Para a segunda fase, foram utilizados 675 juvenis com aproximadamente 14.9 gramas mantidos por 90 dias em 45 aquários (250L). Após este período foram avaliados: parâmetros zootécnicos, perfil hematológico, atividade de enzimas do sistema antioxidante e respostas imunológicas. Para a segunda fase, 192 peixes provenientes da primeira fase experimental, foram infectados por *Aeromonas hydrophila* e submetidos a desafio imunológico por sete dias. Posteriormente, todos os parâmetros mencionados foram reavaliados, exceto parâmetros zootécnicos. Os experimentos foram conduzidos em delineamento inteiramente casualizado, com um tratamento controle (sem suplementação), quatro níveis de quatro níveis de MI (300, 600, 900, e 1200 mg kg^{-1} / L^{-1}) para os experimentos *in vitro* e *in vivo* e quatro níveis de fitase (500, 1500, 2500, e 3500 FTU kg^{-1}). Os resultados do estudo *in vitro* demonstraram que a adição de 900 mg L^{-1} determinou aumento significativo para o burst respiratório, atividade fagocítica e proliferação de leucócitos ($P<0.05$) em relação à cultura celular não suplementada. Resultados semelhantes foram observados no estudo *in vivo* com MI. Baseado nos parâmetros de saúde e desempenho, 900 mg kg^{-1} foi determinado como nível ótimo de suplementação para tilápias. Considerando os efeitos no desempenho produtivo, e parâmetros de saúde avaliados, 500 FTU kg^{-1} foi determinado como nível ótimo de suplementação de fitase para tilápia do Nilo. Portanto, a sequência de estudos conduzidos evidencia os efeitos positivos da suplementação de MI e fitase para a espécie estudada sendo recomendada a suplementação de 900 mg kg^{-1} de MI ou 500 FTU kg^{-1} em dietas vegetais para melhorar o desempenho e aumentar a resistência da tilápia do Nilo à infecção bacteriana.

Palavras-chave: imunoestimulante; capacidade antioxidante; nutrição funcional;

ABSTRACT

This study aimed to evaluate the effects of myo-inositol (MI) in vitro supplementation on respiratory burst and proliferation of leukocytes isolated from Nile tilapia. Additionally, the effects of dietary supplementation of MI and phytase on hematological profile, productive performance, immune and antioxidant system parameters of Nile tilapia subjected to a bacterial infection challenge were assessed. The study was divided into three distinct phases: in vitro assay, productive performance, and bacterial challenge. In the first phase, 60 juveniles weighing approximately 60 grams were maintained for 30 days in 30 tanks (38L). For the second phase, 675 juveniles weighing approximately 14.9 grams were kept for 90 days in 45 tanks (250L). After this period, the following parameters were assessed: zootechnical parameters, hematological profile, antioxidant enzyme activity, and immune responses. In the third phase, 192 fish from the first experimental phase were infected with Aeromonas hydrophila and subjected to an immunological challenge for seven days. Subsequently, all mentioned parameters were re-evaluated, except for zootechnical parameters. The experiments were conducted using a completely randomized design, with one control treatment (no supplementation), four levels of MI (300, 600, 900, and 1200 mg kg^{-1} / L^{-1}) for both in vitro and in vivo experiments, and four levels of phytase (500, 1500, 2500, and 3500 FTU kg^{-1}). The in vitro study results demonstrated that the addition of 900 mg L^{-1} significantly increased respiratory burst, phagocytic activity, and leukocyte proliferation ($P<0.05$) compared to the non-supplemented cell culture. Similar results were observed in the in vivo study with MI. Based on health and performance parameters, 900 mg kg^{-1} was determined as the optimal supplementation level for tilapia. Considering the effects on productive performance and health parameters assessed, 500 FTU kg^{-1} was determined as the optimal phytase supplementation level for Nile tilapia. Therefore, the sequence of studies conducted highlights the positive effects of MI and phytase supplementation for the studied species, recommending the supplementation of 900 mg kg^{-1} of MI or 500 FTU kg^{-1} in plant-based diets to improve growth performance and increase Nile tilapia resistance to bacterial infection.

Keywords: immunostimulant; antioxidant status; functional feed;

LISTA DE ILUSTRAÇÕES

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AquaNutri	Laboratório de Nutrição e Saúde de Peixes
CAT	CATALASE
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
EROS	Espécies reativas de oxigênio
EO	Estresse oxidativo
FAO	Organização das Nações Unidas para a Alimentação e a Agricultura
FMVZ	Faculdade de Medicina Veterinária e Zootecnia
FTU	Unidades de fitase
GPx	Glutationa Peroxidase
MI	Myo-inositol
NRC	National Research Council
RL	Radicais Livres
SOD	Super Oxido Dismutase
NE	No enzyme inclusion
P	Phosphorus
PAE	Polymer associated to enzyme
UNESP	Universidade Estadual Paulista “Júlio de Mesquita Filho’’

SUMÁRIO

CAPÍTULO I

CONSIDERAÇÕES INICIAIS

O Capítulo I introduz brevemente parte dos desafios que intensificação da piscicultura promoveu como aumento em densidades de estocagem, utilização de manejos estressantes para animais como biometrias frequentes, classificações e vacinação. Tais fatores tornam o ambiente produtivo desafiador, favorecendo surtos de doenças de origem viral e bacteriana que resultam em altas taxas de mortalidade e redução de produtividade. Por isso, estratégias nutricionais como o uso de aditivos que aumentam a performance e resistência dos peixes, tais como a enzima fitase e *myo-inositol* tem sido cada vez mais estudadas e empregadas visando mitigar os efeitos deletérios de sistemas intensivos de produção.

O Capítulo II trata sobre um ensaio imunológico *in vitro* que avaliou os efeitos da suplementação de mio-inositol em meios de cultura de leucócitos isolados do rim cranial da tilápia do Nilo intitulado: "Myo-inositol increases respiratory burst, cellular proliferation, and phagocytosis of cultured leukocytes from Nile tilapia". O short communication está redigido de acordo com as normas do periódico Fish and Shellfish immunology (IF 4.7).

O Capítulo III trata sobre um estudo in vivo que avaliou os efeitos da suplementação de mio-inositol no desempenho, respostas antioxidantes, imunológicas e sobrevivência da tilápia do Nilo desafiada por infecção bacteriana. O artigo parcial intitulado "Myo-inositol supplementation: effects on growth performance, immunological status, antioxidant system and its gene-related expression of Nile tilapia subjected to a bacterial challenge'' está redigido de acordo com as normas do periódico Aquaculture (IF 4.2).

O Capítulo IV trata sobre um estudo in vivo que avaliou os efeitos da suplementação de fitase no desempenho, respostas antioxidantes, imunológicas e sobrevivência da tilápia do Nilo desafiada por infecção bacteriana. O artigo parcial intitulado "Dietary phytase improves growth performance, immunological status, antioxidant system and bacterial resistance of Nile tilapia subjected to a bacterial challenge." está redigido de acordo com as normas do periódico Aquaculture (IF 4.2).

1. REVISÃO DA LITERATURA

1.1. INTRODUÇÃO E JUSTIFICATIVA

Dentre as atividades de produção de proteína de origem animal, a aquicultura é a que mais cresce mundialmente, com taxa de até 8% ao ano em algumas regiões (FAO, 2022), sendo o pescado a proteína animal mais consumida no mundo. Neste cenário, o Brasil é um dos países que apresenta maior potencial hídrico para a expansão dessa atividade, possuindo cerca de 10 milhões de hectares de lâmina d'agua (RORIZ et al., 2017). Tal atividade tem crescido, consideravelmente, no Brasil. De acordo com o Instituto Brasileiro de Geografia e Estatística (2019) a produção piscícola brasileira cresceu em aproximadamente 10% nos últimos dois anos, sendo a tilápia do Nilo (*Oreochromis niloticus*) a espécie de água doce mais produzida, correspondendo a 52,6% do total.

A expansão deste setor foi impulsionada por diversos fatores, entre eles a intensificação dos sistemas produtivos. Entretanto, esse processo desafia o bem-estar e a saúde animal. Redução na resistência a doenças, alterações em respostas ao estresse e bem-estar dos animais estão entre as principais consequências associadas à intensificação da aquicultura, além da redução do crescimento sustentável desta atividade (ASHLEY, 2007).

Para evitar surtos de doenças em diferentes sistemas de produção tem-se utilizado medicamentos, geralmente, de maneira inapropriada. O uso de antimicrobianos, para mitigar a ocorrência de doenças infecciosas e melhorar o desempenho dos animais ainda é prática comum em pisciculturas (DAWOOD; KOSHIO; ESTEBAN, 2018). Entretanto, os potenciais riscos à saúde humana fizeram com que doses subterapêuticas destes medicamentos fossem banidas da formulação de dietas visando reduzir e, possivelmente, a longo prazo, eliminar o uso de antimicrobianos como promotores de crescimento para peixes e outros animais (NG; KOH, 2017).

Como alternativa ao uso de antimicrobianos, aditivos com funções imunoestimulantes têm sido considerados potenciais substitutos, aumentando a resistência imunológica de animais aquáticos favorecendo a expansão do setor aquícola de forma sustentável (FUCHS et al., 2015). Pré e probióticos, acidificantes, glucanos, extratos de origem animal e vegetal, enzimas e complexos enzimáticos, são exemplos de aditivos com funções imunoestimulantes para peixes. De fato, diferentes autores verificaram efeitos positivos desses aditivos para o sistema imune (ABO NORAG et al., 2018; BARROS et al., 2014), antioxidante (HOSEINIFAR et al., 2017),

resistência a doenças e/ou desempenho de organismos aquáticos (CARVALHO et al., 2023; WANG; CHEN-YING; LI, 2020).

Algumas enzimas utilizadas como aditivo nutricional, tais como a fitase, podem melhorar o desempenho, a resposta imune e antioxidante de peixes. Compilados de estudos demonstraram que esta enzima além de melhorar, significativamente, a biodisponibilidade de aminoácidos, energia, proteína e minerais contidos na dieta, pode ainda aumentar a eficiência alimentar e o desempenho de peixes (CAO et al., 2007; KUMAR et al., 2012; LEMOS; TACON, 2017), além de reduzir a descarga excessiva de nutrientes no ambiente aquático (MORALES et al., 2016).

O aumento na biodisponibilidade de minerais como fósforo, zinco, ferro e manganês, e outros nutrientes como mio-inositol e aminoácidos, que estão relacionados direta ou indiretamente ao sistema imunológico e antioxidante faz da fitase a enzima com potenciais efeitos positivos nos mecanismos de defesa de peixes. Assim, avaliar possíveis efeitos da aplicação desta enzima no sistema imunológico de peixes possui fundamental importância para a aquicultura (LAZADO et al., 2010). De fato, a suplementação desta enzima promoveu benefícios à resposta imune de tilápias-do-Nilo infectadas por *Aeromonas hydrophila*, otimizando índices fagocitários e reduzindo lesões patogênicas em órgãos como o fígado, baço e intestino (ABO NORAG et al., 2018).

Lazado et al. (2010) verificaram que a adição de fitase foi capaz de aumentar a proliferação de leucócitos isolados do rim cefálico de *Gadus morhua*. Resultados semelhantes foram observados em pesquisas desenvolvidas com frangos de corte, em que também houve aumento significativo na proliferação de leucócitos promovida pela suplementação de fitase (LIU et al., 2008). Os autores dos referidos trabalhos constataram que os efeitos imunoestimulantes da fitase estão relacionados ao aumento na disponibilização de fósforo, mio-inositol e outros ésteres menores de inositol, que são capazes de ativar mecanismos de proliferação celular, melhorar a estrutura de membranas celulares e participar de outros processos fisiológicos.

O mio-inositol é considerado nutriente importante, com diferentes funções no metabolismo celular. É constituinte de membranas celulares, atua como transmissor celular, regulando a atividade de enzimas e transporte de lipídeos (SHIAU; SU, 2004). Níveis inadequados de mio-inositol em dietas para *Cyprinus carpio* (variedade Jian) estão associados à redução da capacidade antioxidante (JIANG et al., 2010) e supressão de respostas

imunológicas (JIANG et al., 2016). A deficiência desse composto foi responsável, ainda, pelo baixo desempenho produtivo de tilápias híbridas (*O. niloticus* × *O. aureus*) (SHIAU; SU, 2005), além de distúrbios no metabolismo lipídico e redução no desempenho de *Paralichthys olivaceus* (LEE et al., 2008).

Devido à importância deste nutriente, a suplementação de mio-inositol em dietas comerciais é usualmente feita para alguns peixes como a tilápia do Nilo, uma vez que a produção endógena desta molécula não é suficiente para suprir as necessidades metabólicas e promover ótimo crescimento (JIANG et al., 2009a; SHIAU; SU, 2005). Entretanto, os efeitos do mio-inositol no sistema imunológico, antioxidante e expressão de genes relacionados ao sistema antioxidante de tilápia do Nilo foram pouco explorados. De fato, pesquisas abordando os efeitos da suplementação de mio-inositol no desempenho e a saúde de tilápias são escassas (PERES; LIM; KLESIUS, 2004; SHIAU; SU, 2005).

1.2. USO DE ENZIMAS NA NUTRIÇÃO DE TILÁPIA DO NILO

As espécies de tilápia são onívoras pertencentes à família dos ciclídeos, nativos da África que foram amplamente introduzidos em todo o mundo, acidental ou deliberadamente (EKNATH; HULATA, 2009). Entre as diferentes espécies de tilápia, a mais cultivada no Brasil e no mundo é a tilápia do Nilo (*Oreochromis niloticus*). Cerca de 17 espécies de tilápia contribuem para a produção global de tilápia, entretanto a tilápia do Nilo é a espécie mais cultivada (SUÁREZ-PUERTO; ARANEDA; GULLIAN-KLANIAN, 2023). A expressiva produção da tilápia do Nilo frente a produção das outras espécies de tilápia pode ser observada na figura 1. No ano de 2020, a tilápia do Nilo foi apontada como a terceira espécie mais produzida mundialmente, contribuindo em cerca de 9% enquanto outras espécies de tilápia contribuíram cem 2,2% da produção mundial de peixes (FAO, 2022). No Brasil, a tilápia continua sendo a espécie de peixe mais produzida nacionalmente, contribuindo em cerca de 64% das 860.355 produzidas no ano de 2023.

A tilapicultura enquanto atividade industrial é dependente de dietas comerciais que atendam às exigências nutricionais fornecendo energia e nutrientes e balanceados adequadamente para que o animal possa expressar seu potencial genético de produção. Fórmulas comerciais de rações para aquicultura em sua maioria são compostas por ingredientes

de origem animal e ou vegetal, considerados coprodutos agroindustriais sendo utilizados como fonte de proteica e ou de energia (LIANG et al., 2022). Assim como em outras atividades de produção animal, na tilapicultura cerca de 70% dos custos de produção são representados por gastos com alimentação.

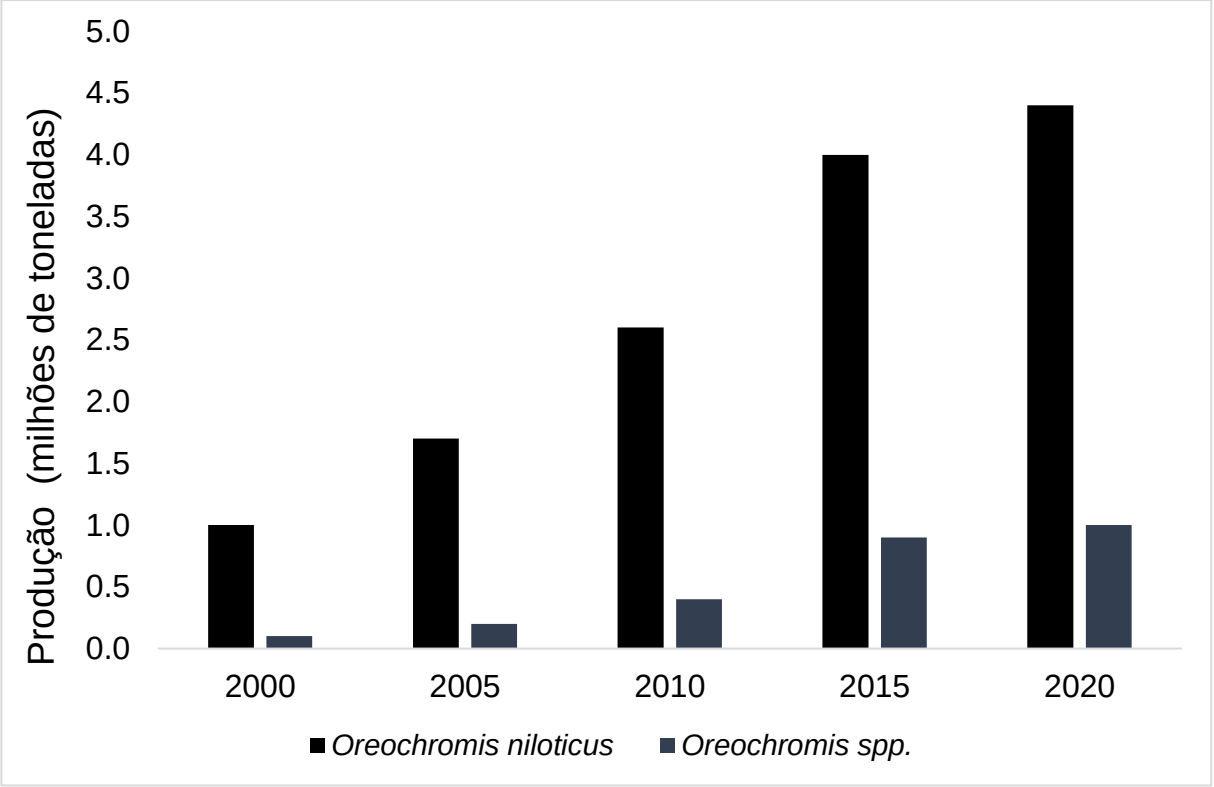

Figura 1. Produção global de *Oreochromis niloticus* e *Oreochromis spp.*

Desta forma, boas práticas de manejo alimentar, determinar exigências nutricionais e utilizar estratégias para aumentar a eficiência alimentar e utilização de nutrientes são fundamentalmente importantes para aumentar a viabilidade econômica da produção de tilápia (GULE; GEREMEW, 2022). Além da viabilidade econômica, também é necessário utilizar estratégias que reduzam a descarga de nutrientes provenientes da produção de peixes para o ambiente aquático, aumentando a sustentabilidade da atividade (TACON; METIAN, 2015). Existem diferentes estratégias utilizadas para melhorar índices produtivos e sustentabilidade, entre elas a adição de enzimas em dietas comerciais, prática comum na produção de peixes.

Enzimas são componentes proteicos que catalisam reações químicas, sendo extraídas de plantas, animais ou obtidos por meio de processos fermentativos microbianos (SANCHEZ; DEMAIN, 2017) sendo amplamente utilizadas na nutrição animal (OJHA; SINGH; SHRIVASTAVA, 2019). Em 2022, o mercado global de enzimas comerciais para alimentação

animal atingiu $1,77 bilhões de dólares e a projeção de crescimento anual entre 2023 e 2032 é de 6,5% (GMI, 2024). O uso de enzimas é importante para o desenvolvimento sustentável da aquacultura (RAVINDRAN; SON, 2011). De fato, enzimas atuam na hidrólise de moléculas complexas presentes em dietas aumentando a biodisponibilidade de nutrientes e energia além de, em determinadas situações, reduzir efeitos deletérios de fatores antinutricionais.

Alguns ingredientes tais como coprodutos industriais de origem vegetal possuem diferentes fatores antinutricionais, como o fitato, que podem ser hidrolisados por enzimas exógenas, como a fitase, reduzindo seu efeito como antinutriente, aumentando a biodisponibilidade de aminoácidos, energia e minerais (KUMAR et al., 2012). Além disso, a adição de fitase em dietas contendo ingredientes vegetais pode reduzir a excreção de fósforo e nitrogênio no ambiente aquático, contribuindo para a sustentabilidade da aquicultura e disponibilizar nutrientes como o mio-inositol que exerce efeitos positivos na fisiologia e saúde de peixes (MORALES et al., 2016). Apesar dos benefícios para o desempenho, saúde e desenvolvimento sustentável, a incorporação de enzimas em dietas extrudadas é um desafio. Isso ocorre por diferentes fatores como lixiviação, e principalmente pela temperatura de processamento.

Rações comerciais utilizadas na aquacultura são fabricadas por peletização ou extrusão (CIAN et al., 2017). Dietas peletizadas geralmente são confeccionadas a temperaturas de até 90°C (CAO et al., 2007), porém, essa técnica é menos utilizada em relação à extrusão. O referido processo é majoritariamente aplicado na aquacultura, visto que as condições de temperatura e pressão durante a extrusão podem aumentar a digestibilidade de nutrientes, flutuabilidade e melhorar características físicas da ração (RIAZ, 2023). Entretanto, a extrusão pode limitar a inclusão pré-processamento de aditivos termosensíveis. Isso ocorre porque durante a extrusão a mistura de ingredientes é submetida a condições de pressão elevada e aumentos de temperatura de até 200°C em condições experimentais (CIAN et al., 2017). Este cenário é um dos principais desafios para a inclusão de enzimas e outros aditivos termosensíveis em dietas extrudadas utilizadas na aquacultura. De fato, as condições citadas de alta temperatura, umidade, e pressão são capazes de inativar ou reduzir a atividade da maioria das enzimas comerciais sendo necessário o emprego de técnicas para estabilizar ou proteger enzimas durante o processamento de dietas, principalmente se elas precisam desempenhar suas funções ao longo do trato gastrointestinal dos peixes (LIANG et al., 2022). Para minimizar a desnaturação de enzimas durante o processamento de dietas extrudadas algumas estratégias podem ser adotadas representadas na Figura 2. Dentre as opções, o pré-tratamento tratamento

de ingredientes com blends enzimáticos ou microrganismos tem sido avaliado como alternativa viável. A fermentação em estado sólido (SSF) de ingredientes tem sido amplamente estudada nos últimos anos. Ingredientes alternativos para a produção de ração como bagaço de uva (WANG et al., 2019), de maçã (RODRÍGUEZ et al., 2017; VENDRUSCOLO et al., 2009) e coproduto da destilação de trigo (SAMAD et al., 2022) tiveram suas propriedades como biodisponibilidade de aminoácidos, e redução de fatores antinutricionais melhoradas.

Apesar dos benefícios promovidos pela ação de enzimas microbianas, a SSF de ingredientes tem sua aplicação comercial reduzida devido ao aumento no custo de produção e equipamentos disponíveis. A aplicação de enzimas comerciais e técnicas de SSF previamente ao processamento de dietas são técnicas pouco utilizadas porque este tipo de estratégia altera propriedades físicas dos ingredientes e pellets, além do risco de contaminação microbiana (LIANG et al., 2022). Outras alternativas mais viáveis são empregadas para fomentar o uso de enzimas na aquacultura. Quitosanas, alginato, e polissacarídeos como xilanas e polímeros vegetais tem sido avaliadas para confeccionar géis e microencapsular ou adsorver enzimas sendo consideradas alternativas promissoras apesar dos efeitos limitados (RODRIGUES et al., 2023; SIRISHA; JAIN; JAIN, 2016).

Adicionalmente, técnicas como para desenvolver enzimas intrinsicamente resistentes, imobilização em nanopartículas, ou por ligações covalentes também são estudadas e consideradas alternativas promissoras (SIRISHA; JAIN; JAIN, 2016). Entretanto, as alternativas mencionadas ainda são onerosas, conferem proteção limitada ou comprometem a atividade da enzima (MRUDULA et al., 2019). A aplicação de enzimas também pode ser realizada pós processamento da dieta por meio de pulverização nos *pellets*. De fato, essa ainda é uma das técnicas mais utilizadas para adicionar enzimas em dietas para peixes. (CAO et al., 2007; LEMOS; TACON, 2017; LIANG et al., 2022). Este método viabiliza o uso de enzimas como a fitase em dietas extrudadas. Pontes et al. (2019) compararam métodos de inclusão de fitase em dietas vegetais para tilápia do Nilo. Os referidos autores adicionaram enzima na mistura de ingredientes pré-processamento e pós-processamento utilizando o método de pulverização. Neste estudo, foi verificado que a enzima adicionada via pulverização não teve sua atividade reduzida e promoveu melhores resultados para ganho de peso, conversão alimentar e digestibilidade de fósforo e cálcio, em comparação ao tratamento com fitase incluída na mistura de ingredientes. Resultados semelhantes foram observados por Rodrigues et al. (2023) que comparou diferentes métodos de inclusão de fitase em dietas extrudadas ou peletizadas para tilápia do Nilo.

