UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

Thamara Luísa Staudt Schneider

PROTEASE EXÓGENA EM DIETAS PARA TILÁPIAS DO NILO
(*Oreochromis niloticus*)

Santa Maria, RS
2024

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Tese apresentada ao Programa de Pós-Graduação em Zootecnia, Área de Concentração em Produção Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Zootecnia**.

Orientador: Prof. Dr. Rafael Lazzari

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Aprovada em 06 de março de 2024:

_____
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(Presidente/Orientador)

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Santa Maria, RS
2024

Este estudo foi financiado em parte pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código Financeiro 001

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001

Schneider, Thamara Luísa Staudt
PROTEASE EXÓGENA EM DIETAS PARA TILÁPIAS DO NILO (*Oreochromis niloticus*) / Thamara Luísa Staudt Schneider. 2024.
137 p.; 30 cm

Orientador: Rafael Lazzari
Coorientadora: Leila Picolli Da Silva
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Zootecnia, RS, 2024

1. produção de peixes; 2. nutrição de peixes; 3. atividade enzimática; 4. expressão de genes.
I. Lazzari, Rafael II. Da Silva, Leila Picolli III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pela autora. Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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AGRADECIMENTOS

A Deus pelo dom da vida e por me fazer depositar fé todos os dias.

À minha base, minha família: Avó Anita, Mãe Janete, Padrasto Luciano, Pai Enio, e irmãs Gabriela e Vitória, pelo amor, incentivo e pela compreensão nos momentos de ausência. E aos falecidos, avós Alfredo Staudt, Ermindo Schneider e avó Semida Staudt, que me guiaram de longe nessa jornada.

Ao meu namorado Jonhatan M. B. e à sua família (Silvia, Luciana, Pitter, Paulo e João Paulo) pelo amor e apoio incondicional.

Ao meu orientador e amigo, Professor Rafael Lazzari, pela confiança, orientação e ajuda de sempre.

Aos professores que compartilharam seu tempo durante as análises em laboratório, confecção das rações e análises de estatísticas, Nilce Peixoto, Daniel Graichen, Bruno Loureiro e Rômulo Rodrigues. A arte de transmitir conhecimentos, é a técnica de saber ensinar. Muito grata!

Aos meus amigos (as) Luiza, Emerson, Maritiele, Alessandra, Lita, Samuel, Edirlene, Antônio, ao grupo 'Friends' e ao time 'Meninas da Bola', que rechearam meus dias com descontração, atividade física, música, pedaladas, futebol, jantas...

Às gurias da Piscicultura, Mara Rubia, Roberta, Giulia, Saionara e Joziane que me acompanharam e auxiliaram nas atividades experimentais. Também, obrigada aos GenEver's (Pedro, Mayara, Kauanne, Tainá, Gilberto, Izael, Renata e Giliane) pelas experiências na biologia molecular.

A toda a UFSM, desde os bens materiais e equipamentos até, principalmente, as pessoas que fazem acontecer a Universidade, desde a limpeza até o reitor. Vocês contribuíram muito para a construção deste trabalho e para a realização desta conquista.

Aos laboratórios de pesquisa, como Laboratório de Piscicultura, Genética Evolutiva (GenEvo) e Histologia que ampliaram meu leque de resultados. Em especial, aos servidores Alexandra, Andrielle, Juliano, Joviana e à aluna Isabela pelo suporte e amizade.

Ao apoio financeiro da CAPES, pois não seria possível a dedicação exclusiva.

Às empresas parceiras, muito obrigada pelas doações e parcerias. Mesmo com todas as dificuldades da pandemia, não deixaram de colaborar com a pesquisa.

Às tilápias pela contribuição para o progresso científico alcançado por este trabalho, e meu respeito aos peixes que foram eutanasiados.

A todos que, de alguma forma ou de outra, torceram pela minha felicidade e contribuíram para a realização deste sonho.

“Por vezes sentimos que aquilo que fazemos não é
senão uma gota de água no mar. Mas o mar
seria menor se lhe faltasse uma gota.”
Madre Teresa de Calcutá

“Give a person a fish and you feed them
for a day; teach them how to grow fish
and you feed them for a lifetime.”
(from a Chinese proverb)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Zootecnia
Universidade Federal de Santa Maria

PROTEASE EXÓGENA EM DIETAS PARA TILÁPIAS DO NILO
(Oreochromis niloticus)