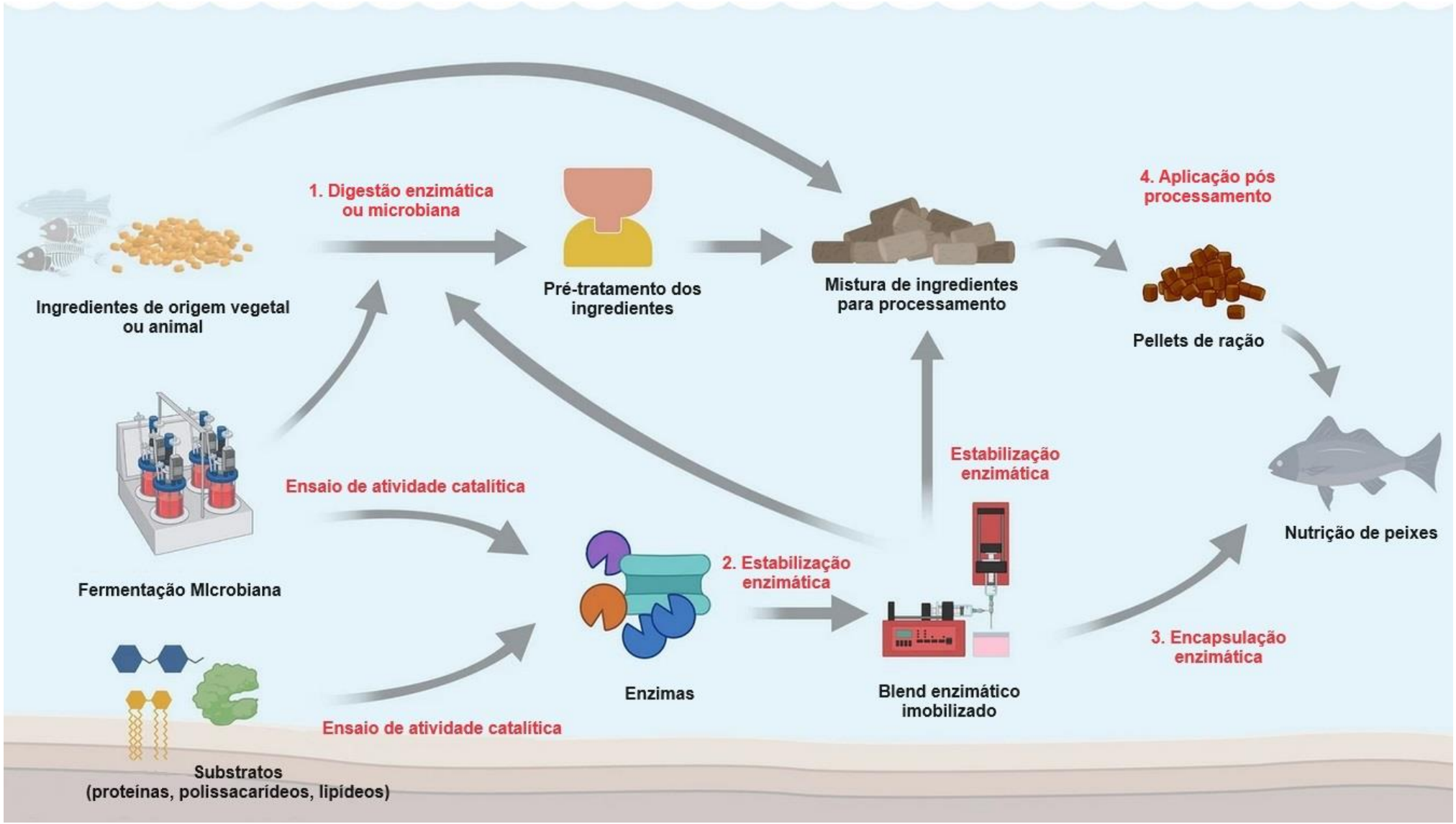

Figura 2. Aplicação de enzimas como aditivo em dietas para aquacultura. Fonte: Adaptado de Liang et al. (2022)

No referido estudo, foram testados 2 métodos de inclusão: fitase adsorvida em polímero vegetal ou pulverizada pós-processamento de dietas. Foi observado que em dietas extrudadas o método de inclusão pós-processamento determinou os melhores resultados para digestibilidade de fósforo, cálcio e cinzas. Em relação a dietas peletizadas foi observo o oposto, o método de adição da enzima adsorvida promoveu melhores resultados para os parâmetros mencionados, devido à possível proteção que o polímero conferiu à fitase contra a hidrólise por proteases endógenas.

De maneira geral, o uso de enzimas adicionadas em níveis e de formas adequadas é benéfico para a nutrição de tilápias, promovendo efeitos positivos para ganho de peso, eficiência alimentar, biodisponibilidade de nutrientes e energia e redução de fatores antinutricionais. Utilizar esse tipo de aditivo tornou-se cada vez mais importante para fomentar o desenvolvimento sustentável do setor produtivo. De fato, o uso de ingredientes vegetais como ingredientes para confecção de dietas impulsionou o uso de enzimas. Isso ocorreu porque nestes ingredientes existem diferentes fatores antinutricionais, como o fitato. Este composto é a principal forma de armazenamento de fósforo, representando até 80% do teor total deste mineral em vegetais e seus subprodutos (KUMAR; ANAND, 2021). O fitato é fonte de fósforo não disponível para peixes podendo se complexar a proteínas, aminoácidos, minerais, reduzindo a biodisponibilidade destes nutrientes e de energia (KUMAR et al., 2012). Estudos demonstram que a suplementação de fitase pode reduzir os efeitos antinutricionais do fitato (CAO et al., 2007). A referida molécula pode se complexar a diferentes nutrientes como mineirais (ferro, cálcio, magnésio, zinco, cobre), proteínas e aminoácidos básicos, carboidratos, bem como reduzir a atividade de enzimas como a tripsina e alfa-amilase (KUMAR et al., 2012). A fitase é uma fosfatase que hidrolisa o fitato, reduzindo seus efeitos deletérios e, portanto, aumentando o aporte de nutrientes e energia biodisponíveis para peixes, organismos não produzem a referida9 enzima em quantidades suficientes (LEMOS; TACON, 2017).

A suplementação de fitase em dietas para tilápias melhora índices zootécnicos como ganho de peso e conversão alimentar. De fato, (ADEOYE et al., 2016), observou que a suplementação de fitase (300 mg kg^{-1}) em dietas vegetais para tilápia do Nilo otimizou a taxa de conversão alimentar, de crescimento específico e ganho de peso final em comparação ao controle. Em outro estudo, Pontes et al. (2021) verificaram que a suplementação de fitase (1500 UF kg^{-1}) aumentou a digestibilidade aparente da energia e proteína bruta, fósforo e outros minerais em relação ao controle. Além disso, a excreção de fósforo reduziu significativamente de 13,83 para 5,25 g kg^{-1} de ganho de peso. Os benefícios da suplementação de fitase na nutrição

de tilápia do Nilo também foi comprovada em condições de produção intensiva. A suplementação de fitase (1500 e 2000 UF kg^{-1}) determinou melhores resultados para biomassa final, taxa de conversão alimentar, e ganho de peso diário e digestibildiade de cinzas, energia e proteína bruta (RODRIGUES et al., 2022). Os efeitos positivos da fitase no desempenho e redução na emissão de poluentes na aquacultura são associados à hidrólise do fitato, reduzindo seu efeito antinutricional, aumentando a biodisponibilidade de fósforo, zinco, ferro, manganês, cálcio, energia e aminoácidos. Tais efeitos foram amplamente estudados com resultados compilados (CAO et al., 2007; KUMAR et al., 2012; LEMOS; TACON, 2017).

Outro grupo de enzimático com uso considerável na produção o de tilápia é representado pelas proteases. Tais enzimas hidrolisam ligações peptídicas específicas no meio (endopeptidases) ou na parte terminal (exopeptidases) da cadeia polipeptídica. Essa classe de enzima é suplementada para suprir a produção de protease endógena e melhorar a utilização de nutrientes em ingredientes de origem vegetal e animal com valores biológicos distintos ((ZHENG et al., 2020). Efeitos positivos da suplementação de proteases em dietas vegetais para tilápia do Nilo tem sido amplamente estudado. A inclusão de protease (175 mg kg^{-1}) em dietas vegetais para tilápia (*Oreochromis niloticus* × *Oreochromis aureus*) aumentou o ganho de peso em 11,1% em relação ao controle (LI et al., 2019). Resultados similares foram observados por (ADEOYE et al., 2016), suplementando protease (200 mg kg^{-1}) em dietas para tilápia do Nilo. Neste estudo a utilização da protease aumentou o ganho de peso dos peixes em 3,6% em comparação ao controle. Em estudo mais recente, (SALEH et al., 2022) verificaram que a suplementação de protease melhorou a conversão alimentar, ganho de peso em 11,5%. Os benefícios da adição de proteases em dietas para peixes foram associados ao maior aporte de nutrientes em ingredientes com valor biológico relativamente baixo e aumento da quantidade de enzimas proteolíticas.

Embora peixes não tenham exigência específica para carboidratos, estes nutrientes estão entre os principais componentes de uma dieta, sendo fonte de energia que contribui para o crescimento e manutenção de funções fisiológicas. Apesar da sua contribuição fundamental, existem diferentes tipos de carboidrato que desempenham funções distintas. Alguns tipos de carboidratos, encontrados em ingredientes vegetais, como os polissacarídeos não amiláceos (PNAs), afetam negativamente a utilização de nutrientes, o desempenho e a saúde animal (FRANCIS; MAKKAR; BECKER, 2001). Com o crescente uso de ingredientes vegetais é fundamental a utilização de carboidrases para melhorar a digestibilidade de ingredientes

vegetais, uma vez que a produção endógena não é suficiente para anular efeitos antinutricionais e disponibilizar os nutrientes desses ingredientes (CASTILLO; GATLIN, 2015).

O grupo das carboidrases incluem enzimas como β-glucanases, amilases, xilanases, celulases e pectinases que catalisam a hidrolise de carboidratos complexos em moléculas como oligo ou polissacarídeos de menor peso molecular (LIANG et al., 2022). As carboidrases com maior representação e uso são as xylanases e glucanases. De fato, as duas classes mencionadas representam cerca de 80% do mercado global das carboidrases (ZHENG et al., 2020). A utilização, número de pesquisas e convergência de resultados sobre os efeitos de carboidrases em peixes são menores do que o observado em outras enzimas como a fitase e geralmente carboidrases são suplementadas em blends enzimáticos (MAAS et al., 2020).

Maas et al. (2021) testaram os efeitos da adição de β-glucanase (500 BGU kg^{-1}) e xylanase (4000 XU kg^{-1}) e fitase (1000 UF kg^{-1}) em dieta vegetal para tilápia do Nilo. Neste estudo, os autores não observaram efeitos positivos isolados ou do blend enzimático no desempenho produtivo dos peixes. Entretanto, efeitos positivos da suplementação de fitase no coeficiente de digestibilidade aparente de fósforo, cálcio e cinzas foram verificados. Os resultados do referido estudo estão em alinhamento com outras pesquisas. (ADEOYE et al., 2016) suplementaram um blend enzimático contendo fitase, xilanase e proteases também não verificou efeitos significativos para o desempenho de tilápia do Nilo. A inclusão de celulase em dietas para tilápia do Nilo também não afetou o desempenho ou a digestibilidade de nutrientes (YIGIT; OLMEZ, 2011).

Outras pesquisas contrastam com os efeitos não significativos de carboidrases, em blends ou isoladas, na nutrição de tilápias. De fato, Lin; Mai; Tan, (2007) verificaram que a suplementação de um blend enzimático contendo proteases, β-glucanase e xilanase, promoveu benefícios para o desempenho e digestibilidade de nutrientes em dietas para tilápia do Nilo. Índices como taxa de crescimento, eficiência alimentar, digestibilidade da matéria seca foram otimizados. Adeoye et al. (2016), também verificaram efeitos positivos com a adição de carboidrase em dietas de tilápia do Nilo. No referido estudo, o peso final e taxa de conversão alimentar dos peixes alimentados com a ração suplementada foi melhor em relação ao controle. Apesar dos resultados disponíveis na literatura serem contraditórios, benefícios associados à suplementação de carboidrases são esperados em dietas com alta inclusão de ingredientes vegetais visto que essas enzimas catalisam a hidrolise de moléculas como PNAs que atuam como fatores antinutricionais restringindo o desempenho e saúde de organismos aquáticos

(CASTILLO; GATLIN, 2015; FRANCIS; MAKKAR; BECKER, 2001; GULE; GEREMEW, 2022).

Outro grupo de nutrientes importante para a nutrição de tilápias, os lipídeos, fornecem energia e são fonte de ácidos graxos essenciais que desempenham papel fundamental no crescimento, sistema imunológico e integridade de membrana. Além disso, lipídeos podem ser utilizados como ferramenta nutricional e promover efeito poupador de proteína em dietas de tilápias (THIRUNAVUKKARASAR et al., 2022). A disponibilidade de lipídios em dietas pode ser aumentada com a suplementação de lipases. Essas enzimas são responsáveis por catalisar a hidrólise total ou parcial de triglicerídeos em moléculas menores como glicerol e ácidos graxos. Apesar da importância deste grupo de nutrientes e enzima, pesquisas sobre a influência da suplementação de lipases em dietas de peixes restritas em comparação à fitases, carboidrases e proteases (ZHENG et al., 2020). De fato, efeitos da inclusão de lipase em dietas para tilápia no Nilo foram pouco explorados. A inclusão de um complexo enzimático contendo lipase exógena em dietas para reprodutores de tilápia do Nilo foi avaliada e efeitos positivos observados (TAHOUN; EL-HAROUN; SULOMA, 2011). A performance reprodutiva foi aumentada e o efeito relacionado com a maior biodisponibilidade energética de fontes não proteicas.

1.3. FITASE

O termo fitase ou mio-inositol-hexafosfato fosfohidrolase refere-se ao grupo de enzimas classificadas como fosfatases que catalisam a hidrólise sequencial do ácido fítico (IP6). O ácido fítico e seus sais mistos também chamados de fitato (Figura 3) são as principais formas de armazenamento de fósforo em vegetais e seus grãos (VINICIUS et al., 2021). Essas moléculas são agentes quelantes considerados antinutrientes que podem se complexar a minerais bivalentes, carboidratos e proteínas, reduzindo a biodisponibilidade para animais não ruminantes (KUMAR et. al., 2012, LEMOS; TACON 2017). O fitato pode ainda potencializar a descarga de nitrogênio e fósforo na água, visto que neste ambiente existem diferentes microrganismos que produzem fosfatases que hidrolisam e tornam biodisponíveis os nutrientes complexados (MORALES et al., 2016).

Além de fosfatases, as fitases podem ainda ser classificadas de acordo com seu mecanismo (Figura 4) e pH ótimo de ação, bem como características estruturais. Considerando a posição em que as reações de desfosforilação se iniciam, as fitases podem ser divididas em dois grupos: 3-fitase (EC 3.1.3.8) e 6-fitase (EC 3.1.3.26) (PRYIA et al., 2023). Segundo os

referidos autores, 3-fitases catalisam a desfosforilação a partir do carbono 1 ou 3 do ácido fítico, enquanto 6-fitases iniciam a hidrólise em carbonos mais próximos ao carbono 5. Grande parte das fitases microbianas são do tipo 3, com exceção à enzima produzida pela bactéria Escherichia coli classificada como tipo 6, enquanto fitases de origem vegetal são do tipo 6, exceto a encontrada nos grãos de soja (tipo 3) (KUMAR; SINHA, 2018).

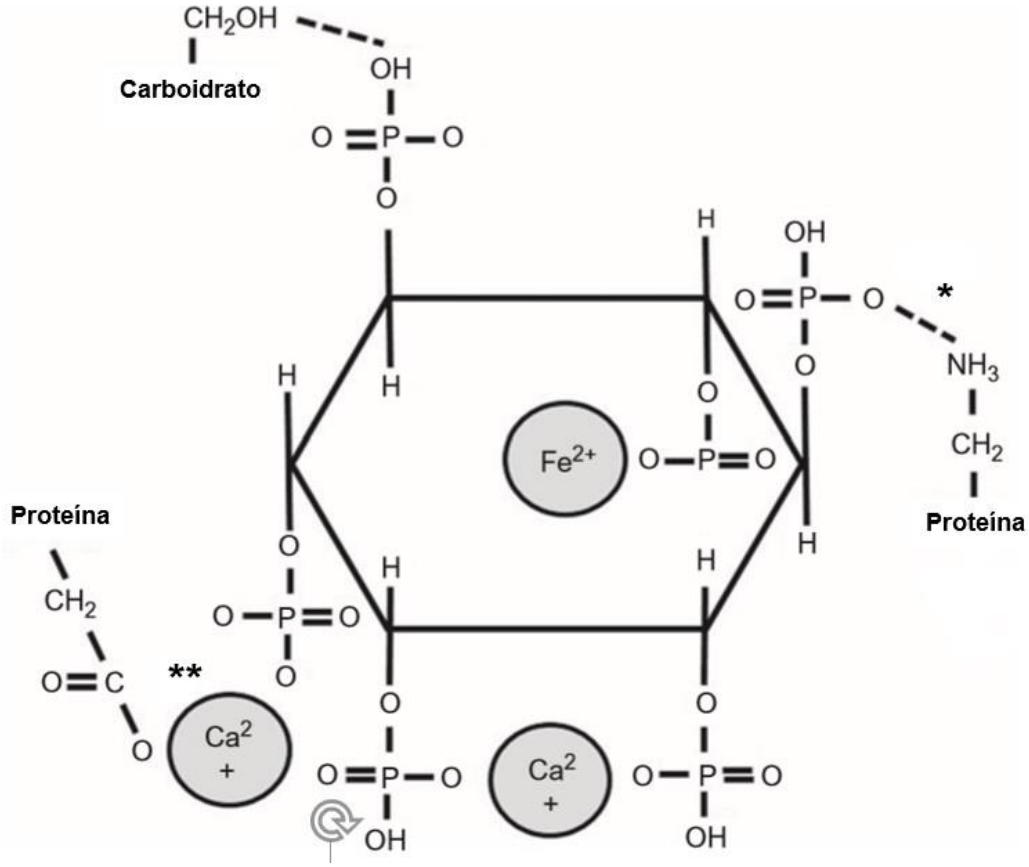

Figura 3. Fitato em vegetais.

Fonte: adaptado de Romano; Kumar (2018).

Considerando as conformações de esturura e catálise, as fitases, podem ainda ser classificadas como: fitase β-hélice (FBA), fosfatases ácidas de histidina (HFA), fosfatase ácido "purple" (FAP) e fosfatase proteína tirosina (KUMAR; SINHA; KAJBAF, 2019). Tal classificação considera a estrutura conformacional, especificidade ao substrato, pH ótimo e mecanismos de ação (VIJAYARAGHAVAN et al., 2013).

Em relação ao pH ótimo de ação, fitases podem ser classificadas em ácidas ou alcalinas. A maioria das fitases 3 e 6 são classificadas como ácidas, com pH ótimo entre 3,0 e 5.5, enquanto fitases alcalinas tem pH ótimo de ação entre 7,0 e 8,0 (VIJAYARAGHAVAN et al.,

2013). A eficiência das fitases ácidas é maior do que fitases básicas, refletindo no produto da reação catalisada pelas enzimas. De maneira geral, fitases catalisam a desfosforilação sequencial do ácido fítico e seus sais mistos de IP6 a IP1 resultando ésteres menores de mio-inositol, biodisponibilização de minerais e outros nutrientes (KUMAR et al., 2012). A ação de fitases ácidas é capaz de hidrolisar até cinco grupos fosfatados, enquanto fitases alcalinas hidrolisam apenas três (Figura 4) sendo consideradas mais eficientes quando comparadas ao grupo de fitases básicas (GREINER et al., 2000; GREINER et al., 2002).

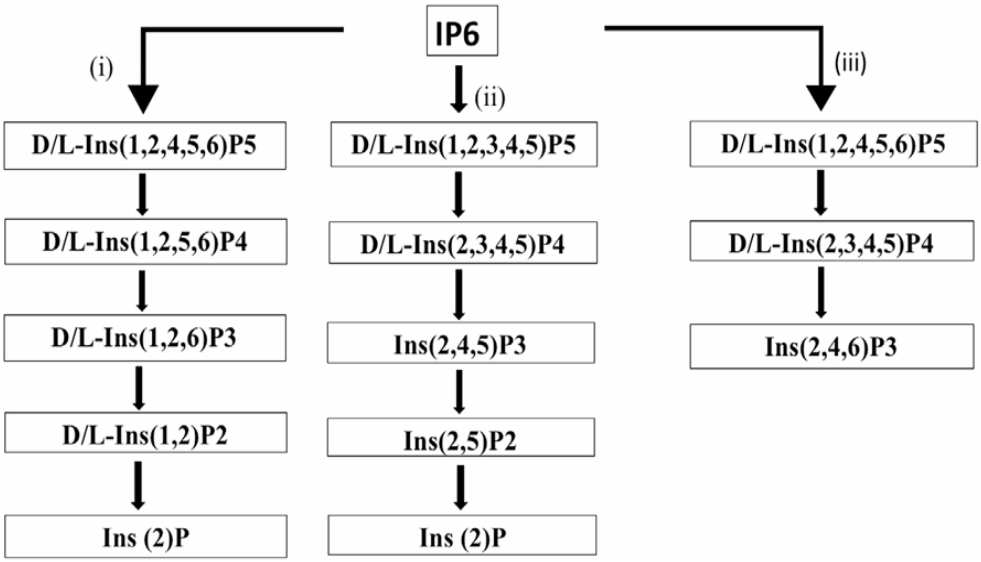

Figura 4. Hidrólise do ácido fítico (IP6) por fitases ácidas (i e ii), e básica (iii).

Fonte: PRIYA et al. (2023)

A ação das fitases também é dependente de outros fatores tais como composição química e de ingredientes da dieta em que a enzima foi suplementada. De fato, níveis dietéticos de minerais bivalentes como o cálcio podem alterar a atividade da fitase. Isso é observado principalmente pela alta afinidade do fitato com o cálcio, bem como as altas concentrações de cálcio em dietas para peixes (CAO et al., 2007). Ao se associar ao cálcio, a molécula de fitato torna-se menos solúvel, portanto, menos disponível para a hidrólise da fitase (KUMAR et al., 2010). Adicionalmente, o cálcio pode ainda aumentar o pH da digesta resultando em condições desfavoráveis para a hidrólise do fitato, portanto, relações entre 1:1 - 1.4:1 (C:P) a fim de otimizar a atividade de fitase em peixes (CAO et al., 2007).

Além dos níveis de cálcio, os ingredientes utilizados para confeccionar a ração também afeta a eficiência da fitase. Esse tipo de influência é relacionado à quantidade de fitato, que

pode ser encontrada em cada ingrediente vegetal variando por diferentes fatores tais como manejo durante cultivo, espécie e grau de desenvolvimento do vegetal (IWAI et al., 2012; GONÇALVES et al., 2007). O processamento da dieta e resistência à hidrólise de enzimas digestivas também são fatores determinantes para a atividade da fitase. Dietas comerciais para tilápia são em sua maioria extrudadas. O processo de extrusão pode atingir temperaturas de até 200°C comprometendo a atividade de fitases comerciais adicionadas junto à mistura de ingredientes (CIAN et al., 2018; MRUDULA et al., 2019). Por isso, a prática mais comum utilizada para suplementar fitase é por aspersão após o processo de extrusão (LEMOS; TACON, 2017).

A suplementação da fitase em dietas vegetais resulta em maior ganho de peso, melhor taxa de conversão alimentar, afeta positivamente a taxa de retenção proteica além de reduzir impactos ambientais promovidos pela tilapicultura. Tais benefícios são resultado da maior biodisponibilidade de nutrientes como aminoácidos, fósforo, cálcio, manganês, ferro, zinco e da energia contida nos alimentos promovida pela ação da fitase (DERSJANT-LI et al., 2015). De fato, benefícios promovidos pela suplementação de fitase em dietas de peixes foram evidenciados e compilados em diferentes trabalhos (CAO et. al., 2007, KUMAR et al., 2012, LEMOS; TACON, 2017).

Recentemente estudos têm sido direcionados para explorar os efeitos extra nutricionais da fitase. A ação da fitase disponibiliza nutrientes essenciais para o funcionamento e modulação de respostas imunológicas (Figura 5) tais como fósforo e mio-inositol (LAZADO, 2010). Akpoilih et al. (2023) verificaram que a suplementação de 1000 FTU kg-1 em dietas vegetais deficientes em fósforo aumentou o *burst* respiratório e a atividade da lisozima da tilápia do Nilo. Tais resultados foram associados ao aumento da biodisponibilidade de fósforo e outros nutrientes como mio-inositol.

Adicionalmente, Abo Norag et al. (2018) observaram que a suplementação de fitase em dietas vegetais deficientes em fósforo normalizaram parâmetros imunológicos de tilápia do Nilo desafiadas por infecção bacteriana. Os resultados deste estudo evidenciaram que a suplementação de 1000 FTU kg^{-1} determinou o mesmo efeito de dietas que atendiam as exigências de fósforo no que se refere a atividade fagocítica dos peixes infectados.

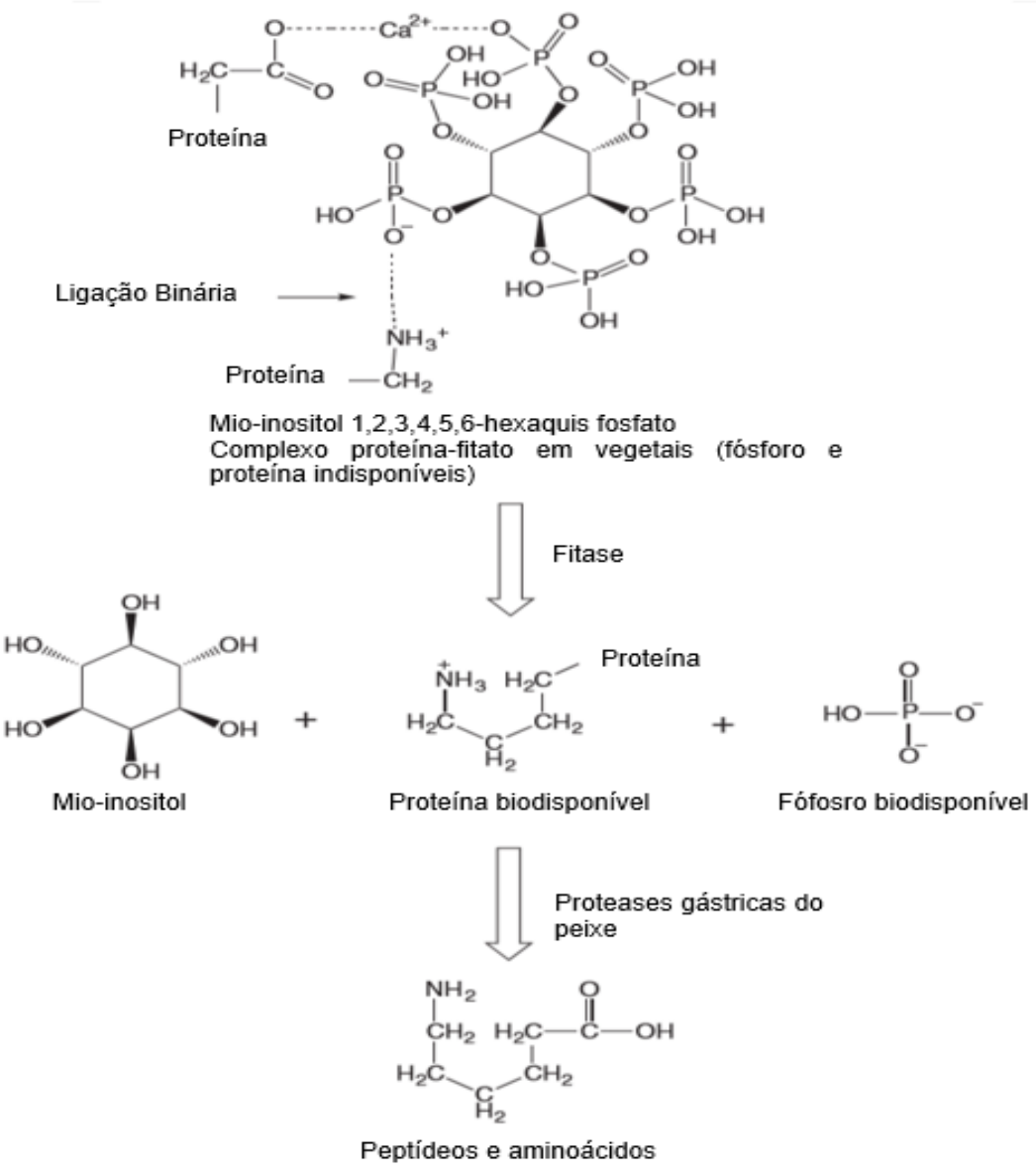

Figura 5. Disponibilização de nutrientes pela ação da fitase.

Fonte: adaptado de Kumar; Kajbaf (2019)

1.4. MIO-INOSITOL, BIOSSÍNTESE E EXIGÊNCIA NUTRICIONAL

Presente em diferentes tecidos, animais e vegetais, o mio-inositol (cis-1,2,3,5-trans-4,6-cyclohexanehexol) é componente estrutural da membrana celular, isômero da molécula de inositol com maior atividade biológica (Figura 6), e de ocorrência predominante (LI et al., 2020). Este poliálcool cíclico de açúcar, pode ser classificado de diferentes maneiras. Alguns autores classificam o mio-inositol (MI) como nutriente tipo vitamina, pertencente ao complexo B (SHIRMOHAMMAD; JOEZY-SHEKALGORABI, 2016), enquanto, outros refutam essa classificação por não considerarem esse composto essencial, visto que pode ser sintetizado em

quantidades nutricionalmente adequadas por alguns animais a partir da glicose-6-fosfato (REGIDOR; SCHINDLER, 2016).

Apesar das classificações divergentes, sabe-se que este composto participa de diferentes processos metabólicos. De fato, o mio-inositol é usado como base para a produção de segundos mensageiros, tais como inositol trifosfato, bifosfato e trifosfato de fosfatidilinositol e fosfoglicanos, que estão associados a respostas celulares frente a estímulos externos como, por exemplo, a ligação da insulina ao seu receptor celular aumentando a sensibilidade ao hormônio (CROZE; SOULAGE, 2013). Este poliálcool também é um osmolito celular compatível e está envolvido na proliferação de células do fígado e enterócitos de peixes, e na regulação osmótica celular conferindo maior proteção em ambientes hipertônicos (CUI et al., 2020; JIANG et al., 2013; SHIAU; SU, 2005).

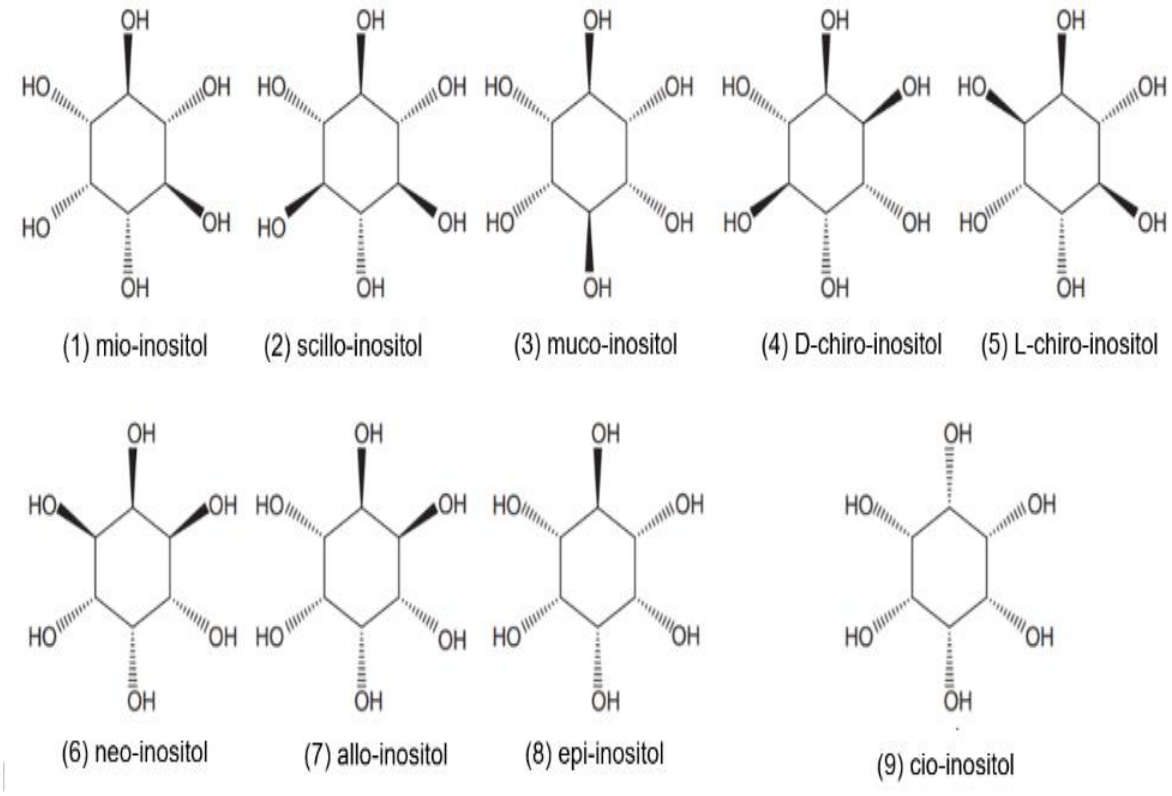

Figura 6. Estrutura química dos nove estéreo isómeros de inositol.

Fonte: adaptado de Palomba (2018)

Por constituir parte de tecidos celulares (animal, vegetal, bacteriano, fúngico) associado a fosfolipídios de membrana e estar presente em maiores quantidades em frutas frescas, vegetais e grãos (CROZE; SOULAGE, 2013), este composto pode ser observado em ingredientes rotineiramente utilizados na confecção de rações animais. Entretanto, a fonte mais comum de mio-inositol em dietas animais é encontrada na forma de ácido fítico ou fitato, principalmente em produtos derivados de cereais e em menores quantidades em vegetais e legumes, assim como em seus grãos (KUMAR; SINHA; KAJBAF, 2019). O fitato não é fonte prontamente disponível de mio-inositol para a maioria dos monogástricos. Grande parte destes animais não produzem a enzima fitase em quantidade ideal para promover sequência de desfosforilações necessárias para disponibilizar o mio-inositol e outros nutrientes complexados ao fitato (DERSJANT-LI et al., 2015; GREINER; KONIETZNY, 2010). Após a hidrólise do fitato, o MI disponibilizado pode ser absorvido no intestino do animal por meio de transporte ativo dos co-transportadores de mio-inositol sódio-dependente 1 e 2 (SMIT1/2) e próton-dependente (H+, HMIT) (Figura 7). Tais co-transportadores sódio-dependentes já foram identificados ao longo do intestino de tilápia do Nilo (SUBRAMANIAM; WEBER; LOEWEN, 2019).

Além da dieta, o mio-inositol pode ser proveniente da produção endógena animal. Diferentes animais, como roedores, são capazes de sintetizar mio-inositol utilizando a glicose-6-fosfato como substrato, em tecidos como o cérebro, testículo, fígado e rim (HAUSER; FINELLI, 1963). A biossíntese deste composto também foi evidenciada em algumas espécies de peixes, no cérebro e fígado. Burtle e Lovell (1989) verificaram a capacidade de síntese de novo de mio-inositol em juvenis de bagre do canal (*Ictalurus punctatus*). Adicionalmente, ao promover a disbiose e não suplementar mio-inositol à dieta, Deng et al. (2002) constataram que o "Sunshine bass" (*Morone chrysops* x *Morone saxatilis*) produz metabolicamente mio-inositol em tecidos como o fígado e cérebro. Tais resultados corroboram com a pesquisa de Shiau e Su (2005), que de maneira semelhante verificaram que a produção de mio-inositol pela microbiota intestinal não interfere, significativamente, na deposição deste nutriente no fígado de tilápias, sugerindo que esta espécie também produz MI de forma endógena em quantidades nutricionalmente insuficientes.

Apesar de não atender às necessidades nutricionais, a biossíntese de mio-inositol em tilápias pode ocorrer em diferentes tecidos e pelos mesmos mecanismos observados em outras espécies. De maneira geral, este processo depende da ação de duas enzimas: mio-inositol fosfato sintetase (MIPS, EC 5.5.1.4) e inositol monofosfatase (IMPase, EC 3.1.3.25). A primeira enzima é responsável pela conversão da glicose-6-fosfostato, fosforilada pela enzima

hexoquinase (HK), em mio-D(L)-inositol-3(1)-fosfato [Ins(3)P]. Enquanto a segunda catalisa a desfosforilação da molécula Ins(3)P originando mio-inositol livre (GEIGER; JIN, 2006). A enzima IMPase pode, ainda, desfosforilar inositol-1-fosfato [Ins(4)P], proveniente de processos fisiológicos como a comunicação celular e renovação de membranas fosfolipídicas (KALUJNAIA; HAZON; CRAMB, 2016; PARTHASARATHY et al., 2006), obtendo mio-inositol livre como produto final (Figura 7). De fato, a atividade destas duas enzimas e até mesmo de algumas variações análogas já foram identificadas em tilápias (*Oreochromis mossambicus*), comprovando a capacidade destes animais sintetizarem mio-inositol (GARDELL, 2013; VILLARREAL; KÜLTZ, 2015).

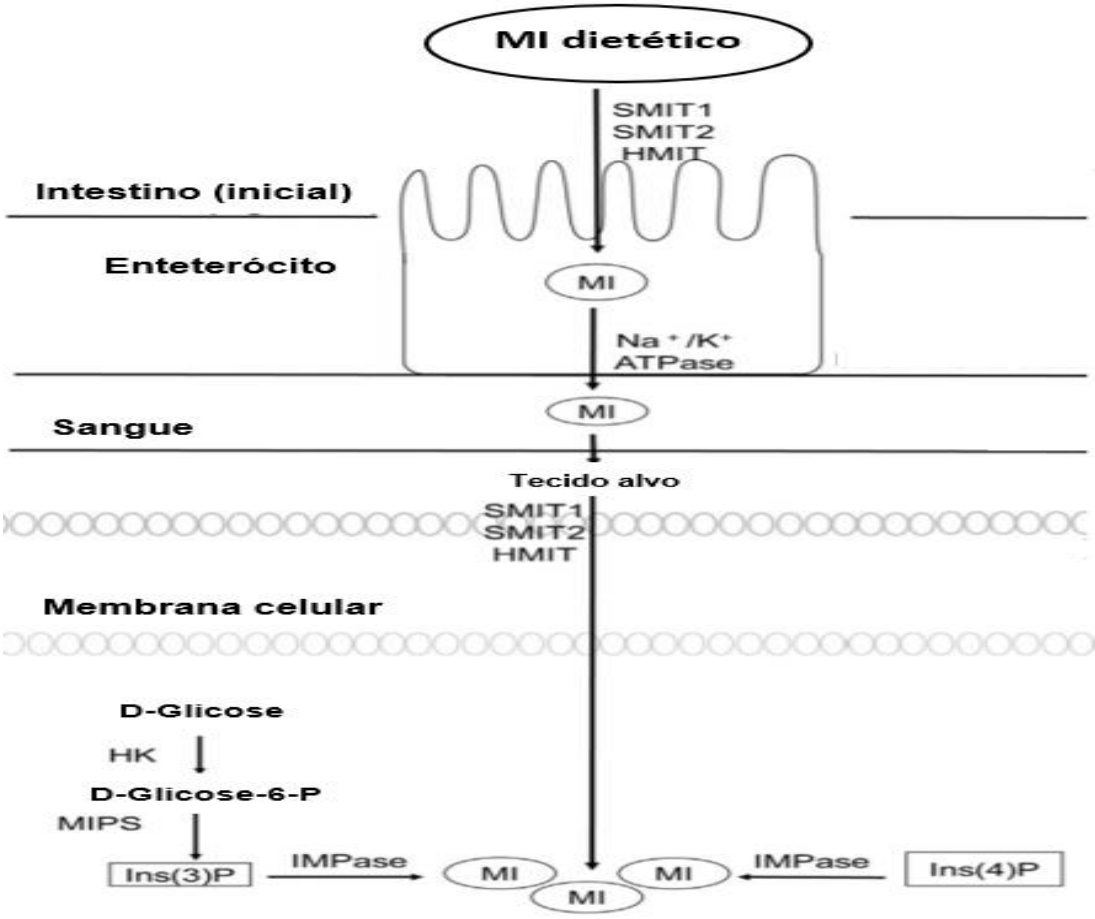

Figura 7. Absorção e biossíntese do mio-inositol em animais.

Fonte: adaptado de Gonzalez-Uarquim et al. (2020)

Adicionalmente, a microbiota intestinal de alguns peixes e outros vertebrados também pode sintetizar mio-inositol (FARRAG et al., 2008), até mesmo em quantidades adequadas para suprir suas necessidades fisiológicas e nutricionais. Peixes como o ‘’Sunshine bass’’ (*Morone chrysops* × *Morone saxatilis*) (DENG; HEMRE; WILSON, 2002) e salmão-do-Atlântico

(*Salmo salar*) (WAAGBØ et al., 1998) não necessitam da suplementação de mio-inositol, fato atribuído à produção de MI pela sua microbiota intestinal. Apesar disso, outras espécies de peixes necessitam de suplementação de mio-inositol para evitar problemas como anorexia, corrosão de nadadeiras, redução no crescimento, eficiência alimentar, movimentos peristálticos, respostas dos sistemas antioxidante e imunológico (COMBS; MCCLUNG, 2017; LI et al., 2018).

A exigência nutricional de mio-inositol para o desempenho produtivo adequado de algumas espécies de peixes tem sido verificada para crescimento satisfatório de: tilápia híbrida (*Oreochromis niloticus* x *Oreochromis aureus*) 400 mg kg^{-1} (SHIAU; SU, 2005), esturjão japonês (*Acipenser schrenckii*) 336,1 mg kg^{-1} (WANG et al., 2018), papagaio (*Oplegnathus fasciatus*) 94,3 mg kg^{-1} (KHOSRAVI et al., 2015), carpa capim (*Ctenopharyngodon idella*) 166 mg kg^{-1} (WEN et al., 2007), carpa jian (*Cyprinus carpio* var. Jian) 518 mg kg^{-1} (JIANG et al., 2009), carpa gibel (*Carassius auratus gibelio*) 165,3 mg kg^{-1} (GONG et al., 2014), *Channa punctatus* 374,1mg kg^{-1} (ZEHRA; KHAN, 2019) e esturjão híbrido (*Acipenser baerii* x *Acipenser schrenckii*) 335,81mg kg^{-1} (LI et al., 2020).

1.5. SISTEMA IMUNE DE PEIXES E MIO-INOSITOL

O sistema imune de peixes, assim como o de mamíferos é composto por dois grupos de respostas: não específicas (RNE) e adaptativas (RA). Repostas não específicas compreendem barreiras epiteliais e de mucosa, mecanismos humorais e celulares, enquanto respostas específicas são promovidas por células especializadas e proteínas, resultando na produção de imunoglobulinas e outros mecanismos que permitem ao organismo responder de forma específica a determinado antígeno (URIBE et al., 2011). Entre os componentes de RNE de peixes temos, macrófagos, granulócitos, “Toll-like receptors’’, células citotóxicas não específicas, lisozima, fosfatase alcalina, sistema complemento, interferons, transferrina, proteína reativa-C e lectinas, enquanto a RA é promovida principalmente por linfócitos e imunoglobulinas por meio de complexo mecanismo de ativação conjunta a RNE (FIRDAUS-NAWI; ZAMRI-SAAD 2016).

Peixes possuem o sistema adaptativo menos desenvolvido em comparação aos mamíferos, sendo o sistema inato o principal responsável pela proteção contra agentes patogênicos (KORDON; KARSI; PINCHUK et al., 2018). Essa diferença ocorre por questões evolutivas, por peixes possuírem menor número de órgãos linfoides que mamíferos, com

ausência de medula óssea e linfonodos (KLOSTERHOFF et al., 2020), menor variedade de imunoglobulinas, baixas taxas de maturação e proliferação de linfócitos (WHYTE, 2007), tornando a resposta adaptativa mais lenta (SECOMBES; WANG, 2012). Apesar da distinção entre a eficiência das respostas imunológicas, ambos atuam de forma conjunta na proteção contra agentes patogênicos (CASTRO; TAFALLA 2015).

O sistema imunológico de peixes pode ser nutricionalmente modulado, prevenindo enfermidades ou aumentando a resistência dos animais a agentes patogênicos (SHIAU; LIN, 2015). Assim, a suplementação dietética de aditivos com propriedades funcionais, como o mio-inositol, pode melhorar a resposta imunológica. A inclusão de 507 mg kg^{-1} de MI aumentou, significativamente, a concentração de proteína total no soro de barramundi (*Lates calcarifer*) (DIAO et al., 2010). Tal resultado pode estar indiretamente associado ao aumento no crescimento de órgãos linfoides como observado em outras espécies de peixe. De fato, a suplementação de 535,8 mg kg^{-1} de MI favoreceu o crescimento dos principais órgãos linfoides (baço e rim cefálico) e a produção de imunoglobulina sérica (IgM) de carpas Jian desafiadas imunologicamente (JIANG et al., 2009a).

Outras respostas imunológicas da carpa Jian também foram afetadas positivamente pela suplementação de 232,7 a 687,3 mg kg^{-1} de MI, com aumento do número e atividade fagocítica de leucócitos e da atividade da lisozima (JIANG et al., 2009a). Em outro estudo foi observado que o MI melhorou a atividade de macrófagos de roedores, por atuar na regulação da homeostase celular, produção de fosfoinositol e aumentar a concentração de taurina intracelular, pontos relacionados à atividade dessa célula (KIM et al., 2003), tal constatação poderia explicar de maneira indireta e parcial os resultados encontrados para carpa Jian.

Pesquisas mais recentes apontam efeitos positivos da suplementação de MI (28,75 mg kg^{-1}) em dietas com níveis insuficientes deste nutriente para propriedades microbiostáticas e microbicidas do muco cutâneo do Taimem siberiano (*Hucho taimen*) (WANG et al., 2020). Fator atribuído a maior concentração de proteínas solúveis no muco da espécie referida, promovida pela suplementação de MI. Níveis inadequados deste nutriente, 27,00 e 163,5 mg kg^{-1} reduziram a eficiência da barreira intestinal de *Ctenopharyngodon idella* (LI et al., 2018), além de deprimir o sistema imune de espécies como carpa Jian, respectivamente (JIANG et al., 2016). A suplementação de MI pode, ainda, modular positivamente a microbiota intestinal, aumentando a colônia de Lactobacillus em detrimento de colônias de *Aeromonas hydrophila* e *Escherichia coli* em carpas (*Cyprinus carpio* Jian) (JIANG et al., 2009b), auxiliando na manutenção da integridade e saúde intestinal.

De maneira geral, os efeitos positivos da suplementação de MI na saúde de peixes estão associados à regulação da expressão gênica de fatores de transcrição relacionados ao sistema imune, como o NF-κB (citocinas), E2F4 (ciclinas) e TOR (crescimento e proliferação celular), aliado à influência do MI na integridade estrutural e fluidez de membranas celulares, fazendo com que células do sistema imune (macrófagos, neutrófilos, linfócitos B) secretem com maior facilidade substâncias bactericidas como fosfatase ácida, lisozima, proteínas do sistema complemento (C3 e C4) e imunoglobulinas (JIANG et al., 2016; LI et al., 2018).

1.6. SISTEMA ANTIOXIDANTE E MIO-INOSITOL

Espécies reativas de oxigênio (EROS) e radicais livres (RL) são produzidos pelo organismo animal durante processos metabólicos como cadeia respiratória, respostas imunológicas entre outros. Tais compostos estão envolvidos na sinalização celular, no combate a agentes patogênicos e produção de energia, desempenhando papel fundamental para a manutenção da homeostase animal. Entretanto, a produção excessiva de EROS e RL pode se tornar prejudicial fazendo com que o organismo entre em estresse oxidativo (EO). Este quadro pode ser definido como o desbalanço entre a produção de EROS, RL, e sistema antioxidante (BILLER; TAKAHASHI, 2018). O EO oxida proteínas e moléculas de DNA, promove peroxidação lipídica em membranas celulares (SIES, 1985) e limita a resposta imunológica de peixes (BILLER-TAKAHASHI et al., 2015).

Manter a homeostase oxidativa é fundamental, principalmente pela relação existente entre sistema antioxidante e imunológico, o qual produz moléculas oxidativas com ação bactericida durante processos fagocitários (BILLER; TAKAHASHI 2018). Existem dois principais mecanismos mitigadores do EO, o enzimático, composto principalmente pelas enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), e o não enzimático, formado por compostos endógenos e dietéticos que estabilizam ou impedem a formação de moléculas oxidativas (PISOSCHI; POP, 2015).

Entre os compostos com ação antioxidante temos o mio-inositol, que pode prevenir a formação de agentes oxidantes e aumentar a expressão de genes relacionados ao sistema antioxidante. Existem três principais mecanismos de ação de antioxidantes: prevenção, interceptação e reparo (SIES, 1993). O mio-inositol pode atuar na prevenção, evitando a formação de agentes oxidantes, como o radical hidroxila, por se ligar a íons de ferro (HU; CHEN; LIN, 1995), tal ação preventiva foi identificada em peixes. De fato, a suplementação de

mio-inositol em dietas para carpas Jian reduziu a formação de radical hidroxila no tecido muscular, sugerindo ação antioxidante preventiva do MI (JIANG et al., 2010).

Adicionalmente, a incubação de enterócitos de carpa Jian em soluções com níveis graduais de MI (0 a 75 mg MI L^{-1}, 72 horas) e posterior exposição ao cobre (6,0 mg Cu L^{-1} de meio de cultura, 24 h) reduziu danos oxidativos ao tecido (JIANG et al., 2011). Em experimento semelhante, Jiang et al. (2013) constataram que o MI também pode atuar como agente antioxidante na interceptação e reparo da oxidação lipídica e proteica, por estabilizar íons de cobre (Cu^{2+}) em mecanismo semelhante ao da glutationa redutase e aumentar atividade de enzimas do sistema antioxidante durante a reparação celular, respectivamente.

Recentemente, a influência do MI na regulação da expressão gênica de fatores de transcrição, como o Nrf2, relacionado ao aumento na expressão de genes de enzimas antioxidantes (SOD, CAT, GPx, glutationa redutase e S-transferase) também foi evidenciado em carpa capim e Jian (JIANG et al., 2016; LI et al., 2018). Assim, torna-se evidente a importância de se investigar os potenciais efeitos do MI e fitase nos mecanismos de defesa contra patógenos e estresse oxidativo da tilápia do Nilo.

1.7. ENSAIOS *IN VITRO*: PARALELO COM ESTUDOS IN VIVO APLICADOS À IMUNOLOGIA DE PEIXES

A pressão pelo aumento na produção fez com o que a atividade tenha se tornado superintensiva, com altas densidades, manejos frequentes, gerando desafios para os animais. Nas condições citadas, os animais encontram-se em desequilíbrio homeostático, lançando mão de estratégias como repartição de nutrientes e energia para entrar em homeostase novamente. Tal estratégia afeta negativamente diferentes funções fisiológicas, como crescimento e respostas imunológicas tornando os animais mais susceptíveis a agentes patogênicos como bactérias e vírus (DAWOOD; KOSHIO; ESTEBAN, 2018). Adicionalmente, surtos de doenças com alta mortalidade se tornaram frequentes gerando prejuízos econômicos consideráveis (DANTAN et al., 2024).