AUTORA: THAMARA LUÍSA STAUDT SCHNEIDER
ORIENTADOR: PROF. DR. RAFAEL LAZZARI

A protease é uma enzima caracterizada pela ação sobre ligações proteicas e pode melhorar assimilação de nutrientes dos ingredientes proteicos. Efeitos positivos de sua inclusão foram identificados em muitas espécies, pela melhora na eficiência alimentar e síntese de proteína. Fatores ligados ao processamento de rações, o nível e a forma de inclusão da protease influenciam na sua eficiência, assim como, a composição das dietas. Dessa forma, o objetivo foi avaliar se há efeitos da inclusão de protease em dietas para tilápias sobre parâmetros de desempenho, metabolismo bioquímico, atividade de enzimas digestivas, digestibilidade dos nutrientes e expressão gênica. Para isto foram realizados dois experimentos com duração de 49 dias. No experimento I, foram testados cinco níveis de protease exógena (controle; 194; 316; 390; 600 mg/kg) em dieta formulada contendo farinha de penas. No experimento II, o desenho experimental foi fatorial 3x2, consistiu na formulação de três dietas e dois níveis de protease exógena (0 e 440 mg/kg). A proporção de proteína da dieta foi aumentada com a inclusão do farelo de soja (SM, sigla em inglês) em substituição a farinha de resíduo de peixe. Os tratamentos foram denominados: SM1-0; SM1-440; SM3-0; SM3-440; SM6-0; SM6-440. Ao final dos experimentos I e II, foram avaliados parâmetros de desempenho e saúde dos peixes. No experimento I, houve melhor desempenho, utilização dos nutrientes e maior expressão do receptor hormônio do crescimento (GHR, sigla em inglês) no fígado com 390 mg/kg de protease na dieta em comparação ao controle. O metabolismo proteico melhorou com o aumento da concentração de proteínas totais e aminoácidos (AA) e menor teor de amônia. A maior inclusão de protease (600 mg/kg) na dieta estimulou o aumento do número de eritrócitos e menor volume corpuscular médio nos peixes. No experimento II, observou-se que o grupo SM1 (SM1-0 e SM1-440) apresentou melhor crescimento, taxa de eficiência proteica e conversão alimentar. A protease exógena, de maneira geral, estimulou a atividade endógena da tripsina, resultando em melhor digestibilidade de proteínas e da morfometria intestinal no grupo com maior SM (SM6-440). Nos peixes que receberam a dieta SM3-440 houve maior teor de albumina e globulina comparada a deita SM1-440, mas não diferiram da dieta SM6-440, indicando uma resposta inata, devido ao aumento da disponibilidade de proteínas e AA. Em conclusão, determinou-se que o nível ótimo de inclusão de protease foi de 440 mg/kg. Entre as dietas, a SM3-440 demonstrou um melhor equilíbrio nutricional e fisiológico. E a inclusão de protease permitiu o aumento de farelo de soja (SM) sem afetar negativamente o crescimento da tilápia do Nilo.

Palavras-chave: atividade enzimática; expressão de genes; nutrição de peixes; produção de peixes.

ABSTRACT

Doctoral thesis
Programa de Pós-Graduação em Zootecnia
Universidade Federal de Santa Maria

EXOGENOUS PROTEASE IN DIETS FOR NILE TILAPIA
(Oreochromis niloticus)

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ADVISOR: PROF. DR. RAFAEL LAZZARI

Protease is an enzyme characterized by its action on protein bonds and can improve the assimilation of nutrients from protein ingredients. Positive effects of its inclusion have been identified in many species, due to improved feed efficiency and protein synthesis. Factors linked to feed processing, the level and form of protease inclusion influence its efficiency, as well as the composition of the diets. Therefore, the objective was to evaluate whether there are effects of its inclusion in tilapia diets on performance parameters, biochemical metabolism, digestive enzyme activity, nutrient digestibility, and gene expression. For this, two feeding trials lasting 49 days were carried out. In feeding trial I, five levels of exogenous protease (control; 194; 316; 390; 600 mg/kg) were tested in a diet formulated with feather meal. In feeding trial II, the experimental design was a 3x2 factorial, consisting of the formulation of three diets and two levels of exogenous protease (0 and 440 mg/kg). The proportion of protein in the diet was increased with the inclusion of soybean meal (SM) replacing fish waste meal. The treatments were named: SM1-0; SM1-440; SM3-0; SM3-440; SM6-0; SM6-440. At the end of the feeding trials I and II, fish performance and health parameters were evaluated. In feeding trial I, there was better performance, nutrient utilization and greater expression of the growth hormone receptor (GHR) in the liver with 390 mg/kg of protease in the diet compared to the control. Protein metabolism improved with increased concentration of total proteins and amino acids (AA) and lower ammonia content. The greater inclusion of protease (600 mg/kg) in the diet stimulated an increase in the number of erythrocytes and a lower mean corpuscular volume in fish. In feeding trial II, it was observed that the SM1 group (SM1-0 and SM1-440) had better growth, protein efficiency rate and feed conversion. In general, the exogenous protease stimulated endogenous trypsin activity, resulting in better protein digestibility, and intestinal morphometry in the group with the highest SM (SM6-440). In fish that received the SM3-440 diet, there was a higher albumin and globulin content compared to the SM1-440 diet, but did not differ from the SM6-440 diet, indicating an innate response, due to the increased availability of proteins and AA. In conclusion, the optimal level of protease inclusion was 440 mg/kg. Among the diets, SM3-440 demonstrated a better nutritional and physiological balance. The inclusion of protease allowed the increase in soybean meal (SM) without negatively affecting the growth of Nile tilapia.

Keywords: enzymatic activity; gene expression; fish nutrition; fish production.

LISTA DE TABELAS

REFERENCIAL TEÓRICO

MANUSCRITO I

MANUSCRITO II

MANUSCRITO III

LISTA DE FIGURAS

REFERENCIAL TEÓRICO

MANUSCRITO II

MANUSCRITO III

SUMÁRIO

1 INTRODUÇÃO GERAL

O crescimento da produção de peixes resulta em demanda por rações. Em 2023, a produção nacional de peixes registrou um incremento de 3,10%, atingindo mais de 887 mil toneladas, enquanto a produção de rações, projetada para 2023, ultrapassou a marca de um milhão de toneladas, refletindo um aumento de 2,8% em relação ao ano anterior (ASSOCIAÇÃO BRASILEIRA DA PISCICULTURA- PEIXE BR, 2024; SINDIRAÇÕES, 2023). A crescente demanda por produtos aquícolas e o fortalecimento da piscicultura destacam a necessidade de formulações de rações mais eficazes, que promovam ciclos produtivos mais curtos. A farinha de peixe e o farelo de soja são os principais ingredientes proteicos usados devido à sua quantidade e qualidade de proteína. A proteína é essencial para o crescimento animal em todas as etapas da vida, sendo tanto valiosa quanto indispensável (ABDEL-TAWWAB et al., 2010; EL-SAYED, 2006). No entanto, a disponibilidade de farinha de peixe tem sido afetada pela escassez decorrente do seu uso intensivo pela indústria, pesca extrativista e a degradação ambiental.

Na piscicultura, uma parte considerável dos custos variáveis de produção está associada à alimentação dos peixes. Estudos têm explorado o emprego de fontes proteicas de origem animal e vegetal em substituição à farinha de peixe, buscando reduzir os custos de produção (AKSNES et al., 2006; HARDY, 2010; OBIRIKORANG et al., 2020). A utilização de fontes proteicas de origem animal; como farinhas e subprodutos de aves e peixes; tem sido justificada pela sua disponibilidade e excelente qualidade nutricional (ABIMORAD et al., 2014; CARDOSO et al., 2021; LEE et al., 2020). A farinha de penas se destaca pelo alto teor proteico e bom teor de aminoácidos, como treonina e arginina (NUTRIENT REQUERIMENT OF FISH-NRC,1993). No entanto, a maior parte dessa proteína é queratina, menos solúvel e mais resistente à digestão. Além disso, a inclusão de altos níveis de subprodutos pode alterar a relação entre os aminoácidos e afetar negativamente o desempenho e a atividade das enzimas digestivas dos peixes (LAZZARI et al., 2010; MAHMOUD et al., 2014; POOLSAWAT et al., 2021). No caso das fontes de origem vegetal, como o farelo de soja, a presença de fatores antinutricionais pode prejudicar o aproveitamento dos nutrientes e o crescimento dos peixes (FRANCIS; MAKKAR; BECKER, 2001).

O uso de proteases exógenas, pelo efeito extraproteináceo, não apenas neutraliza os fatores antinutricionais, mas também, otimiza a utilização de nutrientes

por meio de atividade proteolítica, liberando compostos menores, como aminoácidos livres e peptídeos para a ação das enzimas endógenas (COWIESON; ROOS, 2016; MCDONALD, 1985; VOGELSANG-O'DWYER et al., 2022). Em alguns estudos observou-se redução dos níveis de proteína dietética e redução na utilização da farinha de peixe em dietas com inclusão de protease para as espécies onívoras (RAGAA et al., 2017; SALEH et al., 2021; SHI et al., 2016). Dietas contendo protease resultaram em melhoria na eficiência alimentar, aumento do coeficiente de digestibilidade aparente da proteína e crescimento de peixes, como carpa prussiana (*Carassius auratus G.*) (LIU et al., 2018) e tilápia do Nilo (*Oreochromis niloticus.*) (HASSAAN et al., 2019). Além disso, a incorporação dessa enzima apresentou melhora na qualidade de água do cultivo por meio da redução dos teores nitrogenados (SALEH et al., 2021), contribuindo com o meio produtivo de forma sustentável (BOYD et al., 2020).