Considerando o exposto, pesquisas visando o aumento da resistência de peixes à agentes patogênicos com uso de imunoestimulantes são fundamentais para contribuir com o crescimento sustentável e viabilidade econômica da produção de peixes (VIJAYARAM et al., 2022). Entretanto, estudos convencionais avaliando os efeitos de aditivos e outros componentes com funções imunomoduladoras in vivo são complexos, demandam tempo e considerável investimento financeiro. De fato, estudos in vivo são onerosos, envolvem a avaliação de muitos parâmetros para a investigação, e os resultados são afetados por variáveis ambientais e biológicas não controláveis (LIN et al., 2016). E por se tratar de ensaios longos, com uso de animais sensíveis a diferentes intemperes submetidos a condições estressantes, e dietas deficientes existem chances consideráveis do experimento não ser concluído e precisar ser repetido aumentando custos e tempo-resposta. Além disso, estudos envolvendo peixes e outros animais têm sido restringidos por comitês de ética com a aplicação do princípio dos 3R's: redução, reutilização e reciclagem (SOMA et al., 2019).

Dessa forma, alternativas visando otimizar testes com animais têm sido amplamente estudadas. Neste cenário, a técnica de ensaios in vitro tem sido utilizada como alternativa complementar para estudos in vivo. Resultados de ensaios in vitro não refletem fidedignamente respostas in vivo, entretanto podem reduzir custos, aumentar número de repetições e níveis em experimentos dose-resposta. Avaliações in vitro aumentam a sustentabilidade, permitem controlar efeitos adversos, reduzir custos e estudar mecanismos de ação de maneira isolada (PRIDGEON et al., 2011; SEGNER, 2004) e suas restrições do ponto de vista ético são menores (SLOMAN et al., 2019). Os resultados obtidos em pesquisas in vitro podem ainda guiar e serem correlacionados a resultados de futuros experimentos in vivo (RODRIGUES et al., 2024;

SEGNER, 2004). Além disso, o desenvolvimento de meios de cultura quimicamente definidos e padronizados, como o Leibowitz (L-15) utilizados sinergicamente a antimicrobianos viabilizou o avanço de pesquisas in vitro permitindo que culturas celulares pudessem ser mantidas por maior tempo (BOLS, 1991). Utilizar essa fermenta para desenvolver estudos avaliando o sistema antioxidante, toxicologia ambiental e respostas imunológicas em peixes é um importante passo para reduzir o tempo de avaliações contribuindo para o desenvolvimento sustentável da produção de peixes. A técnica de ensaios in vitro pode ser particularmente interessante para estudos imunológicos, visto que permite avaliar respostas de células de tecidos alvo frente ao estímulo de componentes com efeitos imunomoduladores ou estimulantes além de elucidar mecanismos complexos. Estudos in vitro avaliando respostas imunológicas são conduzidos de duas formas: isolando células de animais que receberam dietas suplementadas com o componente alvo por período determinado ou por meio da suplementação do meio de cultura com o componente estudado (Figura 8).

Por meio do isolamento de células de determinado tecido, pode-se produzir dois tipos distintos de culturas celulares: culturas primárias e subculturas imortalizadas. O primeiro tipo é um grupo de células isoladas diretamente do tecido, não sofreram mutações ou passaram por processos de imortalização, possuindo biologia e fisiologia equiparada ao tecido originário. Culturas primárias são consideradas ideais para estudos in vitro devido à alta representatividade de um modelo in vivo (UYSAL et al., 2018). Subculturas imortalizadas são compostas por linhagens de células que tiveram seus processos de senescência evadidos por mutações ou por indução e que podem ser cultivadas por tempo quase indeterminado (CARTER; SHIEH, 2010).

Comparando as duas formas de culturas celulares, percebe-se que células imortalizadas podem se tornar cancerígenas, perder as propriedades e funções fisiológicas do tecido mãe (CARTER; SHIEH, 2010; KAUR; DUFOUR, 2012; UYSAL et al., 2018). Portanto, a prática que menos destoa de situações in vivo seriam estudos utilizando culturas primárias. Apesar de produzir dados que não refletem o comportamento animal em sua totalidade, resultados de estudos in vitro são validados como ferramentas para avaliar respostas imunológicas de peixes (KIRON, 2012). De fato, estudos in vitro proporcionam a compreensão aprofundada e isolada de interações complexas entre agentes patogênicos e as respostas do sistema imunológico de hospedeiros observadas durante surtos de doenças (VILLENA, 2003).

Para estudos de respostas imunológicas, geralmente são utilizadas células isoladas de órgãos como rim, baço, intestino, pele e brânquias (GOSWAMI et al., 2022). Depois de coletadas e devidamente isoladas, os mecanismos de ação das células podem ser avaliados

simulando infecções bacterianas ou virais. Neste tipo de estudo, parâmetros como fagocitose, *burst* respiratório e a atividade bactericida podem ser avaliados (KIRON, 2012). Além disso, culturas celulares podem ser utilizadas como ferramenta para o desenvolvimento de vacinas contra vírus e bactérias. Infecções bacterianas e virais causam perdas massivas na produção de peixes. Por exemplo, a tilapiacultura tem sido consideravelmente afetada pela proliferação de vírus como o vírus do lago (TiLV) e necrose infecciosa esplênica e renal (ISKNV).

Infecções virais são prevenidas de forma eficiente com o uso de vacinas, porém o desenvolvimento destes produtos é oneroso e demanda longos períodos para testes e validações (EVENSEN; LEONG, 2013; YU et al., 2022). Para o desenvolvimento e teste de eficácia e validação de uma vacina é necessário o uso de muitos animais. Este cenário também pode ser otimizado com pesquisas in vitro. Por meio de técnicas de cultivo celular in vitro é possível isolar vírus purificados para o desenvolvimento e testar a eficiência de vacinas reduzindo o tempo de produção e número de animais utilizados durante o processo (GOSWAMI et al., 2022).

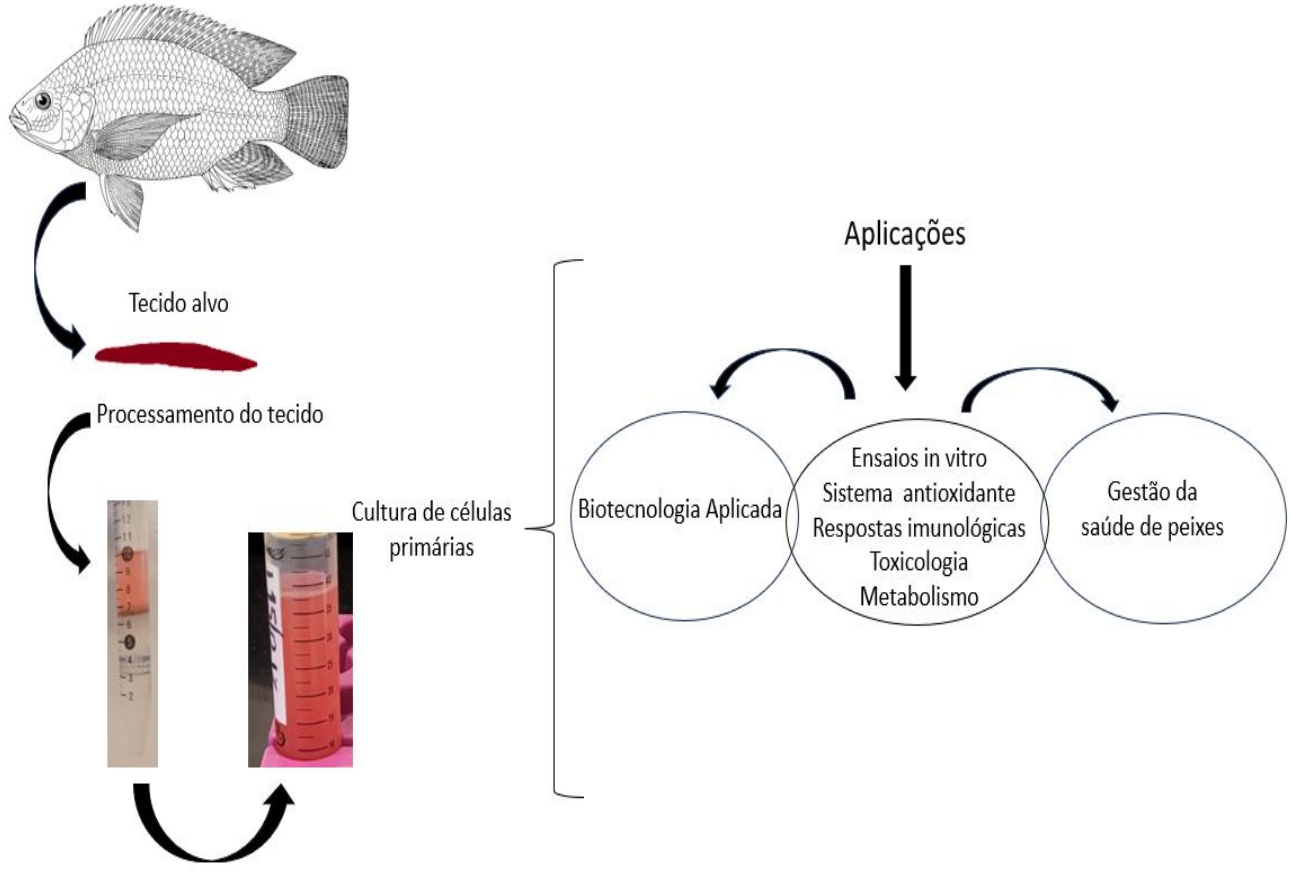

Figura 8. Principais aplicações in vitro para aquacultura.

Apesar do potencial promissor, pesquisas utilizando células de peixes para o desenvolvimento de vacinas ainda são escassas. Células isoladas de outras espécies, como cães, macacos, humanos, roedores e aves são utilizadas com maior frequência para o desenvolvimento de vacinas (GOSWAMI et al., 2022). Entretanto, técnicas in vitro com células de peixe tem sido utilizadas para desenvolver vacinas virais (NAKAJIMA et al., 2002; ZENG et al., 2021) e algumas foram disponibilizadas comercialmente (SATO; OKAMOTO, 2010).

Pesquisas in vitro tem se tornado componentes fundamentais para o avanço da biotecnologia e imunomodulação aplicadas à aquacultura (AARATTUTHODI; DHARAN, 2021). De fato, testes in vitro de suplementação de compostos com função imuno estimulante tem se tornado cada vez mais frequentes. A glutamina (Gln) é um aminoácido funcional para tilápia do Nilo, afetando positivamente respostas imunológicas desta espécie. Este aminoácido é considerado nutriente condicionalmente essencial, sendo utilizado como substrato metabólico durante a proliferação de enterócitos e leucócitos exercendo efeitos positivos em funções de células do sistema imune de tilápia do Nilo, quando suplementada em níveis adequados (CARVALHO et al., 2023).

Foram observados efeitos positivos da suplementação de glutamina em culturas de leucócitos de tilápia do Nilo em estudos recentes (CARVALHO et al., 2018). No referido estudo, foi observado aumento na produção intra e extracelular de ânion superóxido (1,24 e 0,5 mM Gln), atividade fagocítica (1,29 mM Gln), proliferação celular (0,71 mM Gln) e capacidade bactericida de leucócitos (2,50 mM Gln). Neste estudo, foi possível isolar efeitos adversos, observados em estudos in vivo. Baseado nos resultados obtidos no estudo in vitro, Carvalho et al. (2023) replicaram o estudo in vivo. Os referidos autores verificaram efeitos positivos da suplementação de Gln no desempenho, eficiência alimentar e morfologia intestinal. Os efeitos da suplementação de Gln não foram tão expressivos para os parâmetros do sistema imune inato, contrastando significativamente do estudo in vitro. Foram avaliados os seguintes parâmetros: produção de espécies reativas de nitrogênio (NO) e oxigênio (H2O2) e atividade de lisozima. A suplementação de Gln não influenciou a produção das espécies reativas mencionadas, porém, aumentou a atividade da lisozima em 69% em relação ao controle, grupo que não recebeu a suplementação do aminoácido.

A divergência observada entre os resultados dos estudos mencionados pode estar associada a diferentes fatores. Primeiramente, resultados in vitro são mais uniformes visto que a variação entre as células isoladas de determinado tecido é consideravelmente menor do que em modelos animais (GOSWAMI et al., 2022). Além disso, resultados estudos in vitro,

avaliando a suplementação de componentes isolados, não conseguem mimetizar interrelações que ocorrem entre os diferentes nutrientes e componentes de uma dieta. Por isso, variações comparando resultados de modelos in vitro com in vivo são comuns (AARATTUTHODI; VANDANA, 2022).

Em outro estudo contendo um teste in vivo seguido por um in vitro, Jiang et al. (2013) avaliaram os efeitos da suplementação de mio-inositol em parâmetros associados à saúde intestinal e sobrevivência à exposição ao cobre (Cu) de carpa Jian (*Cyprinus carpio* var. Jian), tais como proliferação de enterócitos e capacidade antioxidante. Mio-inositol (MI) é um nutriente que compõe a estrutura de tecidos animais e vegetais e participa ativamente em mecanismos de sinalização celular, evencendo efeitos positivos em funções do sistema imune e antioxidante sendo comumente utilizado como aditivo em dietas para peixes (CUI et al., 2022; SHIAU; SU, 2005).

Jiang et al. (2013) verificaram que a suplementação de 518 mg MI kg^{-1} garantiu uma taxa de sobrevivência de 100% dos animais submetidos ao desafio por exposição ao cobre, enquanto a mortalidade do grupo controle foi de 25%. Além disso, a capacidade antioxidante também foi otimizada com a suplementação de MI. De fato, houve uma redução de 40% da peroxidação lipídica e de 61% oxidação de proteínas e aumento na atividade de enzimas associadas ao estresse oxidativo (catalase, superóxido dismutase, glutationa peroxidase e redutase) no intestino de animais suplementados MI e expostos ao Cu em relação ao grupo não suplementado. Os resultados expostos demonstram os efeitos positivos da suplementação de MI na capacidade antioxidante intestinal de carpas Jian.

No estudo in vitro, também foi observado redução significativa nos marcadores de estresse oxidativo. Jiang et al. (2013) verificaram redução significativa na produção da enzima desidrogenase lática em 36%, 16% para a peroxidação lipídica e 57% da oxidação proteica em cultura de células de enterócitos suplementadas com 75 mg MI L^{-1} submetidas à exposição de Cu. A redução nos indicadores do estresse oxidativo demonstra o efeito positivo da suplementação de MI na capacidade antioxidante de enterócitos da carpa Jian. Comparando os dois estudos conduzidos por Jiang et al. (2013), pode-se observar que os resultados da pesquisa in vitro, mesmo com uso de níveis diferentes de suplementação de MI, são condizentes com os resultados obtidos no estudo in vivo.

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CAPÍTULO II

"Myo-inositol increases respiratory burst, cellular proliferation, and phagocytosis of cultured leukocytes from Nile tilapia"

ABSTRACT

Myo-inositol (MI) is a functional nutrient known to positively affect growth and health parameters when supplemented in appropriate concentrations. This bioactive molecule plays an essential role by modulating several signaling pathways including those related to some immunological responses. Therefore, this study aimed to evaluate the effects of MI supplementation on immunological responses of Nile tilapia head-kidney-derived leukocytes. Primary cell cultures of head-kidney leukocytes were used to evaluate respiratory burst, cell proliferation, and phagocytosis. Five concentrations of MI were supplemented to the culture media: 0, 300, 600, 900, and 1200 mg L^{-1} with 16 replicates per concentration. MI supplementation at 900 mg L^{-1} increased intracellular anion superoxide production up to 15%, whilst 1200 mg L^{-1} boosted extracellular anion superoxide production up to 39% in comparison with the basal group. The phagocytic capacity of cultured cells supplemented with 1200 mg L^{-1} MI was improved by 24% in comparison with the basal group. Proliferative responses also were enhanced by approximately 47% when cells were supplemented with 900 mg L^{-1} of MI. Quadratic regression analysis determined 872 mg L^{-1} to be the optimum supplementation level of MI for leukocyte proliferation. Based on the results of all measured responses, 900 mg L^{-1} of MI was considered the optimal supplementation level for stimulating immunological functions of cultured Nile tilapia leukocytes.

Keywords: immunomodulation, additive, aquaculture

1. INTRODUCTION

The intensification of aquaculture production in various types of intensive systems challenges fish welfare and health. To prevent and/or treat disease outbreaks in aquaculture, antimicrobials have been frequently, and in some cases, inappropriately used. The utilization of such antimicrobials as a strategy to mitigate the occurrence of infectious diseases and improve animal performance is still a noted practice in aquaculture and represents a risk of selecting resistant strains (Dawood et al., 2018)

As an alternative, functional feed additives with immunomodulatory properties have been considered potential substitutes for antimicrobials in disease prevention, in addition to potentially increasing the immune resistance of aquatic animals and, consequently, supporting the sustainable development of the aquaculture industry. Prebiotics and probiotics, glucans, animal and plant extracts, enzymes, and enzymatic blends are examples of feed additives with established immunomodulatory properties when used in fish nutrition (Dawood et al., 2018).

In this regard, myo-inositol (MI) is considered an important nutrient, with different functions in cellular metabolism and a high potential for use as a feed additive in animal nutrition. Myo-inositol is a constituent of cell membranes, acting in cellular signaling and regulating enzyme activity and lipid transport (Shiau and Su, 2005). Moreover, this carboxylic sugar has been shown to positively modulate different immunological responses in fish, such as inflammatory cytokine regulation (Li et al., 2018), reduction of cell apoptosis (Jiang et al., 2016), and increases in lysozyme and alkaline phosphatase mucus concentrations when supplemented in appropriate levels (Wang et al., 2020). Such effects are associated with MI participation in cell signaling pathways (Cui et al., 2022), phosphoinositides production (Fallahi et al., 2018), and cyclins A, E, and D activity which are directly involved in cell proliferation (Jiang et al., 2016).

In fact, it was observed that in common carp (*Cyprinus carpio* var. Jian) immune responses were positively affected by supplementing MI at 232.7 to 687.3 mg kg^{-1} diet, leading to an increase in phagocytosis index, as well as serum lysozyme activity (Jiang et al., 2010). Inappropriate levels of MI in common carp diets were associated with a reduction in lysozyme and acid phosphatase activities, as well as complement 3 and 4 contents in the head kidney and spleen when results were compared with those obtained under optimal dietary MI levels 770.5 mg kg^{-1}(Jiang et al., 2016). The deficiency of this nutrient was further responsible for poor productive performance in hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) (Shiau

and Su, 2005), and other fish species as compiled by Cui et al. (2022). Therefore, MI supplementation in commercial diets is a common and necessary practice for some fish species, such as Nile tilapia, considering that its endogenous production is not enough to meet the metabolic requirements for optimal growth (Jiang et al., 2010; Shiau and Su, 2005; Zaki et al., 2010). Notwithstanding, research investigating the effects of dietary MI supplementation on Nile tilapia health is scarce, but promising (Peres et al., 2004).

Considering the specific characteristics of MI and its important role on the fish immune system, *in vitro* experiments might be an essential tool to understand the complexity of its mechanisms. *In vitro* techniques tend to minimize the external influences promoted by *in vivo* trials and will allow for the assessment of MI effects on specific, isolated components of the fish's immune system. Based on that reasoning, this study aimed to evaluate the effects of MI supplementation in the culture medium of head-kidney-derived leukocytes of Nile tilapia on respiratory burst, phagocytosis and proliferation responses.

2. MATERIAL AND METHODS

2.1. Fish and culture media

A group of Nile tilapia (100 ± 11.43 g) was raised in a recirculation water system composed of 30, 38-L tanks provided with supplementary aeration, and mechanical and biological filtration. Fish were fed a commercial diet containing 32% crude protein and 5% crude fat twice a day to apparent satiation for 30 days. Before sampling, healthy fish were anesthetized using a tricaine methane sulfonate solution (MS-222, 100 mg L^{-1}, Tricaine-S, Western Chemical Inc., Ferndale, WA, USA). Then, blood samples from seven fish were collected with heparinized syringes and used for evaluating neutrophil oxidative radical production based on the reduction of nitroblue tetrazolium (NBT, Cod. 5660, INLAB - Brazil) by superoxide anion (O^{2-}).

After blood sampling, fish were euthanized (MS-222, 500 mg L^{-1}) and head kidneys were aseptically excised and pooled into a homogeneous sample. Then, the head- kidney-derived leukocytes were isolated and cultured according to Secombes (1990), with slight modifications. The culture media was prepared according to Carvalho et al. (2018). Complete culture media (CCM) consisted of MI-free Leibowitz cell culture (L-15, cat #L5520, Sigma Aldrich) media, 50 units mL^{-1} of penicillin and 0.05 mg mL^{-1} of streptomycin (cat #P0781,

Sigma Aldrich) plus 5% of bovine calf serum (BCS, cat # 12133C, Sigma Aldrich) and served as the control medium. Based on literature (Jiang et al., 2010; Peres et al., 2004; Shiau and Su, 2005; Zaki et al., 2010), CCM was supplemented with MI at 0, 300, 600, 900, or 1200 mg L^{-}, resulting in five different media treatments.

2.2. Leukocytes culture

Leukocytes were isolated from the head kidney following the methodology described by Secombes et al. (1990), with modifications. Head kidney tissue was sampled and stored in L-15 supplemented with 2% BCS. After that, samples were homogenized using a glass Potter-Elvehjem tissue grinder and filtered through a 100-μm sterile nylon mesh. The filtered cell homogenate was centrifuged and washed with cold, sterile phosphate buffer saline (PBS, cat #P4417, Sigma Aldrich). Following that, isolated cells were layered on a Percoll (cat #P1644, Sigma Aldrich) gradient (51% v/v) and centrifuged at 400 × *g* for 30 min. The cell layer formed was collected and washed twice with ice-cold PBS at 200 × *g* for 10 min.

The isolated leukocytes were counted using a hemocytometer, and viability was assessed by Trypan blue staining. Cells used in this trial presented a survivability greater than 95%. The cell homogenate was adjusted to 2×10^7 cells mL^{-1} in L-15 with 0.1% BCS. After that, 100-μl aliquots of leukocyte suspension were pipetted in each well of sterile flat bottom 96-well microplates, which were incubated at 27 °C for 2 h for cell attachment. Then, the culture media was substituted with MI-supplemented media, and the microplates were incubated overnight for further analysis.

2.3. Respiratory burst

The respiratory burst activity of head-kidney-derived leukocytes was evaluated by measuring intra- and extra-cellular superoxide anion production, as well as whole-blood NBT reduction as described by Secombes (1990) and Anderson and Siwicki (1995), respectively.

In brief, for the extracellular assay, 100 μl of cytochrome c solution (cat #C2506, Sigma Aldrich) (1.5 mg mL^{-1}, in phenol red-free PBS) mixed with phorbol 12-myristate 13-acetate (PMA, cat #P8139, Sigma, 1 μg mL^{-1}) was added to 16 wells per treatment. Another set of 16 wells per treatment was used as control, adding 100 μl of cytochrome c solution homogenized

with PMA and superoxide dismutase (SOD, 300 U ml^{-1}) (cat #S2515, Sigma Aldrich). After that, a plate reader spectrophotometer was used to measure the absorbance at 545 nm every 15 min. Extracellular O^{2-} production was calculated according to Carvalho et al. (2018): nmol of anion superoxide O^{2-} = [(Δ Absorbance after 45 min × 100) ÷ 6.3].

Intracellular production of O^{2-} was evaluated by quantifying formazan granules formation. Briefly, 100-µl of nitro blue tetrazolium (NBT, cat #N6876, Sigma Aldrich) 0.1% diluted in PBS (PBS, cat #P4417, Sigma Aldrich) was added to 16 wells per treatment and incubated at room temperature for 45 min. The isolated leukocytes were washed twice with 100% methanol (MeOH) and then fixed with 70% methanol. After that, 120 µl of 2 M KOH and 140 µl dimethyl sulfoxide (DMSO, cat #D8418, Sigma Aldrich) were pipetted into the wells to dissolve formazan crystals. The turquoise-blue solution generated was re-suspended, and absorbance was determined in a plate reader spectrophotometer at 620 nm.

Nitroblue tetrazolium (NBT, Cod. 5660, INLAB - Brazil) reduction by superoxide anion (O^{2-}) generated from phagocytes metabolism in fish blood was measured following the methodology described by Anderson and Siwicki (1995) with slight modifications. A 50-µl blood aliquot of each sample was mixed and incubated with 50 µl of CMC for one hour at room temperature. After that, each sample was mixed with 100 µl of NBT solution (0.2%) and incubated at room temperature for 30 min. Then, 50 µL of that mixture was added to 1 mL of N, N-dimethylformamide (Dinâmica, Cod. 1580, SP - Brazil) and centrifuged for 5 min at 3000 rpm. Supernatant was collected and optical density was read at 540 nm. The absorbances were converted to mg ml^{-1} as described by (Li and Gatlin, 2003).

2.4. Phagocytes activity

Cultured Nile tilapia phagocyte activity was assessed by measuring the phagocytosis of stained yeast cells (*Saccharomyces cerevisiae*) as described by Ainsworth and Chen (1990), with modifications. Briefly, 100 µl of leukocyte suspension (1×10^7 cells ml^{-1}) was seeded on a sterile flat bottom 96-well microplate and incubated at 27 ºC for 2 h to allow cell attachment. The culture media was then removed and substituted with MI-supplemented culture media. After a 24-h incubation period, 10 µl of autoclaved dyed yeast suspension (providing a yeast cell:leukocyte ratio of 10:1) was mixed to the leukocyte suspension and incubated at room temperature for 60 min to allow for phagocytosis. Then, the attached cells were carefully washed three times with room temperature PBS after which 100 µl of trypsin-EDTA solution (1.5 g L^{-1} trypsin and 0.4 g L-1 EDTA in PBS) was added to each well and incubated overnight

at 37 °C to solubilize the leukocytes and engulfed yeast, thus resuspending the dye. Absorbance values were read in a plate reader spectrophotometer operating at 620 nm.