A tilápia do Nilo é destaque na produção de peixes cultivados, nacionalmente representa 65,3% da produção total (PEIXE BR, 2024). O Brasil segue em 4º lugar entre os maiores produtores da espécie no mundo (FOOD AND AGRICULTURE ORGANIZATION– FAO, 2022). Os resultados de produtividade são atribuídos ao rápido crescimento, hábito alimentar onívoro, homogeneidade de lote e qualidade de carne, características que tornam a espécie bem aceita pelo mercado consumidor (EL-SAYED, 2006) e um modelo de estudo nacional e mundial. O sistema de produção intensivo, responsável pelo aumento de produção, exige dietas nutricionalmente completas de baixo custo, conforme a fase de cultivo. Para isso, estratégias visando melhorar o desempenho e o aproveitamento de nutrientes de fontes proteicas e mitigar seus efeitos adversos têm sido adotadas, mas resultados inconclusivos ainda existem quanto ao uso de protease em dietas para peixes (GASCO et al., 2016; YIGIT et al., 2018), incluindo a espécie em foco (ADEOYE et al., 2016b; HASSAAN et al., 2019).

Diante disso, objetivou-se avaliar se há efeitos da inclusão de protease em dietas para tilápia do Nilo sobre parâmetros de desempenho, metabolismo bioquímico, atividade de enzimas digestivas, digestibilidade de nutrientes e expressão gênica.

2 REFERENCIAL TEÓRICO

2.1. TILÁPIA DO NILO

A tilápia (*Oreochromis niloticus*) é um peixe teleósteo pertencente à família Cichlidae, sendo originária do continente africano, mais precisamente da Bacia do Rio Nilo. A produção mundial expandiu muito durante o período de 1970 a 1990. A espécie ocupa a 3º posição mundial, com produção de 4,4 milhões de toneladas, perdendo posição apenas para as carpas (carpa capim, *Ctenopharyngodon idellus,* e carpa prateada, *Hypophthalmichthys molitrix*), em 2020 (FAO, 2022).

No Brasil, o primeiro registro da produção de tilápia foi em 1995, em média 12 mil toneladas. Já em 2002, a produção foi de 42,003 toneladas (FAO, 2004). Após 20 anos, o setor de produção da espécie registrou um total de 579,080 toneladas, representação de 65,3% na produção nacional. Considerada o segundo maior grupo de peixes de água doce, desde 2002 tem se um crescimento médio de 5% ao ano. Além disso, a espécie coloca o país em 4º lugar entre os maiores produtores mundiais da espécie (FAO, 2022). Com destaque aos estados da região Sul que lideraram a produção da espécie, representando 33,4% da produção total brasileira (PEIXE BR, 2024).

A tilápia é um dos peixes que apresenta maior potencial para piscicultura, devido ao seu rápido crescimento, rusticidade e ausência de espinhas intramusculares (EL-SAYED, 2006). É uma espécie que apresenta ciclo relativamente rápido em relação às outras espécies, além de se adaptar facilmente às condições de cultivo (DE LEÓN-RAMÍREZ et al., 2022). Nutricionalmente, tem hábito alimentar onívoro, facilidade para a aceitação de rações e a exigência de nutrientes varia de acordo com o peso da espécie (etapa de vida) (Tabela 1).

O pescado de tilápia normalmente é comercializado na forma de filé, entre 35-43% é destinado ao consumo humano (EL-SAYED, 2006). Atualmente, resíduos de peixe processados são utilizados como subprodutos na alimentação animal. Prevê-se que até 2030, a proporção da farinha de peixe obtida a partir desses resíduos aumentará de 27% para 29%. Além disso, a aquicultura deverá atingir mais de 100 milhões de toneladas de produção total de peixes (FAO, 2022).

Tabela 1. Exigência nutricional para tilápia do Nilo

Referência*	A	B	C
Peso (g)	0,015 a 0,087	17 a 21	<100
Proteína bruta (%)	28**	35	30
Energia digestível (kcal/kg)	2500	-	3000
Energia bruta (kcal/kg)	-	4468	-
Aminoácidos essenciais (% da dieta)			
Arginina	1,18	-	1,26
Fenilalanina	1,05	-	0,83
Histidina	0,48	-	0,52
Isoleucina	0,87	-	0,93
Leucina	0,95	-	1,01
Lisina	1,43	-	1,53
Metionina	0,75		0,52
Tirosina	0,50	-	0,83
Treonina	1,05	-	1,18
Triptofano	0,28	-	0,30

*[[A]Santiago; Lovell (1988); [B]Abdel-Tawwab et al. (2010); [C]Furuya et al. (2010)]. **Valor menor que o ideal (35%) para maior utilização do aminoácido limitante.

2.2. FONTES PROTEICAS

A produção da aquicultura mundial aumenta a necessidade de rações para o cultivo de organismos aquáticos. Em 20 anos, a produção de rações para essa atividade aumentou significativamente, passando de 8 para 48 milhões de toneladas (TACON; HASAN; METIAN, 2011). A tilápia, por sua vez, foi responsável por consumir cerca de 17% do total de rações utilizados na aquicultura (HARDY, 2010). No Brasil, a produção estimada de rações para peixes atingiu 1,24 milhão de toneladas em 2023 (SINDIRAÇÕES, 2023). As rações para peixes representam grande parte dos custos variáveis, sendo a proteína um dos nutrientes mais importantes e onerosos em sua alimentação (EL-SAYED, 2006; HARDY, 2010).

A farinha de peixe desempenha um papel importante como fonte de proteína em rações para peixes, contribuindo com aproximadamente 50% da proteína total devido às suas características nutricionais, como elevado valor biológico e equilíbrio de aminoácidos, principalmente provenientes da pesca marinha (MONTOYA-

CAMACHO et al., 2019). A farinha de peixe contém cerca de 60 a 65% de proteína bruta (PB), e fornece uma quantidade equilibrada de aminoácidos essenciais, fosfolipídios, vitaminas, minerais e ácidos graxos essenciais (NRC, 1993). Entretanto, há alguns anos, seu uso contínuo tem sido alvo de críticas por parte de organizações ambientais, tornando-se um recurso escasso e limitado na produção (HARDY, 2010). Essa farinha, além de ser um ingrediente caro, acarreta desafios econômicos associados à sua aplicação na aquicultura e contribui para o esgotamento das populações de peixes selvagens (FAO, 2018).

No Brasil, a produção de farinhas de origem animal totalizou 3,6 milhões de toneladas, mas de 70% destinado para a produção animal, em 2020 (ASSOCIAÇÃO BRASILEIRA DE RECICLAGEM ANIMAL- ABRA, 2020). A farinha de penas é uma fonte promissora, apresenta alto teor proteico (80 a 90% de PB), bom teor de aminoácidos, como treonina e arginina, e disponibilidade, cerca de 7% do peso de abate da ave corresponde às penas (NRC, 1993; ROCHA; SILVA, 2004). Em 2020, a produção dessa fonte representou 16% da produção total (582,3 mil toneladas) (ABRA, 2020). No entanto, a maior parte dessa proteína é queratina, menos solúvel e mais resistente à digestão. O rompimento da queratina e a melhora do valor nutricional da farinha de penas pode ser feita pela hidrólise em alta temperatura e pressão, no entanto, este processo consume energia e destrói aminoácidos essenciais como lisina e metionina (NRC, 1993). Outra alternativa é a inclusão de protease exógena na formulação da ração (LI et al 2015; RAGAA et al., 2017; SHI et al., 2016). Em geral, o uso de subprodutos tem sido uma prática importante na alimentação de peixes em substituição a farinha de peixe (BOSCOLO et al., 2005; CARDOSO et al., 2021; GUIMARÃES; PEZZATO; BARROS, 2008).

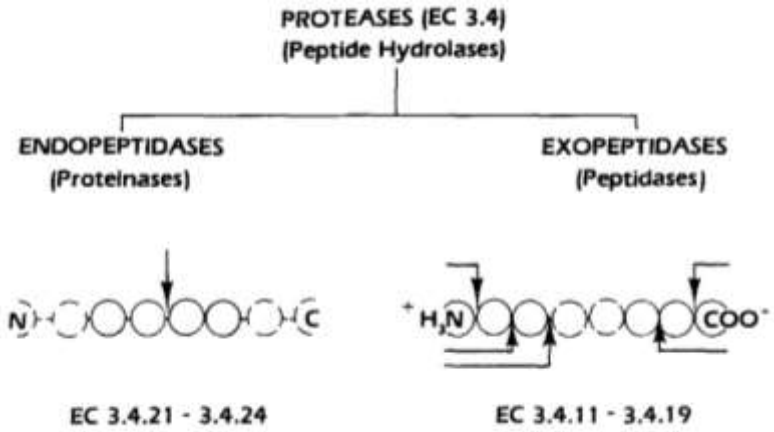
O uso de fontes vegetais como ingredientes nas rações tem sido amplamente estudado devido aos desafios associados aos altos níveis de inclusão e à baixa qualidade, que podem prejudicar a absorção de nutrientes devido aos antinutrientes (FRANCIS; MAKKAR; BECKER, 2001). O farelo de soja é a principal fonte proteica vegetal em rações para peixes e enfrenta desafios, como competição com outras finalidades, oscilação de preço, presença de antinutrientes, como saponinas e inibidores de tripsina, e deficiência em aminoácidos sulfurados (FURUYA, 2010; NRC, 1993). Em rações para peixes, sua utilização em concentrações acima de 40% mostrou impactos negativos no desempenho de espécies como a tilápia do Nilo (MAHMOUD et al., 2014) e na atividade de enzimas endógenas em peixes (LAZZARI

et al., 2010; ZHU et al., 2021). A ampliação do uso de farelo de soja na produção de peixes está relacionada com a disponibilidade e alta digestibilidade (GUIMARÃES; PEZZATO; BARROS, 2008) e, com a possibilidade de melhorar o seu valor nutricional, com a inclusão de protease exógena (DALSGAARD et al., 2012; LEE et al., 2020).