2.5. Leukocytes proliferation

The proliferation of head-kidney-derived leukocytes upon stimulation with a non-specific mitogen was measured using the tetrazolium salt 3-(4,5-dimethyl-2 thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) cat #M2128, Sigma Aldrich, 5 mg mL^{-1}) followed by the colorimetric assay as described by Mosmann (1983), with modifications. Supplemented media were added in sets of 16 wells (for each treatment) of leukocyte primary culture plates. Lipopolysaccharide solution (LPS, cat #L2630, Sigma Aldrich, 0.1 mg mL^{-1}) was used to stimulate proliferation. A set of 16 wells did not receive mitogen stimulation and served as a negative control. The resulting plates were incubated for 18 h at 27 °C. Following that, 10 µl of 5 mg MTT mL^{-1} solution was added to each well and incubated at 27 °C for 4 h. Dimethyl sulfoxide was used to dissolve the precipitated formazan crystals, and the optical density was measured at 570 nm using a plate reader. Leukocyte proliferation capacity was computed as stimulation index (SI = ABS stimulated cells ÷ ABS non-stimulated [control] cells).

3. Statistical analysis

Data obtained from the *in vitro* assays were tested for homogeneity and normality using Browne-Forsythe and Shapiro-Wilk tests. Subsequently, data were subjected to analysis of variance (ANOVA) with the assistance of Minitab 19 software to determine whether MI supplementation levels significantly ($P < 0.05$) affected the parameters evaluated. After that, Tukey's test was applied to compare the means ($p < 0.05$). Additionally, a follow-up trend analysis using orthogonal polynomial contrasts was performed to determine if the significant effects followed linear and/or quadratic patterns. After orthogonal contrast evaluation, proliferative response data was submitted to a second-order polynomial model ($y = ax^2 + bx + c$) to determine the optimal supplementation level of MI.

4. Results

4.1. Respiratory burst

Overall, the intra- and extra-cellular production of superoxide anion by Nile tilapia head-kidney-derived leukocytes was positively affected by myo-inositol (MI) graded levels in culture media (Table 1; $P<0.05$). MI supplementation at 900 and 1200 mg L^{-1} significantly increased intra- and extra-cellular anion superoxide production when compared to the non-supplemented group. After subjecting both extra- and intra-cellular anion superoxide production data to a follow-up trend analysis (Figures 1 and 2, respectively), it was observed that MI supplementation manifested a linear effect (Table 1; $P<0.05$). Conversely, NBT reduction by Nile tilapia's whole blood leukocytes was not affected ($P<0.05$) by MI-rich culture media resulting in a reduction of 6,19; 6,33; 6,29; 6.01; 5,91 mg ml^{-1} to 0; 300; 600; 900 and 1200 mg L^{-1} respectively.

4.2. Leukocytes proliferation capacity

The proliferation capacity of Nile tilapia head-kidney-derived leukocytes was positively affected by graded MI levels (Table 2; $P<0.05$). Supplementing MI at 900 mg L^{-1} to culture media produced a higher proliferation response in comparison with the non-supplemented group. After subjecting cell proliferation capacity to a follow-up trend analysis, a quadratic trend effect of MI supplementation was observed (Table 2; $P<0.05$). A second-order regression analysis determined 872 mg MI L^{-1} as the optimum supplementation level for optimal cell proliferation (Figure 3).

4.3. Phagocytosis activity

The phagocytosis activity of Nile tilapia head-kidney-derived phagocytes was positively affected by MI supplementation (Table 2.; $P>0.05$). Higher levels of MI supplementation (900 and 1200 mg L^{-1}) significantly enhanced the phagocytic activity (Table 2. $P<0.05$) when compared with the other MI levels. A follow-up trend analysis determined a linear effect of MI supplementation on phagocytosis activity (Table 2, $P<0.05$), as observed in Figure 4.

5. Discussion

Myo-inositol effects on growth performance (Peres et al., 2004; Shiau and Su, 2005; Zaki et al., 2010), influence on the immunological system (Jiang et al., 2016, 2010; Li et al., 2018; Peres et al., 2004; Wang et al., 2020) and physiological functions compiled by Cui et al. (2022) of aquatic species have been evaluated. However, the effects of MI on some immunological functions in fish species, including Nile tilapia, require further investigation.

Compared with other vertebrates, fish immunological defense relies mainly on innate responses to eliminate pathogens with subsequent activation of adaptative responses. Respiratory burst represents the activity of leukocytes when eliminating invading microorganisms. After engulfing pathogens, the leukocytes produce several substances such as reactive oxygen species and nitrogen that not only will damage the cellular membrane of pathogenic microorganisms but also trigger adaptative responses depending on the severity of the infection (Biller and Takahashi, 2018). MI plays a significant role in several immunological mechanisms including phagocytosis, lymphocyte proliferation, and non-specific immunological responses (Cui et al., 2022). To our knowledge, there are limited studies

evaluating the effects of MI supplementation on respiratory burst, phagocytosis, and leukocyte proliferation of Nile tilapia *in vivo* and *in vitro*. Therefore, this pioneer study evaluated the effects of MI supplementation on the aforementioned parameters.

There are different methodologies to evaluate the respiratory burst of leukocytes. In this study, this parameter was assessed by quantifying extracellular and intracellular superoxide anion production, as well as whole-blood nitro blue tetrazolium (NBT) reduction. Overall, higher levels of MI supplementation to the culture media positively modulated extra- and intra-cellular anion superoxide production of Nile tilapia leukocytes compared with the non-supplemented group. In fact, extra- and intra-cellular anion superoxide production was enhanced by 15% and 39% when MI was supplemented at 900 and 1200 mg L^{-1}, respectively. Overall, these results corroborate those from the phagocytosis assay in which MI supplementation increased phagocytosis up to 24% when compared with the non-supplemented culture media. Conversely, the whole blood NBT reduction was not affected by MI supplementation. This result may be related to the methodology used to evaluate the NBT reduction instead of the MI effect on the mentioned parameter. The authors hypothesized that the incubation time of whole blood leukocytes with MI was not enough to promote significant effects on NBT reduction.

Myo-inositol has been associated with enhancements in phagocyte activity of fish (Jiang, et al., 2010) and other animals (Chen et al., 2015). It was observed that dietary supplementation of 400 mg kg^{-1} of MI improved Jian carp (*Cyprinus carpio* var. Jian) phagocyte activity against *Aeromonas hydrophila* infection (Jiang, et al., 2010). Accordingly, positive effects of MI supplementation on phagocytosis also were found in the present study and, to the best of our knowledge, are the first reports on the influence of MI on Nile tilapia phagocytic activity. There are different mechanisms in which MI could be involved in the stimulation of phagocytosis. This molecule promotes the depolarization of the leukocyte membrane, increasing the potential membrane difference between the host defense cell and the pathogen, which in turn increases cellular attraction and optimizes phagocytosis (Chen et al., 2015). Moreover, myo-inositol is the precursor of biosynthetic components of several phosphoinositides (Fallahi et al., 2018). These phospholipids are involved in plasma membrane distortion promoted by actin polymerization and contraction, thus being essential for pathogen phagocytosis (Swanson, 2014).

Besides affecting respiratory burst and phagocytosis, MI supplementation to the culture media of Nile tilapia leukocytes also improved cell proliferation. Such proliferation was most affected by 900 mg MI L^{-1}, resulting in an increase of 47% compared with the non-

supplemented cell culture. Similar results were obtained by Lazado et al. (2010). Those authors observed that crude phytase supplementation to head-kidney cultured leukocytes of Atlantic cod (*Gadus morhua*) increased leukocyte proliferation due to phytate hydrolysis by phytase, which yields myo-inositol and other inositol phosphates. Similar associations were described in previous studies with other fish species. The correlation between MI supplementation and leukocyte proliferation was further described for fish species by Jiang et al. (2016). In that study, dietary MI supplementation to juvenile Jian carp up-regulated the gene expression in head-kidney and spleen of the E2F4 signaling factors, cyclins D1, A, and E which are involved in cell proliferation with different degrees and roles. Bearing this in mind, we conclude that this study offers scientific evidence of the importance of MI for Nile tilapia immunological parameters. Further studies including *in vivo* assessments and disease-resistance trials should be performed to consolidate the results found herein and expand our understanding of the immune-boosting characteristics of MI and its potential use in functional and environmentally friendly aquafeeds.

6. Conclusion

Overall, myo-inositol (MI) enhanced the immunological activity and proliferation of Nile tilapia cultured leukocytes, and 900 mg L^{-1} is considered the optimum supplementation level to improve phagocytic activity, killing capacity, and cell proliferation of cultured Nile tilapia leukocytes. The findings of this *in vitro* study are promising and warrant further studies on MI effects on Nile tilapia's immunological responses.

Ethics statement

All procedures performed were approved by the Institutional Animal Care and Use Committee at Texas A&M University under IACUC 2022-0162.

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8. Tables

Table 1. Respiratory burst activity of head-kidney-derived leukocytes incubated with graded levelsof myo-inositol (mg L^{-1})

CCM	0	300	600	900	1200	P-value	P.S.E.	Linear trend	Quadratic trend
Extracellular (nmol)	2.01^{B}	2.00^{B}	2.26^{AB}	2.31^{A}	2.26^{AB}	0.005	0.192	0.0011	0.3090
Intracellular (Abs)	507^{B}	669^{A}	616^{A}	656^{A}	704^{A}	0.000	109	0.0002	0.0946
Phagocytosis activity (abs)	0.217^{B}	0.217^{B}	0.205^{B}	0.253^{A}	0.270^{A}	0.000	0.031	0.0001	0.0011

Values represent means of 16 replicate wells. Means within the same row that share a common letter do not differ statistically (Tukey's test, $p < 0.05$). CCM, Complete culture media, P.S.E., pooled standard error.

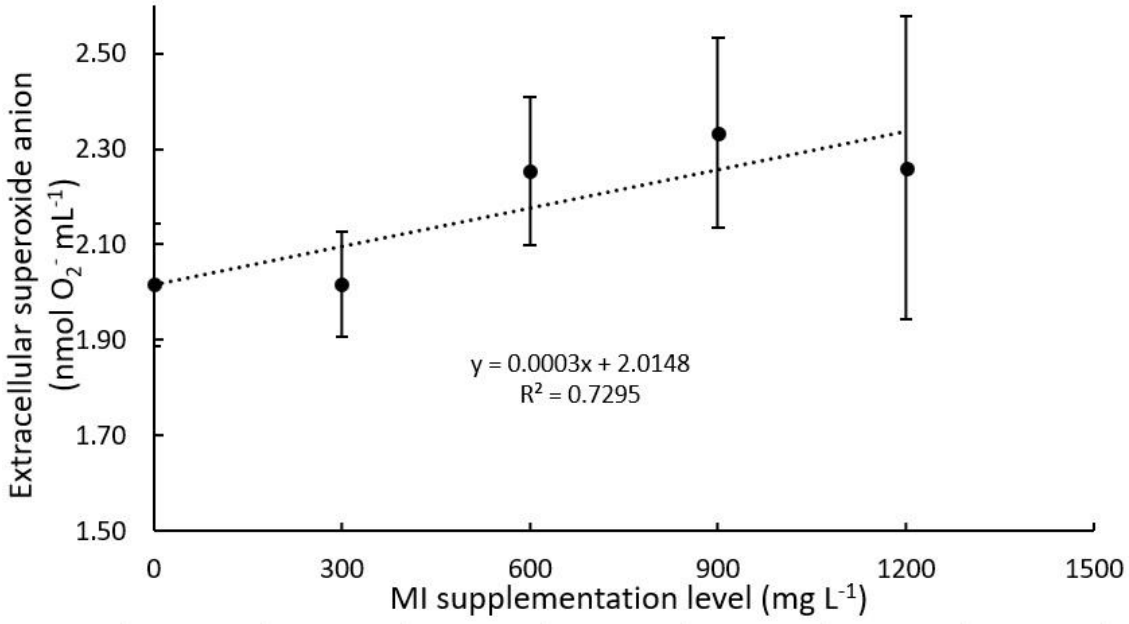
Table 2. Proliferation capacity upon non-specific stimulation of head-kidney-derived leukocytes incubated with graded levels of myo-inositol (mg L^{-1})

CCM	0	300	600	900	1200	P-value	P.S.E.	Linear trend	Quadratic trend
Head-kidney leukocytes	1.46 ^{D}	1.86 ^{C}	1.99 ^{B}	2.14 ^{A}	2.02 ^{B}	0.000	0.113	0.0001	0.0000

Values represent means of 16 replicate wells. Means within the same row that share a common letter do not differ statistically (Tukey's test, $p < 0.05$). CCM, Complete culture media, P.S.E., pooled standard error.

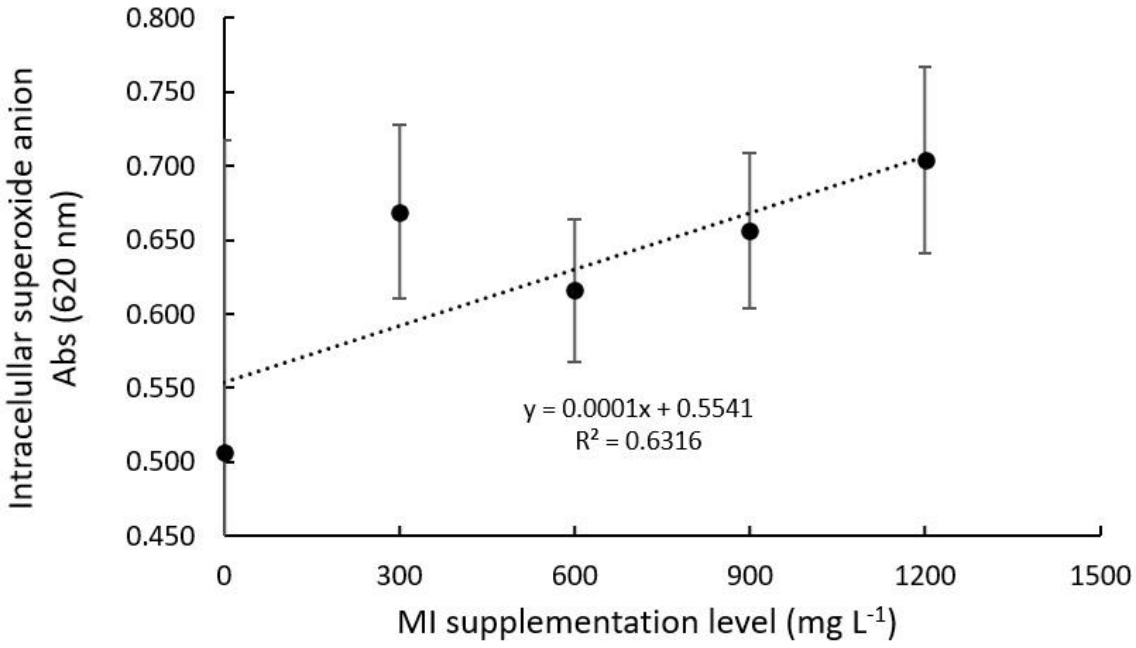
9. Figures

Figure 1 Extracellular superoxide anion production by Nile tilapia head-kidney derived leukocytes incubated with graded levelsof myo-inositol (mg L^{-1})



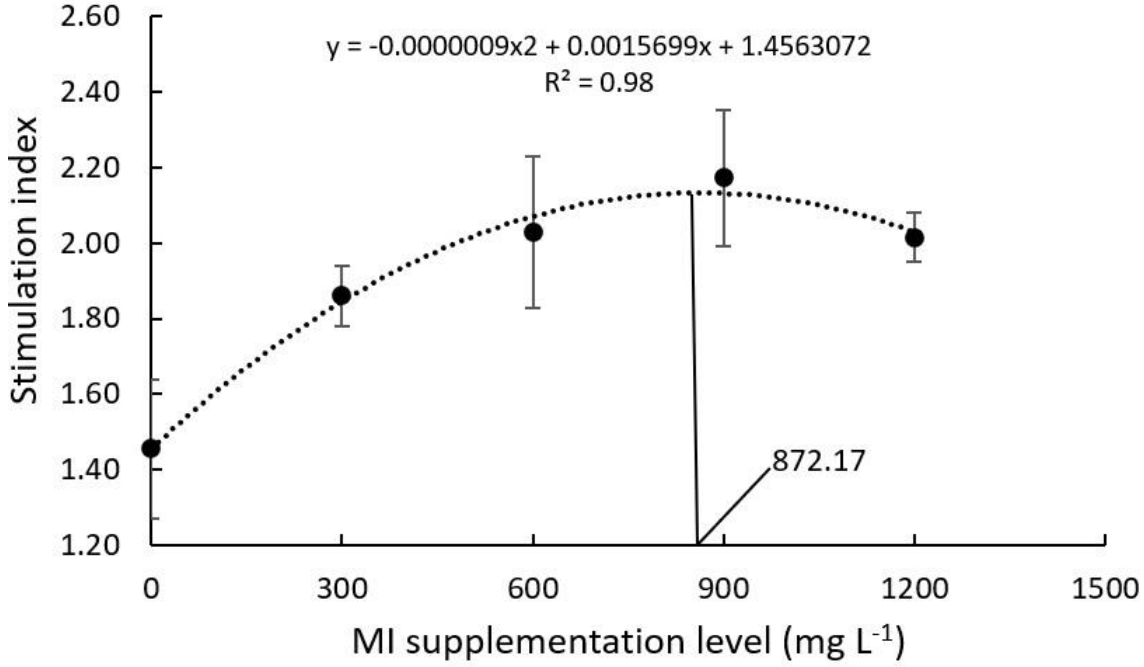
Values represent the means of 16 replicate wells.

Figure 2. Intracellular superoxide anion production by Nile tilapia head-kidney derived leukocytes incubated with graded levels of myo-inositol (mg L^{-1})



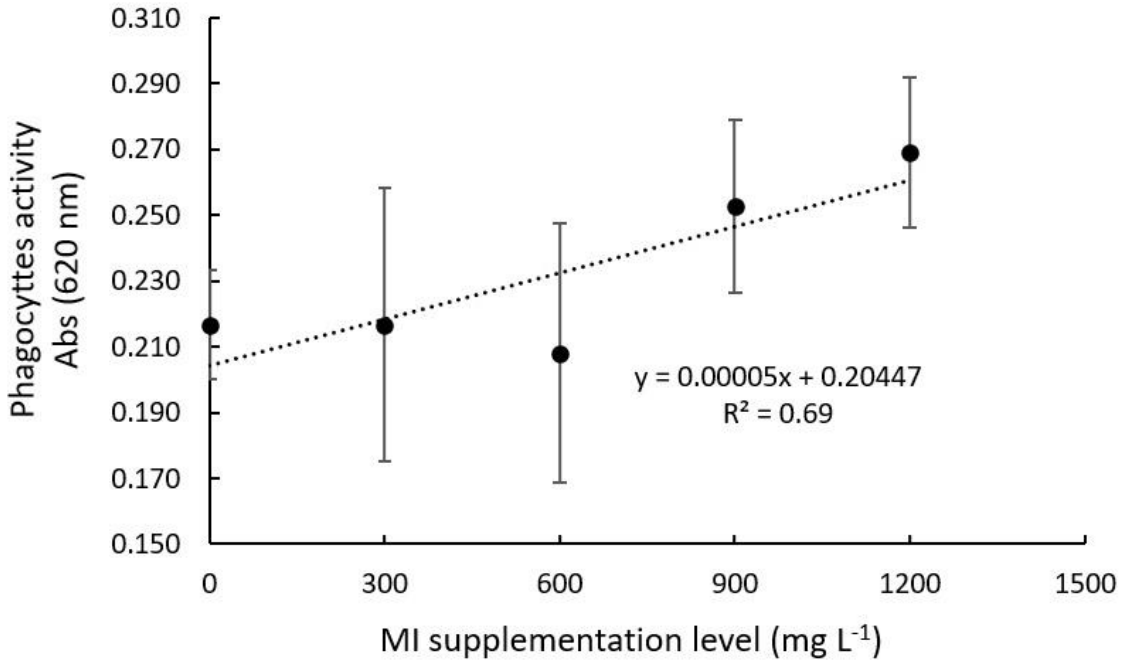
Values represent the means of 16 replicate wells.

Figure 3. Proliferation of head-kidney-derived leukocytes of Nile tilapia incubated with graded levelsof myo-inositol (mg L^{-1})



Values represent the means of 16 replicate wells.

Figure 4. Phagocytes activity of Nile tilapia head-kidney-derived leukocytes incubated with graded levels of myo-inositol (mg L^{-1})



Values represent the means of 16 replicate wells.

CAPÍTULO III

"Myo-inositol supplementation: effects on growth performance, immunological status, antioxidant system and its gene-related expression of Nile tilapia subjected to a bacterial challenge."

ABSTRACT

The study was carried out in a completely randomized design with five treatments and five replicates as follows: control (no supplementation), four and four myo-inositol dietary levels levels (300, 600, 900, and 1200 mg kg^{-1}). Juvenile Nile tilapia (375 fish, 14.9 ± 0.18 g average initial weight) was randomly distributed in 25 250-L aquaria with a density of 15 fish/aquaria. The practical diets were formulated to contain 27% of digestible protein and 14.5 MJ kg^{-1} of 4200 kcal kg^{-1}. Fish were fed their respective diets for 90 days. After this period, growth performance was assessed. Subsequently, five fish per treatment were sampled to evaluate: hematological and blood biochemical profile, innate immune parameters, and antioxidant enzyme activity. The data obtained were used as a parameter for comparison before stress. A 7-day bacterial challenge was conducted with a group of those fish and the same parameters were evaluated to generate the after-stress data. Myo-inositol (MI) supplementation improved weight gain and final weight in comparison with the control group, regardless the dietary level. Fish fed MI-supplemented diets presented an increase in serum cholesterol levels before the bacterial challenge. Additionally, MI supplementation increased the activity of superoxide dismutase and glutathione peroxidase before the bacterial challenged and reduced the malonaldehyde levels after bacterial challenge when supplemented at 900 and 300 mg kg^{-1}, respectively. MI supplementation determined limited effects on innate immunological parameters, increasing lysozyme activity after the bacterial challenge when supplemented at 300 mg kg^{-1} and did not significantly affect survival rate or respiratory burst activity. Considering a polynomial regression analysis of weight gain, 793 mg kg^{-1} of MI is the dietary level recommended for supplementation in Nile tilapia diets.

Keywords: *Aeromonas hydrophila*; feed additives; fish resistance; *Oreochromis niloticu*s.

1. Introduction

The increase in global population worsens the concern of food shortage pressuring the food production sectors. In this sense, aquaculture plays an important role in providing animal protein of high biological value contributing for food shortage attenuation (Boyd et al., 2022). Fish farming is one of the fastest-growing segments in the global food industry. In fact, in 2022 aquatic animal production reached the historical mark of 185 million tonnes, increasing four percent compared with 2020 production, and aquatic animals farming exceeded for the first-time capture fisheries, holding a 51% share of the total produced (FAO, 2024). Besides the present scenario, the prediction is that aquatic animal farming will continue to grow pushed by market demand, which will increase in about 18% by 2030 (FAO, 2022). Despite its considerable expansion, fish farming can only effectively contribute to society and food security through the responsible use of resources and awareness of environmental impacts (Verdegem et al., 2023).

The rising demand for aquaculture farming products stimulated the intensification of productive systems and threatens aquaculture sustainability. Such process was based on using higher densities of monocultures, which can reduce dissolved oxygen, increase nitrogen concentration on water, promote chronical stress and increase fish susceptibility to pathogens leading to disease surges (Boyd et al., 2020; Haenen et al., 2023). In fact, disease outbreaks are major constrains not only to profitability but also for aquaculture sustainable development (David et al., 2021) considering that antimicrobials have been used in excessive amounts to control and prevent diseases rising the risk of antimicrobial resistance (Caputo et al., 2023; Ringø et al., 2007).

There are several approaches to increase fish farming sustainability, including the use of functional feed additives. In fact, prebiotics, probiotics, and enzymatic blends can improve nutrient utilization, reducing waste discharge in the water and environmental impacts (Boyd et al., 2020). Other molecules such as nucleotides, mannooligosaccharides, peptidoglycans, chitosans (Vijayaram et al., 2022) and functional nutrients such as amino acids, minerals and vitamins (Vijayaram et al., 2023) can increase disease resistance mitigating the inadequate use of antimicrobials.

In this context, myo-inositol (MI) could contribute to aquaculture sustainable development and fish resistance against pathogens. MI is a nutrient with a potential yet to be exploited as a functional feed additive in animal nutrition. This molecule is considered a vitamin-like nutrient which composes the structure of both animal and plant tissues, participates

in transmembrane signaling, stimulates the activity of immunological and antioxidants components (Cui et al., 2022; Shiau and Su, 2004). Indeed, MI supplementation positively affected phagocytosis activity of Jian carp (*Cyprinus carpio* var. Jian) as well as serum lysozyme (Jiang et al., 2010). Similar results were observed by Jiang *et al.* (2016). In that study, MI supplementation increased C4 and C3 concentrations, lysozyme and phosphatase activity in the head kidney and spleen of juvenile Jian carp.