2.3. PROTEASES EM DIETAS PARA PEIXES

As proteases são enzimas que podem ser classificadas em endo e exopeptidases, com base na especificidade posicional (MCDONALD, 1985). As endopeptidases hidrolisam nas ligações internas dos polipeptídeos e as exopeptidases atuam perto das extremidades no terminal C ou N (Figura 1).

Figura 1. Classificação das proteases



Fonte: McDonald (1985)

As endopeptidases clivam as proteínas em peptídeos de vários tamanhos, enquanto as exopeptidases liberam um único aminoácido, um dipeptídeo ou um tripeptídeo, conforme revisado recentemente por Vogelsang-O'Dwyer et al. (2022). Além disso, as proteases também podem ser classificadas de acordo com a sua origem, ou seja, de origem microbiana, vegetal ou animal (EUROPEAN FOOD SAFETY- EFSA, 2009). Contudo, as proteases também são classificadas de acordo com o principal grupo químico responsável pela catálise no sítio catalítico, como exemplo: serina proteases. A serina protease (3.4.21) desempenha um papel importante sobre a digestão de dietas mistas, contendo proteínas de origem vegetal e animal (RAGAA et al., 2017; YIGIT et al., 2018).

A inclusão de proteases na alimentação de peixes pode ser dividida em dois momentos distintos: inicialmente, como aditivo alimentar; e, posteriormente, como medida preventiva diante de estratégias alimentares que substituem a farinha de peixe. O uso de proteases exógenas não apenas inativa antinutrientes, mas também melhora a utilização de nutrientes por meio da atividade proteolítica, liberando compostos menores para a ação das enzimas endógenas (MCDONALD, 1985). A avaliação dos efeitos diretos da enzima no desempenho dos animais é dificultada quando a protease está como componente de um complexo enzimático (BOYD et al., 2020). Por outro lado, em dietas para juvenis de tilápia do Nilo, a inclusão de protease, apenas, demonstrou benefícios na disponibilidade de nutrientes e no desempenho dos peixes (ADEOYE et al., 2016a; HASSAAN et al. 2019; RAGAA et al., 2017).

O crescimento dos peixes está relacionado com a expressão de muitos genes, com destaque para o hormônio do crescimento (GH) que se liga ao receptor (GHR), desencadeando a liberação do fator de crescimento semelhante à insulina (IGF-1) no fígado e em outros tecidos (AKSNES et al., 2006; MA et al., 2020). O eixo somatotrópico, influenciado pelo estado nutricional, destaca a importância da disponibilidade de nutrientes e aminoácidos na manutenção do IGF-1, essencial para os efeitos promotores de crescimento do GH (MORIYAMA; AYSON; KAWAUCHI, 2000). No estudo realizado por Hassaan et al. (2019) sobre a substituição de farinha de peixe por farelo de algodão e inclusão de protease exógena na dieta de tilápia do Nilo, observou-se uma correlação negativa entre os marcadores GH e IGF-1. Os peixes alimentados sem protease apresentaram pior conversão alimentar e aumento da expressão do GH, enquanto aqueles alimentados com protease exibiram maior expressão de IGF-1. Essa disparidade no crescimento e na expressão do GH pode ser atribuída às fontes proteicas vegetais, que possuem antinutrientes causadores de desequilíbrio de aminoácidos. Resultados semelhantes foram previamente observados em truta arco-íris e dourada (*Sparus aurata*) (AKSNES et al., 2006; GÓMEZ-REQUENIA et al., 2004).

O impacto da protease (175 mg/kg) na digestibilidade de ingredientes proteicos foi avaliado em truta arco-íris (LEE et al., 2020), revelando uma melhora na digestibilidade da matéria seca e energia, assim como, melhora do aproveitamento de pelo menos um aminoácido de cada ingrediente. Por outro lado, níveis superiores a 1 g/kg de protease não resultaram em benefícios no crescimento e na utilização de nutrientes em truta arco-íris quando comparado a dieta controle (sem inclusão de

protease) (YIGIT et al., 2018). Da mesma forma, em robalos alimentados com dietas contendo farinha de tenébrio (250 g/kg) e protease exógena (0,2 g/kg), não se observou aprimoramento na digestibilidade da matéria seca e proteína (GASCO et al., 2016). A falta de impacto das enzimas exógenas pode estar relacionada hábito alimentar dos peixes, composição das dietas ou ao nível de inclusão, destacando que a quantidade de enzima baseada em outros animais de produção não é recomendada.