The benefits of MI are often linked to upregulation of genes associated to immunological responses such as Nrf2 and E2F4 cycling signaling factors (Jiang et al., 2016),and participation in PI3K-Akt pathway which results in AKT activation. The mentioned kinase modulates the activity and production of target proteins such as NF-κB, TOR, and caspase involved in immunological responses (Cui et al., 2022).Contrastingly, deficient dietary levels of myo-inositol decreased immunological responses of different fish species ((Jiang et al., 2016; Li et al., 2018; Wang et al., 2020) and also productive performance (Jiang et al., 2010; Li et al., 2017; Shiau and Su, 2005; Zaki et al., 2010).

Recent studies verified that supplementing dietary MI enhanced productive performance (Shiau and Su, 2005; Zaki et al., 2010) and carbohydrate metabolism of Nile tilapia (Zhu et al., 2022). Although MI presented positive effects productive performance, studies evaluating MI influence on immunological parameters of Nile tilapia are scarce. Graded dietary levels of MI (0, 50, 100, 200, 400 or 800 mg/kg diet) did not significantly affect Nile tilapia survival rate after *Streptococcus iniae* bacterial challenge and determined limited effects on health status (Peres et al., 2004). The mentioned study considered that endogenous production of MI was sufficient to meet Nile tilapia requirements for growth and health performance. Comparing to other fish species, the number of studies evaluating dietary myo-inositol effects on Nile tilapia immune system are limited and the results are controversial.

Based on the data presented, this study evaluated the effects of myo-inositol supplementation on growth performance, antioxidant, and immunological competence of Nile tilapia subjected to *Aeromonas hydrophila* infection.

2. Material and Methods

2.1. Ethics Statement

All experimental procedures were approved by the Animal Ethics Committee of the Veterinary and Animal Science College, São Paulo State University (protocol CEUA 0134/2020).

2.2.Feed processing and growth performance

State University, Botucatu, SP – Brazil). Five isonitrogenous and isoenergetic plant-based diets were formulated to contain 26.81% digestible protein (DP) and 3036 kcal kg^{-1} digestible energy (DE) meeting the nutrient requirements of Nile tilapia (Furuya, 2010; NRC, 2011), as shown in Table 1. Diets were supplemented with graded levels of myo-inositol (myo-inositol, I5125, Sigma-Aldrich, USA): 0; 300; 600; 900, and 1200 mg kg^{-1}. Feed ingredients were ground into fine particles (0.5 mm), weighed, and homogenized. After that, water was added to the ingredient mixture in a proportion of 25% of the dry weight. The feed mixture was submitted to the extrusion process in a simple screw experimental extruder (4.0 mm pellets, Exteec, Ribeirão Preto, SP, Brazil). Then, the experimental diets were dried in a forced circulation oven at 45°C for 24 hours and then stored in a cold chamber (4º C).

The feeding trial was set in a completely randomized design consisting of five treatments and five replicates. A group of 375 male Nile tilapia (~14.93 g) was randomly distributed in 25 250-L aquaria (15 fish/tank) composing a recirculation aquaculture system (RAS). Fish were fed experimental diets for 90 days four times a day to apparent satiation (8:30; 11:30 am; 2:30; 5:30 pm). Water quality parameters were kept under the recommended levels for the species and monitored using a multi-probe system (YSI PRO 1020®, YSI Environmental, Yellow Springs, OH, USA) once a day: Temperature (26.5 ± 0.5 °C); pH (6.42 ± 0.28); and dissolved oxygen (5.51 ± 0.81 mg L−1). The ammonia concentration was determined using a commercial test kit (Alcon®, Camboriú, SC, Brazil).

Subsequently to the 90-day growth performance trial, the biomass of each experimental unit (tank) was harvested and the productive parameters were recorded and calculated as follows:

Weight gain (WG) = final weight (g) – initial weight (g).

Feed efficiency (FE) = weight gain (g) / dry feed intake (g).

Survival (%) = (Surviving fish / initial number of fish stocked) × 100.

2.3.Chemical Bromatological Analyzes

Samples of diets were ground and analyzed in triplicate following standard methods (AOAC, 2000) for dry matter (DM), crude energy (CE), crude protein (CP), crude fiber (CF), crude lipid (CL) and Ash. Dry matter (DM) was determined by oven-drying at 105°C for 24 h. Crude energy (CE) was assessed using an adiabatic bomb calorimeter (C200, IKA, Staufen, BW, Germany). The Kjeldahl methodology was used in to quantify the nitrogen content, and crude protein (CP) was then calculated as %N × 6.25. Crude lipid (CL) concentration was assessed by Soxhlet extraction with petroleum ether. Ash content was determined by burning samples at 550°C for 24 h.

2.4. Bacterial challenge with *Aeromonas hydrophila*

Prior to subjecting the experimental fish to the bacterial challenge, the lethal dose 50% (LD_{50}) was determined using a different group of fish raised for that purpose. The LD_{50} of *Aeromonas hydrophila* in Nile tilapia was determined by intracelomatic injection following the methodology described by Vaneci-Silva et al. (2022), with slight modifications. In brief, *A. hydrophila* was cultured in brain heart infusion (BHI) (28 °C for 24 hours). After that, bacteria seeds were inoculated in BHI broth, and incubated for 24 hours at 28 °C. Then, the bacteria solution was washed three time in phosphate-buffered saline solution (PBS, pH 7.4), and centrifuged (3000 × g, 4 °C / 10 min). Subsequently, the lethal does (LD50) was determined corresponding to 1.41×10^8 CFU mL^{-1}.

The bacterial assay was carried out with 96 fish from the growth performance trials (16 fish/treatment + a negative control injected with PBS) were anesthetized in benzocaine solution (100 mg mL^{-1}) and injected intraperitoneally with *A. hydrophila* solution (0.1 ml fish $^{-1}$). Fish were randomly stocked into 24 40-L aquaria and mortality was recorded for 7 days. After that, fish were sampled for further analysis.

2.5.Hematological profile

For hematological profile assessment, five fish per treatment (one per tank) were anesthetized with benzocaine solution (100 mg L^{-1}) and blood was sampled from the caudal vasculature using a 1 ml syringe coated with anticoagulant (3% EDTA, Vetec, Química Fina Ltda, Duque de Caxias, RJ, Brazil). Red blood cell count (RBC) was determined by dilution and enumeration using a hemocytometer. Hemoglobin (Hb) was determined by the

cyanmethemoglobin colorimetric method using a commercial kit (Gold Analisa Diagnóstica, Belo Horizonte MG, Brazil) according to Collier (1944). Hematocrit (Htc) was measured by the microhematocrit method (Goldenfarb et al., 1971). The mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) were calculated as MCV = (10 × Htc)/RBC and MCHC = (100 × Hb)/Htc (Wintrobe, 1934). Total plasma protein (TPP) was measured using a manual Goldberg refractometer. The albumin concentration (ALB) was determined by the bromocresol method using a commercial kit (Ref. 19, Labtest Diagnóstica S.A., Brazil). The albumin:globulin ratio (A:G) was assessed using albumin and total plasma protein values, whilst globulin (GLOB) was calculated as follows: GLOB = TPP – ALB. Serum glucose, cholesterol and triglyceride concentrations were determined by spectrophotometry assay using commercial kits (Ref. 137, Ref. 76, Ref. 87, Labtest Diagnóstica S.A., Brazil).

2.6. Immunological parameters

The innate immune responses were evaluated in five fish per treatment (one per tank). Fish were blood-sampled using 1.0-ml syringes, coated or not with anticoagulant, according to the target analysis.

Serum lysozyme activity (LYZ) was determined using the turbidimetric reduction assay described by Ellis (1990). This assay evaluates lysis of suspended *Micrococcus lysodeikticus* cells by measuring the reduction of optical density throughout lysis of the bacterial cell wall. In brief, a cell suspension of *Micrococcus lysodeikticus* (M3770, Sigma-Aldrich) was prepared in autoclaved PBS (0.2 mg/ml). Thereafter, 100 μL of cell suspension was mixed with serum in a flat-bottomed 96-well plate. Optical density was read with a microplate reader at 530 nm.

Respiratory burst activity was evaluated by measuring the reduction of Nitroblue Tetrazolium (NBT, Cod. 5660, INLAB - Brazil) by superoxide anion (O^{2-}) generated from phagocytes metabolism in fish blood following the methodology described by Anderson and Siwicki (1995) with slight modifications. Briefly, each blood sample was mixed with a NBT solution (0.2%) and incubated at room temperature for 30 min. After that, 50 μL of that mixture was added to 1 mL of N, N-dimethylformamide (Dinâmica, Cod. 1580, SP - Brazil) and centrifuged during 5 min at 3000. Supernatant was collected and optical density read at 540. The absorbances were converted to mg ml-1 as described by Li and Gatlin (2003).

2.7. Liver antioxidant capacity

Liver samples were homogenized in phosphate buffer (0.05 M pH 7.0) in 1:5 ratio (g/ml). After that, the resulting homogenate was centrifuged (2800 g; 20 min; 4 °C), and the supernatant was collected for total protein content and enzymatic activity analysis. Samples total protein concentration was assessed using the Bradford method (Bradford, 1976) with coomassie brilliant blue G-250 as dye and bovine serum albumin as standard, absorbance reading was proceeded at 595 nm.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was evaluated using the nitroblue tetrazolium (NBT) reduction assay at 560 nm (Beauchaump & Fridovich, 1971). Catalase (CAT; EC 1.11.1.6) activity was measured following the methodology described by Sinha (1972), in which dichromate in acetic acid is reduced to chromic acetate in the presence of H2O2 and heat (100°C) generating perchromic acid which is quantified in spectrophotometer at 610 nm. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was evaluated following the methodology described by Flohè & Günzler (1984) with absorbance reading at 320 nm. Enzyme activities were expressed in U.mg $^{-1}$ of protein.

2.8. Liver lipid peroxidation (MDA)

Concentration of malonaldehyde (MDA) was as described by Buege & Aust (1978). Supernatant from the liver homogenate (200 μL) was homogenized in 500 μL of a solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA), 80% (v/v) hydrochloric acid 0.25 N and 0.01% (w/v) butylated hydroxytoluene. The resulting solution was heated to 100 °C for 15 min and centrifuged at 1500 g for 10 min in 27°C. After that, supernatant was collected, and absorbance reading was proceeded at 535 nm. Concentration was expressed as nmol MDA as g tissue^{-1} using a calibration curve to convert the data.

2.9. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test to determine possible significant differences among treatments. All analyses were conducted using Minitab® 16.1.1.0 software. Differences among treatments were considered significant at $P < 0.05$. Each data was expressed as mean ± SD. The Student t-test was used to compare non-parametric data.

3. Results

3.1. Growth performance

Myo-inositol (MI) dietary levels enhanced growth performance parameters of Nile tilapia after a 90-day supplementation period (Table 2, Fig. 1). FBW and WG were positively affected by myo-inositol supplementation, which determined higher values compared to the control diet, regardless the MI dietary level ($P<0.05$). Orthogonal contrast analysis determined a significant quadratic trend for final body weight (FBW; $R^2=0,37$; optimal level 750 mg kg^{-1}) and weight gain (WG; $R^2=0,43$; optimal level 793 mg kg^{-1}; Figure 1.). Feed intake, feed efficiency and survival rate were not affected by MI supplementation ($P>0.05$).

3.2. Hematological and biochemical blood parameters

Myo-inositol dietary levels did not affect hematological parameters before the bacterial challenge ($P>0.05$). After the challenge, there was an increase in HTC ($P<0.05$) in fish fed 900 mg kg^{-1} compared to the control diet. The other parameters were not affected by MI supplementation after the BC ($P>0.05$). Comparing the results before and after, there was a decrease in RBC count, Hb level, and HTC determined by MI dietary levels ($P<0.05$). Contrastingly, MI supplementation 600 and 300 mg kg^{-1} determined an increase in MCV and MCHC, respectively ($P<0.05$).

Regarding the biochemical parameters, MI affected only the cholesterol level of fish fed supplemented diets before the BC. MI supplementation increased cholesterol levels in comparison to the control diet, regardless of dietary level ($P<0.05$). The remaining parameters before the BC were not affected ($P>0.05$). Comparing before and after data, there was a decrease in triglicerides and glucose levels of fish fed MI in comparison with the control diet ($P<0.05$). Contrastingly, there was an increase in cholesterol level of fish fed fed 900 mg kg^{-1} of MI ($P>0.05$).

3.3. Immunological parameters

There were no effects of MI supplementation on lysozyme activity and NBT reduction after or before the BC ($P>0.05$). Comparing the before and after the BC data, the NBT reduction was not affected by MI supplementation ($P>0.05$). Lysozyme activity was enhanced the most by 300 mg kg^{-1} of MI. There were no significant ($P>0.05$) effects of MI supplementation on Nile tilapia survival rate after bacterial challenge (Figure 2.).

3.4. Antioxidant capacity and malondialdehyde concentration in liver

Myo-inositol determined a significant effect on antioxidant capacity of Nile tilapia ($P>0.05$). Dietary levels of MI enhanced GPx activity before the BC in comparison with the control diet, regardless the dietary level ($P<0.05$). The other parameters were not affected before the BC ($P>0.05$). After the BC, MI determined a reduction in MDA production when supplemented at 300 mg kg^{-1} in comparison with the control diet. Comparing before and after the BC data, MI supplementation determined a reduction in SOD and GPx activity ($P<0.05$) whilst the control diet did not affect the mentioned parameters ($P>0.05$). MI supplementation also reduced MDA production when supplemented at 300 mg kg^{-1}, in comparison with the control diet ($P<0.05$).

4. Discussion

Myo-inositol is a nutrient with a range of physiological functions including enhancement of growth performance, immunological status, antioxidant capacity, salinity and stress coping (Cui et al., 2022). In fact, some of these regulatory effects were observed in this study aiming to evaluate the effects of MI dietary levels on growth performance, antioxidant capacity and immunological responses of Nile Tilapia subjected to a bacterial challenge.

Data obtained in this study demonstrated that MI dietary supplementation significantly improved growth performance of Nile tilapia in comparison with the control group regardless the level and regression analysis determined 793 mg kg^{-1} as optimal dietary level for WG. This dietary level is higher compared to the finding of previous studies with *Oreochromis spp.*: 408 mg kg^{-1} (Shiau and Su, 2005), 500 mg kg^{-1} (Zaki et al., 2010) and 400 mg kg^{-1} (Bu et al., 2021). These differences may be related to dietary composition, life stage or experimental design considering that most of the mentioned studies used semi-purified diets. The results of this study may be related to the MI capacity of modulating gut microbiome, increasing the concentrations of commensal bacteria while reducing pathogenic populations that disrupt intestinal function. Even though microbiome composition was not evaluated in this study, there are scientific evidence in literature corroborating with this hypothesis. The supplementation of MI, (232.7 up to 990.3 g kg^{-1}) positively modulated gut microbiota of Jian carp (*Cyprinus carpio* var. Jian) in comparison with the non-supplemented group (Jiang et al., 2009a). The authors observed a reduction in both *A. hydrophila* and *Escherichia coli* and an increase in *Lactobacillus spp.* concentrations in fish fed MI supplemented diets. Additionally, MI supplementation increased the most weight gain of Jian Carp at 518 mg kg^{-1} and this effect was related to gut microbiome, intestinal morphology modulation and enhanced endogenous enzymes activity.

Besides microbiome modulation, MI also improves fish intestinal barrier function against reactive oxygen species, reducing tissue damage which positively affects nutrient absorption and growth performance. It is well known that fish growth performance is strongly dependent of gut health status and the latter is often associated to intestinal barrier (Feng et al., 2022; Refstie et al., 2010). In this study, MI supplementation increased antioxidant capacity of Nile tilapia. These findings enforce the hypothesis that MI supplementation positively affected intestinal barrier of Nile tilapia. In fact, Li et al. (2018) observed that MI deficiency reduced the intestinal barrier function of Grass carp (*Ctenopharyngodon idella*) resulting in poor growth performance. In the mentioned study, Grass carp fed MI deficient diets (< 276.7 mg kg^{-1}) presented a reduction in intestinal antioxidant capacity compromising enterocytes integrity among other adverse effects. The opposite effect was observed in Grass carp fed MI supplemented diets.

The effects of MI supplementation on both hematological and biochemical parameters were limited. In fact, MI improved serum cholesterol levels before the bacterial challenge, increasing energy substrate for fish metabolism. Such result was expected due to MI capacity of modulating metabolism of lipids and increasing its utilization in Nile tilapia (Zhu et al., 2023). After the bacterial challenge, fish presented a decrease in the number of red blood cells, hematocrit percentage, and hemoglobin levels. However, the decrease in these parameters was not sufficient to cause anemia in the fish, as the values remained within the normal range for the species (Weiss; Wardrop, 2010). This decrease in the evaluated parameters likely occurred due to the pathogen ability to induce hemorrhage, as *A. hydrophila* produces hemolysin, a toxin that causes red blood cell lysis (Groff and Zinkl, 1999; Campbell and Ellis, 2007).

Regarding the antioxidant data, MI supplementation was able to mitigate oxidative damage of fish after bacterial challenge. Reactive oxygen species (ROS) are metabolically generated in health individuals, however, if ROS production suppress the antioxidant capacity of the organism it can lead to physiological and structural damage in animals cells, such as lipid, protein, and DNA oxidation (Scherz-Shouval and Elazar, 2017). In this study, a slight increase in the antioxidant activity of the enzymes SOD and GPx was observed before bacterial challenge in fish that received diets supplemented with myo-inositol. The authors hypothesize that the observed increase is possibly related to MI ability of stimulating the expression of the Nrf2 factor, which upregulates the expression of antioxidant enzymes SOD, CAT, and GPx (Jiang et al., 2016; Li et al., 2018). In fact, similar results were observed in other fish species. MI dietary supplementation enhanced the expression of NrF2, antioxidant enzymes such as SOD, CAT, GPx and glutathione reductase (GR) as well as their activity on fish intestine (Li et

al., 2018). Additionally, the activity of SOD, CAT and GPx were enhanced in Jian carp (*Cyprinus carpio* var. Jian) fed diets supplemented with MI in levels up to 838,8 mg kg^{-1} (Jiang et al., 2009b).

Contrastingly, after bacterial challenge, fish that received diets supplemented with myo-inositol presented a reduction in SOD and GPx activity. This decrease may have occurred due to a shift in MI antioxidant mechanism of action. Instead of stimulating NrF2 expression to further increase antioxidant enzymes, MI could have enhanced fish anti-superoxide anion (ASA), anti-hydroxyl radical (AHR) scavenging capacity (Nordberg and Arner, 2001; Kohen and Nyska, 2002). Indeed, this effect was observed in Jian carp fed diets supplemented with 567.94 mg kg^{-1} of MI which resulted in an increase in both ASA and AHR and reduction of oxidative damage (Jiang et al., 2009b). Similar effects were observed by Jian et. al (2011), in which Jian carp were fed MI supplemented diets (518 mg kg^{-1}) and exposed to copper induced oxidative stress. It was verified that MI supplementation increased survival rate, ASA and AHR capacity as well as SOD, CAT, GPx and GR activities. The benefits of MI supplementation were associated with its participation in the stabilization of free radicals by either donating electrons or participating in Fenton-like reaction.

After bacterial challenge, there was a decrease in MDA levels in fed 300 mg kg^{-1} of MI. MDA is a common biomarker associated with lipid peroxidation (Jian et al 2009a). Once again, this result reflects the ability of the studied compound to scavenge free radicals, particularly superoxide anion and hydroxyl radicals, which are highly reactive and damaging to cells (Nordberg and Arner, 2001; Kohen and Nyska, 2002). This finding is consistent with the findings of Jiang et al. (2009). In the mentioned study it was observed that *Cyprinus carpio* fed diets supplemented with 518 mg kg^{-1} of myo-inositol showed reduced lipid peroxidation (MDA). Increased MI dietary levels did not affect MDA concentration in this study, and the reason for that is unknown, but could be associated with the high PSD values observed in the data.

The effects of MI supplementation on immunological parameters were limited but promissory to some extent. In this study, respiratory burst was measured by NBT reduction and was not affected by MI supplementation. Lysozyme activity was increased after the bacterial challenge in fish fed 300 mg kg^{-1} of MI. Similar results were obtained in a study with Jian carp (Jiang et al.,2010). The authors verified that supplementing 232 mg kg^{-1} enhanced but higher dietary levels did not affect serum lysozyme activity of fish subjected to a bacterial challenge. Additionally, the activity of lysozyme in the head kidney and spleen of Jian carp was also enhanced by supplementing 232.7 and 545.8 mg kg^{-1} of MI (Jiang et al., 2016). It is well known

that serum lysozyme is derived from macrophages acting as a main component of fish innate system possessing a direct antibacterial effect on gram-positive bacteria (Magnadóttir, 2006). The increase in lysozyme activity in the mentioned studies may be attributed to the role of MI in stimulating leukocytes proliferation via the expression of E2F4 and cyclins which directly affect lysozyme production and activity (Jiang et al., 2016).

Regarding the resistance of fish subjected to the bacterial challenge, MI supplementation increased fish survival rate in comparison to the control group. Indeed, supplementing 900 mg kg^{-1} of MI increased Nile tilapia survival in about 13%. The number of studies evaluating the effects of MI as feed additive on the Nile tilapia survival to bacterial challenges are limited. Peres et al. (2004) verified that MI supplementation, up to 800 mg kg^{-1} determined no significant effects on Nile tilapia survival rate after subjection to a bacterial challenge. However, it is worth noting that in the mentioned study 800 mg kg^{-1} of MI determined a survival rate15.5% higher than the non-supplemented group. These effects may be associated with MI ability to modulate immunological responses after an infection, such as complement C4 and C3, serum lysozyme and acid phosphatase activities via gene expression pathways (Jiang et al., 2016) or enhancement of intestinal barrier, cell integrity, intercellular junctions and reduction of hemorrhage (Li et al., 2018) observed in other fish species.

5. Conclusion

The findings of this study demonstrate that MI enhances not only growth performance but also antioxidant and immunological parameters and resistance against *A. hydrophila* infection. Based on the data presented, 900 mg kg^{-1} of MI is recommended to improve the above-mentioned parameters of Nile tilapia.

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Tables

Table 1. Chemical Ingredient composition of the experimental diets.

Ingredients (%)	*Myo*-inositol (mg kg^{-1})				
	0	300	600	900	1200
Soybean meal	56.80	56.80	56.80	56.80	56.80
Corn	31.86	31.83	31.80	31.77	31.74
Wheat middlings	6.00	6.00	6.00	6.00	6.00
Soybean oil	2.2	2.2	2.2	2.2	2.2
DL – Methionine	0.35	0.35	0.35	0.35	0.35
L - Threonine	0.35	0.35	0.35	0.35	0.35
L - Tryptophan	0.02	0.02	0.02	0.02	0.02
Dicalcium Phosphate	2.05	2.05	2.05	2.05	2.05
Vitamin C[1]	0.09	0.09	0.09	0.09	0.09
Vitamin E[2]	0.010	0.010	0.010	0.010	0.010
Premix Vit/Min[4]	0.15	0.15	0.15	0.15	0.15
BHT[5]	0.02	0.02	0.02	0.02	0.02
NaCl	0.1	0.1	0.1	0.1	0.1
Myo-inositol[6]	0	0.03	0.06	0.09	0.12
Analyzed composition (% dry weight basis)					
Dry matter (%)	93.07	94.00	95.19	95.05	95.12
Crude Energy (kcal/kg^{-1})	4225	4261	4370	4345	4331
Crude protein (%)	30.91	32.01	31.87	32.18	31.80
Crude fiber (%)	3.07	3.31	3.71	3.61	3.64
Crude lipid (%)	3.64	2.12	2.53	2.48	2.29
Ash (%)	5.76	5.82	5.83	6.00	5.96

[1]Vitamin C – Stay – C® 35 – DSM. Nutritional Products. Switzerland. [2]Vitamin E – Vitamin E 50% – Zhejiang Tianxin Pharmaceutical CO. LTD. [4]Vitamin and Mineral Premix – DSM. Nutritional Products. Switzerland. (kg of product): vitamin A = 1.750.000 IU; vitamin D3 = 375.000 IU; vitamin K3 = 500 mg; vitamin B1 = 2.000 mg; vitamin B2 = 2.500 mg; vitamin B6 = 2.500 mg; vitamin B12 = 5.000 mg; vitamin B3 = 8.750 mg; vitamin B5 = 7.500 mg; folic acid = 625 mg; calcium pantothenate= 12.000 mg; biotin = 50 mg; vitamin C = 37.500 mg; Fe = 15.000 mg; Cu = 4 mg; Mn = 3.750 mg; Co = 50 mg; I = 100 mg; Se = 75 mg; Zn = 13.33 g. [5]Butylated hydroxytoluene – antioxidant. Myo-inositol – myo-Inositol – Sigma-Aldrich. USA.

Table 2. Growth performance of Nile tilapia fed graded levels of dietary myo-inositol for 90 days^{-1}.

	Diets							
	Control	**300**	**600**	**900**	**1200**	***PSD***	***P* value**	***Trend***
IBW (g)	14.99	14.89	14.89	14.86	14.84	0.17	0.799	-
FBW (g)	218.96 b	232.62 a	231.85 a	232.57 a	230.07 ab	6.01	0.019	Quadratic*
WG (g)	204 b	217.51 a	217 a	217.70 a	215.22 ab	5.95	0.017	Quadratic**
FI (g)	285.25	294.90	293.22	295.32	290.70	7.87	0.385	-
FE	0.71	0.74	0.74	0.74	0.74	0.02	0.401	-
SUR (%)	100	98.66	100	100	100	1.19	0.431	-

Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$). Abbreviations mean: IBW - Initial body weight, FBW - final body weight, WG - weight gain, FI - feed intake, FE - feed efficiency, SUR – survival; $^{*}y=-0.0000245x^2+0.0367381x+220.38$ ($R^2 = 0.37$); $P<0.01$; $^{**}y=-0.00016x^2+0.25362x+1376.2$ ($R^2 = 0.43$); $P<0.01$.