Em resumo, a abordagem da nutrição de precisão está cada vez mais focada na melhoria da qualidade das fontes de proteína, visando otimizar os recursos econômicos, sociais e ambientais. É reconhecido que as necessidades nutricionais dos peixes estão ligadas à quantidade e a proporção adequada de aminoácidos, em vez da quantidade total de proteína. A combinação de estratégias complementares, como, o uso de subprodutos em substituição à farinha de peixe, por exemplo a farinha de penas, e a inclusão de protease na dieta visando melhorar a eficiência alimentar. Neste contexto, o uso de protease exógena, apesar de ser influenciado pela qualidade da proteína dos ingredientes e pelo seu nível de inclusão, pode potencializar essas fontes através da liberação de aminoácidos.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar se há efeitos da inclusão de protease em dietas contendo subprodutos de origem animal e aumento do farelo de soja para tilápias sobre parâmetros de crescimento, alterações digestivas e respostas metabólicas.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar se têm efeitos da protease em dietas contendo subprodutos de origem animal sobre a utilização dos nutrientes dietéticos, índices digestivos, composição corporal e deposição de nutrientes;

- Identificar o nível ótimo de protease na dieta sobre os parâmetros de desempenho;

- Observar se há alterações bioquímicas, hematológicas e de marcadores moleculares relacionados ao crescimento (GHR e IGF-1) com o aumento da inclusão de protease exógena;

- Avaliar se existem efeitos da protease em dietas contendo aumento de farelo de soja sobre os parâmetros de crescimento, utilização dos nutrientes dietéticos e índices digestivos;

- Identificar se possui modificações nas características bromatológicas, retenção e deposição de nutrientes em tilápias alimentadas com dietas sem e com protease;

- Avaliar se a inclusão de protease em dietas contendo subprodutos proteicos de origem animal altera a atividade de enzimas digestivas e o aproveitamento dos nutrientes dietéticos;

- Observar se têm respostas metabólicas, hematológicas e da morfometria intestinal em tilápias alimentadas com dietas contendo aumento do farelo de soja em substituição a farinha de resíduo de peixe.

4 DESENVOLVIMENTO

Esta tese foi desenvolvida em três manuscritos:

I. Nutritional implications of exogenous proteases in fish feeding (REVIEW)

II. Protease improves performance, GHR gene expression, nutrient deposition, hematological and biochemical indicators of Nile tilapia (*Oreochromis niloticus*)

III. Protease in Nile tilapia diets: growth, chemical composition, nutrient retention, digestibility, digestive enzymes, intestinal morphometry, and blood-biochemical responses

MANUSCRITO I

Nutritional implications of exogenous proteases in fish feeding (REVIEW)*

*Artigo publicado na revista Pesquisa Agropecuária Gaúcha, doi.org/10.36812/pag.202228170-93

PESQUISA AGROPECUÁRIA GAÚCHA

doi: https://doi.org/10.36812/pag.202228170 -93

REVIEW

Nutritional implications of exogenous proteases in fish feeding

Thamara Luísa Staudt Schneider[1], Rafael Lazzari[2]

Abstract - The increase in the demand for fish production drives the search for food strategies to maximize productivity. In this review, the nutritional implications of the use of exogenous proteases in fish growth are described. Exogenous proteases help in digestive processes, acting in the hydrolysis of macromolecules and in the release of smaller particles, such as amino acids and peptides. Dietary supplementation improves fish growth, mainly because of the availability and greater use of nutrients. The action of proteases is directly linked to better intake, feed efficiency and protein synthesis. On the other hand, enzyme activity depends on the substrate and dietary composition as there are limitations on digestibility. Furthermore, in feed processing, thermal stability and the form of inclusion of the protease influence its efficiency. However, the increase in fish weight gain can offset the cost of including the enzyme in the diet. However, there are still gaps regarding the effects of protease in fish feeding, for example, enzyme: specific substrate and enzyme: digestive system ratio; and, stabilization technologies (mainly extruded diets), so further studies are needed.

Keywords: Antinutrients. Growth. Enzyme. Nutrition. Fish farming.

Implicações nutricionais de proteases exógenas na alimentação de peixes

Resumo - O aumento na demanda da produção de peixes impulsiona a busca por estratégias alimentares a fim de maximizar a produtividade. Nesta revisão foram descritas as implicações nutricionais do uso de proteases exógenas sobre o crescimento dos peixes. As proteases exógenas auxiliam nos processos digestivos atuando na hidrólise de macromoléculas e na liberação de partículas menores, como aminoácidos e peptídeos. A suplementação dietética melhora o crescimento dos peixes, principalmente pela disponibilidade e maior aproveitamento dos nutrientes. A ação de proteases está ligada diretamente a melhor ingestão, eficiência alimentar e síntese de proteína. Por outro lado, a atividade da enzima depende do substrato e da composição dietética já que há limitações na digestibilidade. Além disso, no processamento de rações, a estabilidade térmica e a forma de inclusão da protease influenciam na sua eficiência. Contudo, o aumento no ganho em peso dos peixes pode compensar o custo de inclusão da enzima na dieta. No entanto, ainda existem lacunas quanto aos efeitos da protease na alimentação de peixes, como exemplos, relação enzima: substrato específico e enzima: sistema digestório; e, tecnologias de estabilização (principalmente, dietas extrusadas), por isso, são necessários estudos adicionais.

Palavras-chave: Antinutrientes. Crescimento. Enzima. Nutrição. Piscicultura.