Table 3. Hematological parameters of Nile tilapia fed graded levels of dietary myo-inositol and subjected to bacterial challenge.

		Diets						
		0	**300**	**600**	**900**	**1200**	***PSD***	***P* value**
RBC (10^6 mL^{-1})	Before	2.14	2.29 A	2.46 A	2.28	2.35 A	0.19	0.235
	After	1.92	1.64 B	1.87 B	1.74	1.71 B	0.30	0.678
	***P* value**	0.349	0.000	0.000	0.057	0.044		
Hb (g dL^{-1})	Before	7.04	7.70	7.88 A	7.78	8.13 A	0.53	0.103
	After	7.43	6.74	7.17 B	7.52	6.15 B	0.98	0.077
	***P* value**	0.416	0.058	0.029	0.425	0.047		
Htc (%)	Before	28.00	33.10 A	32.10 A	31.00	33.70 B	2.85	0.076
	After	28.10 ab	24.50 bB	28.30 abB	30.38 a	25.88 ab A	4.27	0.047
	***P* value**	0.970	0.005	0.018	0.634	0.002		
MCV (fL)	Before	131.16	149.49	131.12	137.42	145.10	11.82	0.234
	After	149.8	148.76	151.88	155.22	137.93	16.97	0.674
	***P* value**	0.237	0.632	0.075	0.131	0.352		
MCHC (%)	Before	25.43	23.39 B	24.53	25.16	23.81	1.97	0.557
	After	26.51	27.71 A	25.40	24.75	26.66	2.08	0.330
	***P* value**	0.452	0.030	0.289	0.749	0.196		
A:G	Before	0.36	0.35	0.35	0.36	0.38	0.05	0.951
	After	0.36	032	0.33	0.32	0.34	0.24	0.910
	***P* value**	0.997	0.305	0,444	0.488	0.385		

RBC - Red blood cell count; Hb- Hemoglobin; Htc - Hematocrit; MCV - Mean corpuscular volume; MCHC - Mean corpuscular hemoglobin concentration; A:G - Albumin:globulin ratio.Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$).

Table 4. Biochemical blood parameters of Nile tilapia fed graded levels of dietary myo-inositol and subjected to bacterial challenge.

		Diets						
		0	**300**	**600**	**900**	**1200**	***PSD***	***P* value**
Triglicerides	Before	391	669 A	424.4 A	323.3 A	414	249.60	0.144
	After	138.3	176.2 B	154.8 B	121.8 B	70.8	68.43	0.415
	***P* value**	0.098	0.005	0.023	0.021	0.057		
Cholesterol	Before	100.15b	146.8 a	131.25 ab	109.48 b B	131.38 ab	19.46	0.008
	After	142.30	148.6	157.20	176.61 A	148.6	32.09	0.667
	***P* value**	0.102	0.922	0.173	0.000	0.922		
Glucose (mg dL^{-1})	Before	45.85	63.25 A	51.14 A	68.10 A	63.25 A	18.14	0.221
	After	35.36	29.07 B	35.21 B	39.97 B	29.07 B	13.33	0.208
	***P* value**	0.099	0.044	0.006	0.032	0.040		

Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$).

Table 5. Immunological blood parameters of Nile tilapia fed graded levels of dietary myo-inositol and subjected to bacterial challenge.

		Diets						
		0	**300**	**600**	**900**	**1200**	***PSD***	***P* value**
LYZ (µg ml $^{-1}$)	Before	11.44	10.95 B	10.91	9.92	12.07	2.91	0.144
	After	13.19	14.55 A	9.89	16.64	13.43	5.48	0.415
	***P* value**	0.292	0.014	0.556	0.212	0.799		
NBT (mg/ml)	Before	4.60	5.33	5.10	5.07	5.07	0.97	0.837
	After	5.31	4.63	4.93	5.73	4.86	0.83	0.516
	***P* value**	0.339	0.282	0.810	0.399	0.713		

LYZ - Serum lysozyme; NBT – Nitro Blue Tetrazolium. Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, P < 0.05).

Table 6. Antioxidant enzyme activity and malondialdehyde concentration of Nile tilapia fed graded levels of dietary myo-inositol for 90 days.

		Diets						
		0	**300**	**600**	**900**	**1200**	***PSD***	***P* value**
SOD (U ml^{-1})	Before	64.07	79.93 A	87.25 A	80.37 A	74.82 A	14.67	0.185
	After	43.89	42.74 B	39.22 B	45.39 B	46.36 B	10.06	0.853
	***P* value**	0.120	0.009	0.02	0,006	0.003		
CAT (U g prot^{-1})	Before	1.50	2.30	2.44	1.80	1.10	0.83	0.138
	After	1.50	1.42	1.57	1.78	1.82	0.43	0.051
	***P* value**	0.997	0.349	0.243	0,980	0.084		
GPx (U g prot^{-1})	Before	5.91 b	7.43 ab A	8.31 ab A	9.14 a A	7.25 ab A	1.44	0.024
	After	5.25	4.58 B	5.31B	5.17 B	4.92 B	1.23	0.878
	***P* value**	0.548	0.016	0.009	0,005	0.013		
MDA (nmol g^{-1})	Before	1.94	2.09 A	1.91	1.96	1.97	0.29	0.878
	After	2.08 ab	1.71 b B	2.36 ab	2.84 a	2.13 ab	0.53	0.043
	***P* value**	0.658	0.040	0,232	0.062	0.377		

SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; and MDA: malondialdehyde. Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, P < 0.05).

Figures

Figure 1. Regression analysis of weight gain (g) of Nile tilapia fed graded levels of dietary myo-inositol for 90 days. Black dots represent five replicates of each dietary treatment. Lines were used to mark the optimum dietary level determined by regression analysis.

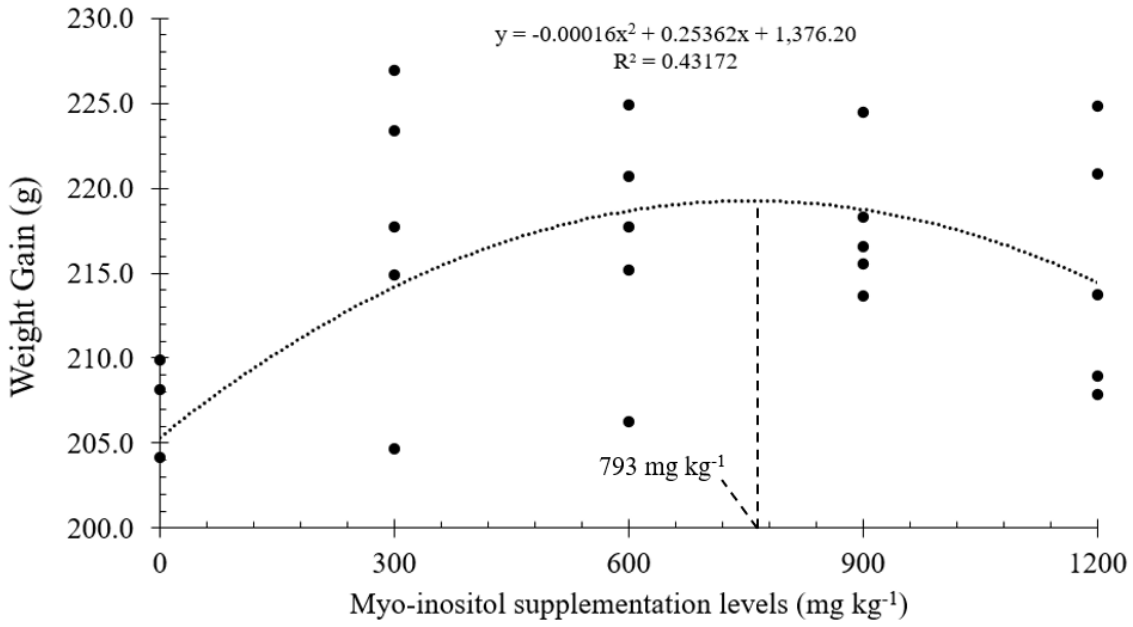
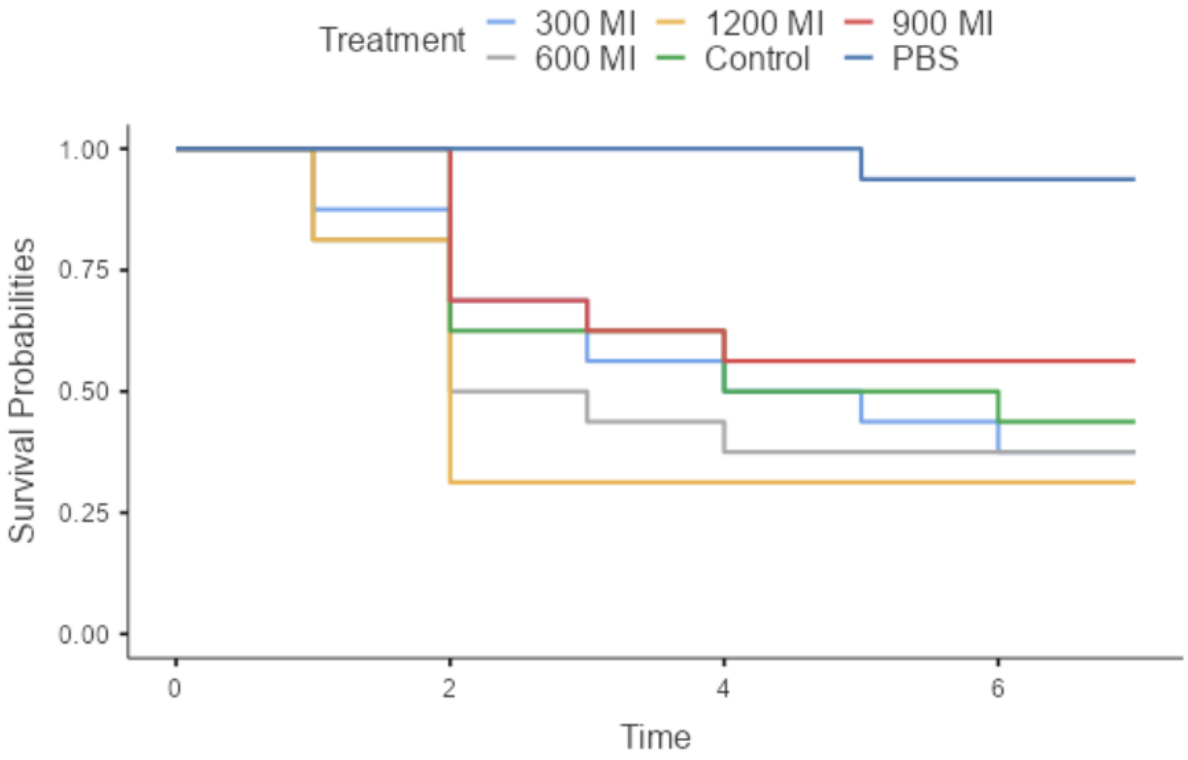


Figure 2. Bacterial challenge assay of Nile tilapia infected with *Aeromonas hydrophila* after a 90-days period feeding trial. Kaplan-Meier survival curve of Nile tilapia fed experimental diets in response to *A. hydrophila* for 15 days (Kaplan-Meier survival analysis $P < .05$).



CAPÍTULO IV

"Dietary phytase improves growth performance, immunological status, antioxidant system and bacterial resistance of Nile tilapia subjected to a bacterial challenge."

ABSTRACT

This study assessed the effects of phytase on growth performance, antioxidant status, and health of Nile tilapia subjected to Aeromonas hydrophila infection. The study was carried out in a completely randomized design with five treatments and five replicates as follows: control (no supplementation), four phytase levels (500, 1500, 2500, and 3500 FTU kg $^{-1}$). Juvenile Nile tilapia (375 fish, 14.9 ± 0.18 g average initial weight) was randomly distributed in 25 250-L aquaria with a density of 15 fish/aquaria. Fish were fed their respective diets for 90 days. After this period, growth performance was assessed. Subsequently, five fish per treatment were sampled to evaluate: the hematological profile, innate immune parameters, antioxidant enzymes activity. The data obtained were used as a parameter for comparison before stress. A 7-day bacterial challenge was conducted with a group of those fish and the same parameters were evaluated to generate the after-stress data. Phytase supplementation improved weight gain, final weight and feed efficiency in comparison with the control group, regardless the dietary level ($P<0.05$). Phytase supplementation determined no effects on hematological profile ($P>0.05$). Regarding the antioxidant capacity, phytase dietary levels increased the most superoxide dismutase activity after the bacterial challenge when supplemented at 2500 FTU kg^{-1}. Additionally, phytase supplemented at 500 FTU kg^{-1} significantly reduced malondialdehyde concentration in comparison with the control group($P<0.05$). Respiratory burst was positively affected by phytase supplemented at 500 FTU kg^{-1} this dietary level also increased survival rate in 31% in comparison with the control diet. Considering a broken line linear model for weight gain, and the immunological parameters evaluated, 500 FTU kg^{-1} of phytase is considered the recommended dietary level for Nile tilapia diets.

Keywords: *Aeromonas hydrophila*; immunomodulation; antioxidant capacity; *Oreochromis niloticu*s

Introduction

Aquaculture production has been growing faster than capture fisheries in the past years, reaching a share of 49% of the total produced in 2020 (FAO 2022). Such growth has been boosted mainly by inland waters fish farms. In that scenario, Brazil is one of the countries with the largest potential for expansion of this activity, with around 10 million hectares of freshwater surface (RORIZ et al., 2017). Brazilian aquaculture production has grown 5% compared with 2021, and Nile tilapia (*Oreochromis niloticus*) is the most produced freshwater species, representing 63.5% of the total national production (PEIXE BR, 2024).

Alongside the increase in productivity lays the challenge that aquaculture sector faces in reducing diseases outbreaks. Due to the high fish density, water quality, temperature oscillation, and hypoxia frequently observed on productive systems, disease outbreaks became a major concern for aquaculture development causing high mortality rates and economic loss (SUBASINGHE et al., 2023). In fact, it has been estimated that at least ten percent of global aquaculture production is lost due infectious diseases resulting in 10 billion USD lost in revenue annually (SHINN et. Al., 2015). Several alternatives have been adopted aiming at reducing the impacts of disease and promote a sustainable development of the aquaculture sector. Immunomodulation via feed additives is one of the most feasible strategies used to increase fish resistance and performance.

Among the mentioned feed additives, enzymes have been studied to exploit their potential as immunomodulators. Phytase is an enzyme widely used as feed additive in non-ruminant production as performance booster. Compiled studies verified that phytase considerably improves amino acids, energy, protein, and minerals bioavailability, which potentially benefits feed efficiency and fish growth performance (CAO *et al.*, 2007; KUMAR *et al.*, 2012; LEMOS; TACON, 2017). Besides its nutritional application, phytase can also enhance antioxidant and immunological responses. It is well known that phytase increases phosphorus, zinc, iron, manganese, and other nutrients such as *myo*-inositol and amino acids related to both immunological and antioxidant responses (PRIYA *et al*., 2023).

In fact, phytase supplementation in phosphorus-deficient diets improved immunological responses of Nile tilapia subjected to *Aeromonas hydrophila* infection (ABO NORAG *et al*., 2018). In that study, phagocytic activity was improved and pathogenic lesions on the liver, spleen, and intestine were mitigated by supplementing 1000 UF kg^{-1}. The authors associated

these findings with an increase in phosphorus availability as well other nutrients such as minor inositol esters and *myo*-inositol.

Based on the data presented, this study aims to evaluate the effects of phytase supplementation on growth performance, antioxidant, and immunological competence of Nile tilapia subjected to *Aeromonas hydrophila* infection.

1. Material and Methods

2.1 Feed processing and growth performance

The feeding trial was conducted at the Laboratory of Fish Nutrition and Health – AquaNutri (Sao Paulo State University, Botucatu, SP – Brazil). Five isonitrogenous and isoenergetic plant-based diets were formulated to contain 26.81% digestible protein (DP) and 3036 kcal kg^{-1} digestible energy (DE) meeting the nutrient requirements of Nile tilapia (Furuya, 2010; NRC, 2011), as shown in Table 1. Diets were supplemented with graded levels Phytase (Quantum Blue® phytase, AB Vista, USA): 0; 500; 1500; 2500, and 3500 FTU kg^{-1}. In this study, FTU was considered as the amount of enzyme that liberates 1 micromole of inorganic orthophosphate per minute from 0.0051 mol L^{-1} sodium phytate at 37° C and pH 5.50 (ENGELEN et al., 1994). Feed ingredients were ground into fine particles (0.5 mm), weighed, and homogenized. After that, water was added to the ingredient mixture in a proportion of 25% of the dry weight. The feed mixture was submitted to the extrusion process in a simple screw experimental extruder (4.0 mm pellets, Exteec, Ribeirão Preto, SP, Brazil). Then, the experimental diets were dried in a forced circulation oven at 45°C for 24 hours and then stored in a cold chamber (4º C).

The feeding trial was set in a completely randomized design consisting of five treatments and five replicates. A group of 375 male Nile tilapia (~14.93 g) was randomly distributed in 25 250-L aquaria (15 fish/tank) composing a recirculation aquaculture system (RAS). Fish were fed experimental diets for 90 days four times a day to apparent satiation (8:30; 11:30 am; 2:30; 5:30 pm). Water quality parameters were kept under the recommended levels for the species and monitored using a multi-probe system (YSI PRO 1020®, YSI Environmental, Yellow Springs, OH, USA) once a day: Temperature (26.5 ± 0.5 °C); pH (6.42 ± 0.28); and dissolved oxygen (5.51 ± 0.81 mg L−1). The ammonia concentration was determined using a commercial test kit (Alcon®, Camboriú, SC, Brazil).

Subsequently to the 90-day growth performance trial, the biomass of each experimental unit (tank) was harvested, and the productive parameters were recorded and calculated as follows:

Weight gain (WG) = final weight (g) – initial weight (g).

Feed efficiency (FE) = weight gain (g) / dry feed intake (g).

Survival (%) = (Surviving fish / initial number of fish stocked) × 100.

2.2 Chemical Bromatological Analyzes

Samples of diets were ground and analyzed in triplicate following standard methods (AOAC, 2000) for dry matter (DM), crude energy (CE), crude protein (CP), crude fiber (CF), crude lipid (CL) and Ash. Dry matter (DM) was determined by oven-drying at 105°C for 24 h. Crude energy (CE) was assessed using an adiabatic bomb calorimeter (C200, IKA, Staufen, BW, Germany). The Kjeldahl methodology was used in to quantify the nitrogen content, and crude protein (CP) was then calculated as %N × 6.25. Crude lipid (CL) concentration was assessed by Soxhlet extraction with petroleum ether. Ash content was determined by burning samples at 550°C for 24 h.

2.3 Bacterial challenge with *Aeromonas hydrophila*

Prior to subjecting the experimental fish to the bacterial challenge, the lethal dose 50% (LD_{50}) was determined using a different group of fish raised for that purpose. The LD_{50} of *Aeromonas hydrophila* in Nile tilapia was determined by intracelomatic injection following the methodology described by Vaneci-Silva et al. (2022), with slight modifications. In brief, *A. hydrophila* was cultured in brain heart infusion (BHI) (28 °C for 24 hours). After that, bacteria seeds were inoculated in BHI broth, and incubated for 24 hours at 28 °C. Then, the bacteria solution was washed three time in phosphate-buffered saline solution (PBS, pH 7.4), and centrifuged (3000 × g, 4 °C / 10 min). Subsequently, the lethal does (LD50) was determined corresponding to 1.41×10^8 CFU mL^{-1}.

The bacterial assay was carried out with 96 fish from the growth performance trials (16 fish/treatment + a negative control injected with PBS) were anesthetized in benzocaine solution (100 mg mL^{-1}) and injected intraperitoneally with *A. hydrophila* solution (0.1 ml fish $^{-1}$). Fish

were randomly stocked into 24 40-L aquaria and mortality was recorded for 7 days. After that, fish were sampled for further analysis.

2.4 Hematological profile

For hematological profile assessment, five fish per treatment (one per tank) were anesthetized with benzocaine solution (100 mg L^{-1}) and blood was sampled from the caudal vasculature using a 1 ml syringe coated with anticoagulant (3% EDTA, Vetec, Química Fina Ltda, Duque de Caxias, RJ, Brazil). Red blood cell count (RBC) was determined by dilution and enumeration using a hemocytometer. Hemoglobin (Hb) was determined by the cyanmethemoglobin colorimetric method using a commercial kit (Gold Analisa Diagnóstica, Belo Horizonte MG, Brazil) according to Collier (1944). Hematocrit (Htc) was measured by the microhematocrit method (Goldenfarb et al., 1971). The mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) were calculated as MCV = (10 × Htc)/RBC and MCHC = (100 × Hb)/Htc (Wintrobe, 1934). Total plasma protein (TPP) was measured using a manual Goldberg refractometer. The albumin concentration (ALB) was determined by the bromocresol method using a commercial kit (Ref. 19, Labtest Diagnóstica S.A., Brazil). The albumin:globulin ratio (A:G) was assessed using albumin and total plasma protein values, whilst globulin (GLOB) was calculated as follows: GLOB = TPP – ALB. Serum glucose, cholesterol and triglyceride concentrations were determined by spectrophotometry assay using commercial kits (Ref. 137, Ref. 76, Ref. 87, Labtest Diagnóstica S.A., Brazil).

2.5 Immunological parameters

The innate immune responses were evaluated in five fish per treatment (one per tank). Fish were blood-sampled using 1.0-ml syringes, coated or not with anticoagulant, according to the target analysis.

Serum lysozyme activity (LYZ) was determined using the turbidimetric reduction assay described by Ellis (1990). This assay evaluates lysis of suspended *Micrococcus lysodeikticus* cells by measuring the reduction of optical density throughout lysis of the bacterial cell wall. In brief, a cell suspension of *Micrococcus lysodeikticus* (M3770, Sigma-Aldrich) was prepared in autoclaved PBS (0.2 mg/ml). Thereafter, 100 μL of cell suspension was mixed with serum in a flat-bottomed 96-well plate. Optical density was read with a microplate reader at 530 nm.

Respiratory burst activity was evaluated by measuring the reduction of Nitroblue Tetrazolium (NBT, Cod. 5660, INLAB - Brazil) by superoxide anion (O^{2-}) generated from phagocytes metabolism in fish blood following the methodology described by Anderson and Siwicki (1995) with slight modifications. Briefly, each blood sample was mixed with a NBT solution (0.2%) and incubated at room temperature for 30 min. After that, 50 μL of that mixture was added to 1 mL of N, N-dimethylformamide (Dinâmica, Cod. 1580, SP - Brazil) and centrifuged during 5 min at 3000. Supernatant was collected and optical density read at 540. The absorbances were converted to mg ml-1 as described by Li and Gatlin (2003).

2.6 Liver antioxidant capacity

Liver samples were homogenized in phosphate buffer (0.05 M pH 7.0) in 1:5 ratio (g/ml). After that, the resulting homogenate was centrifuged (2800 g; 20 min; 4 °C), and the supernatant was collected for total protein content and enzymatic activity analysis. Samples total protein concentration was assessed using the Bradford method (Bradford, 1976) with coomassie brilliant blue G-250 as dye and bovine serum albumin as standard, absorbance reading was proceeded at 595 nm.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was evaluated using the nitroblue tetrazolium (NBT) reduction assay at 560 nm (Beauchaump & Fridovich, 1971). Catalase (CAT; EC 1.11.1.6) activity was measured following the methodology described by Sinha (1972), in which dichromate in acetic acid is reduced to chromic acetate in the presence of H2O2 and heat (100°C) generating perchromic acid which is quantified in spectrophotometer at 610 nm. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was evaluated following the methodology described by Flohè & Günzler (1984) with absorbance reading at 320 nm. Enzyme activities were expressed in $U.mg^{-1}$ of protein.

2.7 Liver lipid peroxidation (MDA)

Concentration of malonaldehyde (MDA) was as described by Buege & Aust (1978). Supernatant from the liver homogenate (200 μL) was homogenized in 500 μL of a solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA), 80% (v/v) hydrochloric acid 0.25 N and 0.01% (w/v) butylated hydroxytoluene. The resulting solution was heated to 100 °C for 15 min and centrifuged at 1500 g for 10 min in 27°C. After that, supernatant was collected, and absorbance reading was proceded at 535 nm. Concentration was expressed as nmol MDA as g $tissue^{-1}$ using a calibration curve to convert the data.

2.8 Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test to determine possible significant differences among treatments. All analyses were conducted using Minitab® 16.1.1.0 software. Differences among treatments were considered significant at $P < 0.05$. Each data was expressed as mean ± SD. The Student t-test was used to compare non-parametric data.

3 Results

3.1 Growth performance

Phytase dietary levels improved growth performance parameters of Nile tilapia after a 90-day supplementation period (Table 2, Fig. 1). After subjecting WG data to a linear broken-line regression model a breakpoint were found at 427.7 FTU kg^{-1}. FBW and WG were enhanced by phytase supplementation determining greater values in composition with control diet regardless the enzyme level ($P<0.05$). Feed efficiency was positively affected by dietary phytase supplementation phytase supplementation. Phytase at 1500 FTU kg^{-1} increased the most FE compared with the other dietary levels ($P<0.05$). Fish fed diets supplemented with phytase presented higher FI in comparison with the control diet ($P<0.05$). Survival rate was not affected by dietary levels of phytase ($P>0.05$).

3.2 Hematological and biochemical blood parameters

Phytase dietary levels did not affect hematological parameters before or after the bacterial challenge ($P>0.05$). Comparing the results before and after, there was a decrease in RBC count, Hb level, and HTC determined by phytase supplementation levels ($P<0.05$). Contrastingly, phytase supplementation at 500 FTU kg^{-1} determined an increase in MCV ($P<0.05$).

Regarding the biochemical parameters, phytase supplementation determined no effects on triclicerides, cholesterol and glucose before or after the bacterial challenge. Comparing before and after data, there was a decrease in triglicerides levels of fish fed phytase-supplemented diets in comparison with the control group ($P<0.05$). A similar pattern was observed in glucose data, fish fed phytase-supplemented diets presented a decrease in that parameter ($P<0.05$) with exception for those fed 3500 FTU kg^{-1} dietary which promoted no effects ($P>0.05$).

3.3 Immunological parameters

Phytase dietary supplementation determined no effects on lysozyme activity and NBT reduction before or after the BC ($P>0.05$). Comparing before and after the BC data, lysozyme activity was not affected by phytase dietary levels ($P>0.05$). In contrast, NBT reduction was positively affected by phytase supplementation ($P<0.05$), 500 FTU kg^{-1} increased the most NBT reduction in comparison with the other dietary levels.

3.4 Antioxidant capacity and malondialdehyde concentration in liver

Dietary levels of phytase determined a significant effect on antioxidant capacity of Nile tilapia to some extent. Phytase supplementation did not affect SOD, CAT, GPx activities or MDA levels before the bacterial challenge. After the bacterial challenge, only SOD activity and MDA levels were affected by phytase supplementation ($P>0.05$). In fact, 2500 FTU kg^{-1} increased the most SOD activity while 500 FTU kg^{-1} determined reduced the most MDA concentration in comparison to other dietary groups. GPx and CAT activities were not affected by phytase supplementation after the bacterial challenge ($P>0.05$). Comparing before and after the BC data, phytase supplementation resulted in a reduction in SOD activity, regardless the dietary level ($P<0.05$). Additionally, fish fed 500 and 3500 FTU kg^{-1} presented a reduction in GPx activity ($P<0.05$). The other parameters were not affected by phytase dietary supplementation ($P>0.05$).

4. Discussion

The findings of this study shows that dietary phytase supplementation significantly enhanced the growth performance and feed efficiency of Nile tilapia. Indeed, Nile tilapia fed phytase-supplemented diet presented a higher growth performance regardless the dietary level. This data is in accordance with previous studies conducted in similar conditions (Abo Norag et al., 2018; Li et al., 2019; Maas et al., 2021; Pontes et al., 2021, 2019) and under commercially intensive rearing conditions (Rodrigues et al., 2023). These results can be attributed to phytate hydrolysis catalyzed by dietary phytase which reduces antinutritional effects of phytate. In fact, phytate can bind itself to cationic groups of amino acids, proteins, starch, lipids, calcium, zinc and trace minerals reducing the bioavailability of these nutrients (Kumar and Anand, 2021). Besides that, phytate can also bind to fish digestive enzymes such as trypsin, pepsin, amylopsin and amylase which considerable worsen the bioavailability of amino acids and energy (Kumar et al., 2012). Dietary phytase counter these antinutritional effects hydrolyzing phytate into

orthophosphates and esters of myo-inositol phosphates, rendering bioavailable phosphorus and other nutrients previously complexed to phytate.

In this study, the breakpoint of phytase supplementation for weight gain was found at 427.7 FTU kg^{-1}. This level is lower than the established in previous studies which ranged from 700 to 2000 FTU kg^{-1} (Adeshina et al., 2023b; Furuya et al., 2001; Negm et al., 2024; Rodrigues et al., 2022). These differences can be attributed to enzyme origin, experimental conditions but mainly to diet formulation and ingredient composition. Most of the mentioned studies evaluated phytase effects on P-deficient diets, therefore, it is expected that higher enzymes are set as optimum levels for growth performance. The optimum phytase level for weight gain found in this study is similar to the one observed by Maas et al. (2020) evaluating practical diets: 660 FTU kg^{-1}. Additionally, the growth performance findings of this study are in accordance with (Abo Norag et al., 2018). The authors evaluated the same commercial enzyme used in the present study and verified that 500 FTU kg^{-1} affected the most Nile tilapia weight gain, even though the diet was 50%-deficient in phosphorus.

The effects of phytase dietary supplementation on hematological and biochemical parameters were limited but are in accordance with the findings of previous studies (Abo Norag et al., 2018). In the mentioned study, phytase did not affect serum biochemical profile of Nile tilapia after and before a bacterial challenge. Subsequently to the bacterial challenge, fish there was a reduction in the number of red blood cells, hematocrit percentage, and hemoglobin levels. The mentioned decrease in the hematological parameters did not cause anemia in the fish, as the values remained within the normal range for the species (Weiss; Wardrop, 2010). In fact, these findings might be related to the pathogen capacity of inducing hemorrhage, as it is well known that *A. hydrophila* produces hemolysin, a toxin that causes red blood cell lysis (Groff and Zinkl, 1999; Campbell and Ellis, 2007).

The antioxidant data obtained in this study showed a potential effect of phytase in modulating antioxidant capacity of Nile tilapia. Antioxidant enzymes have been used as indicators of the antioxidant status of organisms and as biomarkers to assess oxidative stress (Ayhan and Zeliha 2009; İbrahim et al. 2011; Kakoolaki et al. 2013). Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and malondialdehyde (MDA) are amongst the most evaluated biomarkers of antioxidant capacity. In fact, SOD is a major antioxidant enzyme catalyzing the dismutation of superoxide anion free radical into oxygen and hydrogen peroxide (Younus, 2018), GPx and CAT plays crucial roles in scavenging free radicals and derivatives, such as hydrogen peroxide, reducing oxidative damage (Kim et al., 2015) while MDA is metabolite generated from lipid peroxidation reactions (Pan et al., 2017).

To the authors knowledge, there are limited studies evaluating the effects of phytase supplementation on antioxidant capacity of Nile tilapia. In this study, phytase supplementation increased the activity of SOD after the bacterial infection, with a posterior decrease when comparing the groups supplemented groups before and after the bacterial challenge. Phytase supplementation at 500 FTU kg^{-1} also determined a reduction in MDA concentration, showing a penitential modulator effect of reducing lipid peroxidation. Similar results were observed in other fish species fed phytase supplemented diets. In fact, (Xu et al., 2022) observed an increase in SOD and GPx activities and a reduction in MDA concentration in the liver of Gibel carp (*Carassius auratus gibelio*) fed 2000 FTU kg^{-1}. Similarly, Adeshina et al. (2023) verified a positive linear effect of phytase supplementation in African catfish soybean meal-based diets in levels ranging from 250 up to 1000 FTU kg^{-1}. These findings may have occurred due to the effects of phytase on increasing the availability of micronutrients such as Zn, Cu, and Mn, as the SOD activity is affected by the mentioned minerals (Younus, 2018).

Regarding the innate immunological parameters data, phytase supplementation presented a potential modulatory. Although lysozyme activity was not affected by phytase supplementation, respiratory burst, measured by NBT reduction, was enhanced the most in fish fed 500 FUT kg^{-1} after the bacterial challenge. Similar results were observed on survival rates after the infection challenge. Fish 500 FUT kg^{-1} presented a survival rate 31% higher in comparison with the non-supplemented group. Positive effects of phytase supplementation on Nile tilapia survival rate after a bacterial challenge were previously reported. In fact, Abo Norag et al. (2018) verified that supplementing 500 or 1000 FTU kg^{-1} in 50%-phosphorus deficient diets increased survival rate in 14 and 20% respectively, in comparison with the fish fed the non-deficient and non-supplemented diet. The mentioned findings were associated with an increase in phosphorus and could be related to an increase in myo-inositol bioavailability which is an expected effect of phytase. Both nutrients have influence on fish immunological responses and health status. Phosphorus deficiency can lead to decreased immune function on fish organs, induce oxidative damage and apoptosis considering that this mineral regulates NF-κB, TOR, Nrf2, p38 MAPK and MLCK signaling pathways in fish organism (Chen et al., 2017). Added to that, the positive effects of phytase on immunological parameters may be associated with myo-inositol role in stimulating leukocytes proliferation via the expression of E2F4 and cyclins which affects the lysozyme production and activity and other metabolites produced by leukocytes (Jiang et al., 2016; Pietsch et al., 2008; Zheng et al., 2019).

5. Conclusion

In this study the authors investigated the effects of phytase in growth performance, hematological parameters, antioxidant capacity and immunological responses of Nile tilapia fed graded levels of phytase. Considering the mentioned parameters, 500 FTU kg^{-1} can be recommended as the optimum dietary level of phytase to increase performance and fish resistance.

Ethics Statement

All experimental procedures were approved by the Animal Ethics Committee of the Veterinary and Animal Science College, São Paulo State University (protocol CEUA 0134/2020).

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Tables

Table 1. Chemical Ingredient composition of the experimental diets.

Ingredients (%)	Phytase (FTU kg^{-1})				
	0	500	1500	2500	3500
Soybean meal	56.80	56.80	56.80	56.80	56.80
Corn	31.86	31.86	31.86	31.86	31.86
Wheat middlings	6.00	6.00	6.00	6.00	6.00
Soybean oil	2.2	2.2	2.2	2.2	2.2
DL – Methionine	0.35	0.35	0.35	0.35	0.35
L - Threonine	0.35	0.35	0.35	0.35	0.35
L - Tryptophan	0.02	0.02	0.02	0.02	0.02
Dicalcium Phosphate	2.05	2.05	2.05	2.05	2.05
Vitamin C[1]	0.09	0.09	0.09	0.09	0.09
Vitamin E[2]	0.010	0.010	0.010	0.010	0.010
Premix Vit/Min[4]	0.15	0.15	0.15	0.15	0.15
BHT[5]	0.02	0.02	0.02	0.02	0.02
NaCl	0.1	0.1	0.1	0.1	0.1
Analyzed composition (% dry weight basis)					
Dry matter (%)	93.07	95.31	94.08	94.14	94.68
Crude Energy (kcal/kg^{-1})	4225	4354	4354	4354	4354
Crude protein (%)	30.91	32.64	32.31	32.16	32.59
Crude fiber (%)	3.07	3.43	3.62	3.39	3.36
Crude lipid (%)	3.64	2.35	1.96	1.89	2.21
Ash (%)	5.76	6.06	5.98	5.95	5.54

[1]Vitamin C – Stay – C® 35 – DSM. Nutritional Products. Switzerland. [2]Vitamin E – Vitamin E 50% – Zhejiang Tianxin Pharmaceutical CO. LTD. [4]Vitamin and Mineral Premix – DSM. Nutritional Products. Switzerland. (kg of product): vitamin A = 1.750.000 IU; vitamin D3 = 375.000 IU; vitamin K3 = 500 mg; vitamin B1 = 2.000 mg; vitamin B2 = 2.500 mg; vitamin B6 = 2.500 mg; vitamin B12 = 5.000 mg; vitamin B3 = 8.750 mg; vitamin B5 = 7.500 mg; folic acid = 625 mg; calcium pantothenate= 12.000 mg; biotin = 50 mg; vitamin C = 37.500 mg; Fe = 15.000 mg; Cu = 4 mg; Mn = 3.750 mg; Co = 50 mg; I = 100 mg; Se = 75 mg; Zn = 13.33 g. [5]Butylated hydroxytoluene – antioxidant. Phytase – Quantum Blue – AB Vista, USA.

Table 2. Growth performance of Nile tilapia fed graded levels of phytase for 90 days^{-1}.

	Diets						
	Control	**500**	**1500**	**2500**	**3500**	***PSD***	***P* value**
IBW (g)	14.99	14.95	14.84	14.97	14.93	0.16	0.738
FBW (g)	218.96 b	239.48 a	237.63 a	239.56 a	232.26 a	6.09	< 0.001
WG (g)	204 b	224.54 a	222.79 a	224.59 a	217.33 a	6.11	< 0.001
FI (g)	285.25 c	307.90 ab	296 bc	313.30 a	294 bc	7.44	< 0.001
FE	0.71 b	0.73 ab	0.75 a	0.72 b	0.74 ab	0.01	0.038
SUR (%)	100	100	100	100	100	-	0.431

Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$). Abbreviations mean: IBW - Initial body weight, FBW - final body weight, WG - weight gain, FI - feed intake, FE - feed efficiency, SUR – survival; $^{*}y=-0.0000245x^2+0.0367381x+220.38$ ($R^2 = 0.37$); $P<0.01$; $^{**}y=-0.00016x^2+0.25362x+1376.2$ ($R^2 = 0.43$); $P<0.01$.

Table 3. Hematological parameters of Nile tilapia fed graded levels of phytase and subjected to bacterial challenge.

		Diets						
		0	**500**	**1500**	**2500**	**3500**	***PSD***	***P* value**
RBC (10^6 mL^{-1})	Before	2.14	2.37 A	2.47 A	2.33 A	2. 50 A	0.28	0.419
	After	1.92	1.86 B	1.81 B	1.60 B	1.77 B	0.26	0.223
	***P* value**	0.349	0.036	0.003	0.010	0.014		
Hb (g dL^{-1})	Before	7.04	8.13 A	8.13	8.31 A	7.88	0.60	0.053
	After	7.43	6.15 B	7.50	7.01 B	6.71	0.98	0.077
	***P* value**	0.416	0.033	0.321	0.038	0.054		
Htc (%)	Before	28.00	30.90	33.80 A	31.80 A	34.70 A	3.22	0.056
	After	28.10	29.30	26.30 B	26.00 B	26.30 B	2.58	0.328
	***P* value**	0.970	0.557	0.003	0.016	0.003		
MCV (fL)	Before	131.16	131.30 B	137.19	137.73	141.63	11.95	0.672
	After	149.8	158.09 A	146.90	166.7	152.28	20.25	0.656
	***P* value**	0.237	0.020	0.112	0.120	0.268		
MCHC (%)	Before	25.43	24.79	24.56	24.79	24.06	2.02	0.595
	After	26.51	25.64	27.45	25.86	26.14	2.27	0.810
	***P* value**	0.452	0.364	0.104	0.321	0.258		
A:G	Before	0.36	0.35	0.39	0.36	0.35	0.05	0.835
	After	0.36	034	0.30	0.34	0.34	0.07	0.869
	***P* value**	0.997	0.802	0,247	0.531	0.906		

RBC - Red blood cell count; Hb- Hemoglobin; Htc - Hematocrit; MCV - Mean corpuscular volume; MCHC - Mean corpuscular hemoglobin concentration; A:G - Albumin:globulin ratio. Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$).

Table 4. Biochemical blood parameters of Nile tilapia fed graded levels of phytase and subjected to bacterial challenge.

		Diets						
		0	**500**	**1500**	**2500**	**3500**	***PSD***	***P* value**
Triglicerides	Before	391	482.7 A	517 A	522 A	823 A	209.70	0.303
	After	138.3	113.6 B	73.3 B	90.84 B	108.4 B	34.38	0.321
	***P* value**	0.098	0.021	0.017	0.019	0.036		
Cholesterol	Before	100.15	132.42	121.00	123.30	138.10	31.63	0.292
	After	142.30	149.10	165.00	138.60	167.10	26.30	0.590
	***P* value**	0.102	0.094	0.065	0.470	0.364		
Glucose (mg dL^{-1})	Before	45.85	70.31 A	66.70 A	67.96 A	60.28	18.77	0.149
	After	35.36	32.70 B	43.32 B	36.54 B	42.21	7.94	0.187
	***P* value**	0.099	0.014	0.005	0.008	0.079		

Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$).

Table 5. Antioxidant enzyme activity and malondialdehyde concentration of Nile tilapia fed graded levels of dietary myo-inositol for 90 days

		Diets						
		0	**500**	**1500**	**2500**	**3500**	***PSD***	***P* value**
SOD	Before	64.07	91.21A	84.69 A	82.49	69.45	16.68	0.097
(U ml^{-1})	After	43.89 ab	44.33 ab B	41.16 b B	59.40 a	38.77 b	8.65	0.013
	***P* value**	0.120	0.000	0.011	0.024	0.017		
CAT (U g $prot^{-1}$)	Before	1.50	2.37	2.39	0.75 B	0.58	1.57	0.364
	After	1.50	1.57	1.31	2.11 A	1.65	0.45	0.127
	***P* value**	0.997	0.528	0.328	0.010	0.134		
GPx (U g $prot^{-1}$)	Before	5.91	8.61 A	7.51	7.24	6.91 A	1.45	0.324
	After	5.25	4.65 B	4.79	5.96	4.10 B	1.16	0.166
	***P* value**	0.548	0.000	0.056	0.218	0.040		
MDA (nmol g^{-1})	Before	1.94	1.96	1.78	1.87	1.90 B	0.31	0.886
	After	2.08 ab	1.67 b	2.25 ab	2.23 ab	2.99 a A	0.50	0.014
	***P* value**	0.658	0.767	0.137	0.132	0.031		

SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; and MDA: malondialdehyde. Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$).

Table 6. Immunological blood parameters of Nile tilapia fed graded levels of dietary myo-inositol and subjected to bacterial challenge.

		Diets						
		0	**500**	**1500**	**2500**	**3500**	***PSD***	***P* value**
LYZ (µg ml $^{-1}$)	Before	11.44	11.85	10.62	10.52	14.22	2.89	0.303
	After	13.19	19.01	11.49	13.82 b	14.16	4.38	0.321
	***P* value**	0.292	0.274	0.799	0.301	0.976		
NBT (mg/ml)	Before	4.60	4.92	5.22 A	4.58	4.52	0.89	0.292
	After	5.10 ab	5.84 a	4.71 B ab	4.40 b	3.84 b	0.47	0.005
	***P* value**	0.339	0.186	0.004	0.613	0.082		

LYZ - Serum lysozyme; NBT – Nitro Blue Tetrazolium. Values are means ± PSD (pooled standard deviation) of five replicates. Letters indicate significant differences between dietary treatments (ANOVA, Tukey's test, $P < 0.05$).

Figures

Figure 1. Broken line analysis of weight gain (g) of Nile tilapia fed graded levels of dietary phytase for 90 days. Black dots represent five replicates of each dietary treatment. Lines were used to mark the optimum dietary level determined broken line model.

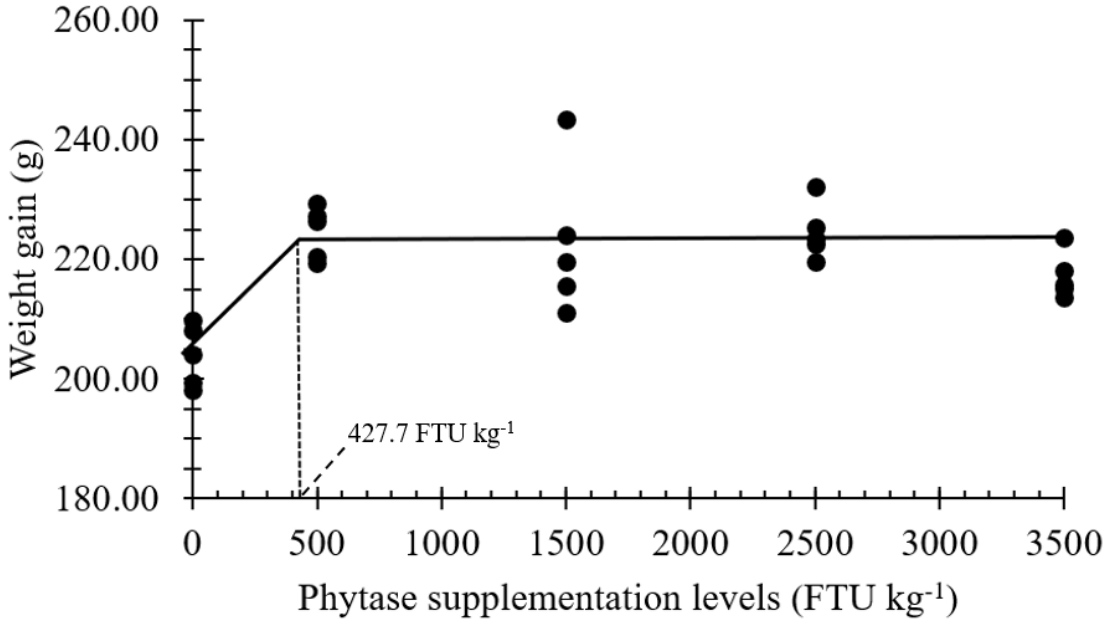
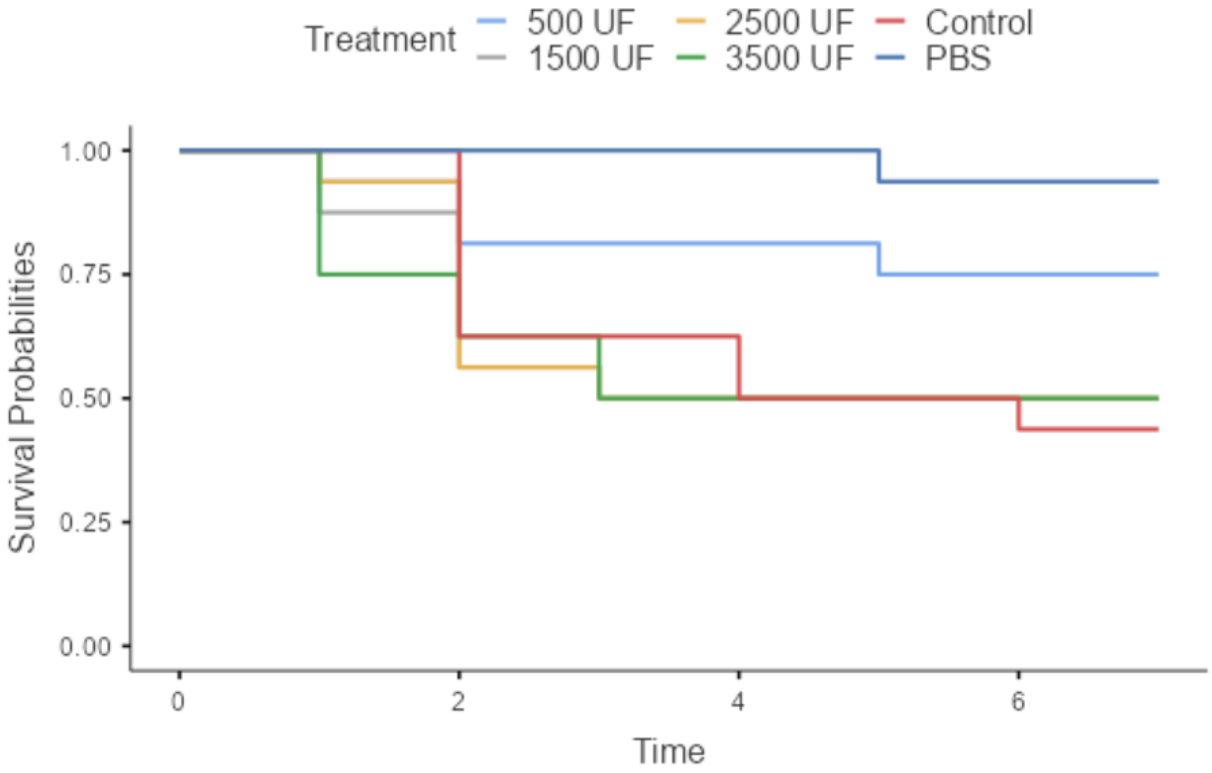


Figure 2. Bacterial challenge assay of Nile tilapia infected with *Aeromonas hydrophila* after a 90-days period feeding trial. Kaplan-Meier survival curve of Nile tilapia fed experimental diets in response to A. hydrophila for 15 days (Kaplan-Meier survival analysis P < .05).



CAPÍTULO V

IMPLICAÇÕES

Utilizar aditivos alimentares que melhorem não somente a performance produtiva animal, mas também aumente a resistência frente a infecções por agentes patogênicos, biodisponibilização de nutrientes em rações e reduzam a excreção de poluentes para o ambiente comtempla os objetivos do desenvolvimento sustentável da ONU fome zero e agricultura sustentável; indústria, inovação, infraestrutura e vida na água.

De fato, a fitase e o mio-inositol estão entre os aditivos comerciais que promovem os benefícios mencionados contribuindo para a sustentabilidade da aquacultura. A fitase aumenta o uso de ingredientes vegetais, fontes renováveis de fósforo, reduz a excreção deste mineral e nitrogênio no ambiente aquático. Adicionalmente, melhora a biodisponibilidade de aminoácidos, energia, e minerais para os animais promovendo efeitos positivos para o desempenho produtivo. O potencial indireto imunoestimulante da fitase tem sido avaliado visto que essa enzima também aumenta a disponibilidade de mio-inositol.

Este nutriente afeta positivamente o desempenho produtivo bem como respostas imunológicas de peixes devido as diferentes funções fisiológicas que desempenha como elucidado ao longo deste trabalho. Estimular o uso dos aditivos mencionados pode ainda reduzir o uso inadequado de antimicrobianos, realidade comum no setor aquícola. Os resultados obtidos neste estudo demonstram que a fitase e o mio-inositol apresentam potencial como melhoradores de desempenho e imunoestimulantes. Sendo recomendado a suplementação de 900 mg kg^{-1} ou 500 FTU kg^{-1} de mio-inositol ou fitase, respectivamente para melhorar respostas produtivas e de resistência à infecção bacteriana. Além disso, esta tese pode ser utilizada como guia para estudos futuros ou servir como referência durante a escolha de níveis de suplementação em dietas práticas, contribuindo para a sustentabilidade da aquacultura.