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Introduction

Aquaculture is a significant activity for the world's food supply, being recognized as the fastest growing agribusiness sector. In 2020, this sector kept production in line with market demand, guaranteed supply and provided record exports (BRAZILIAN FISH FARMING ASSOCIATION - PEIXE BR, 2021). Worldwide, fish is the most consumed animal protein in human food. The increase in consumption is stimulated by increased production and improved distribution channels, associated with population growth, urbanization, and rising incomes (FAO, 2018; 2020).

In 2018, aquaculture grew by 3.2% over the previous year, with fish production accounting for 46% (82.1 million tons) of the world's aquaculture (FAO, 2020). The increase in production is accompanied by an increase in demand for ingredients and feed. The choice of ingredients for the acquisition of rations depends mainly on the feeding habit of the species (PORTZ; FURUYA, 2012). Carnivorous fish require diets with a higher amount of protein and use of animal sources in the diets when compared to herbivorous and omnivorous fish. This difference is directly related to the morphology of the digestive tract of each species (RODRIGUES *et al*., 2012).

The obstacles in the fish feeding indirectly stimulate the search for nutritional strategies that improve the use of these rations, notably, protein, which is one of the most important and most expensive nutritional fractions. The low availability and high cost of animal sources means that the amount of these sources in the rations is reduced and plant sources and/or by-products are used in fish feeding. Plant sources contain antinutrients that can impair fish growth (FRANCIS; MAKKAR; BECKER, 2001). On the other hand, the use of exogenous enzymes can improve the nutritional growth of animals, in addition to optimizing the cost/benefit ratio of feeding, survival and growth of animals (CASTILLO; GATLIN, 2015; KEMIGABO *et al*., 2019; KUMARI *et al*., 2013).

Enzymes are biological catalysts that accelerate biochemical reactions using alternative pathways, this allows for greater availability of nutrients for absorption by the body, which under normal conditions would not occur (GOMES *et al*., 2019). In animal production, commercial enzymes began to be used in the 1990s. Since then, animal production associated with biotechnology has advanced in levels, sources, efficacy and substrate specificity (BOYD *et al*., 2020). The presence of enzymes allows for changes in the plant base and greater inclusion of dietary ingredients (HASSAAN *et al*., 2019; SALEH *et al*., 2021). In fish feed, the most used enzymes are proteases, lipases, phytase and carbohydrates (CASTILLO; GATLIN, 2015; ZHENG *et al*., 2019).

Exogenous proteases can compensate for the deficiency of endogenous enzymes and help break down macromolecular proteins that are difficult to digest (SALEH *et al*., 2021; SHI *et al*., 2016). The use of exogenous proteases has allowed the reduction of protein and fish meal levels in diets for Nile tilapia (*Oreochromis niloticus*) (LIU *et al*., 2016; RAGAA *et al*., 2017; SALEH *et al*., 2021) and prussian carp (*Carassius auratus gibelio*) (SHI *et al*., 2016). Nutritional implications were also observed in rainbow trout (*Oncorhynchus mykiss*), prussian carp (*C. auratus* gibelio) and Nile tilapia (*O. niloticus*)

as better feed efficiency, higher apparent protein digestibility coefficient and higher growth, respectively (DREW *et al*., 2005; GODA *et al*., 2020; LIU *et al*., 2016).

There are few studies on the use of exogenous proteases in fish diets compared to other enzymes such as phytase and carbohydrase (BOYD *et al*., 2020). Thus, the objective was to compile existing studies and describe the nutritional implications of exogenous proteases on fish growth responses. In addition to pointing out issues that have not yet been elucidated and the additional studies necessary for an adequate use of proteases in the fish diet.

Exogenous enzymes used as fish additives

Enzymes are proteins that accelerate reactions and break the bonds between molecules, providing nutrients and allowing greater action of digestive enzymes on a given substrate (GOMES *et al*., 2019). In animal production, the use of commercial enzymes started in the 1990s, consequently, this technology has advanced a lot in terms of levels, sources, efficacy, and substrate specificity (BOYD *et al*., 2020). The main categories of enzymes used in fish feed include proteases; lipases; phosphatases (phytase) and carbohydrases (xylanase, β-glucanases, etc). In addition to improving the availability of nutrients, they are also considered nutritional additives that improve the utilization of low-quality food in the diet of aquatic animals (HASSAAN *et al*., 2019).

Anti-nutritional factors

Anti-nutritional or anti-nutrient factors are components present in plant ingredients, cereals and legumes, which can hinder the activity of digestive enzymes and the absorption of nutrients from food (FRANCIS; MAKKAR; BECKER, 2001; LAZZARI *et al*., 2010). Antinutrients include trypsin inhibitors, hemagglutinating agents, phytic acid, gossypol, alkaloids, thiaminase, among others. These antinutrients are present in plant ingredients commonly used in fish nutrition, such as soybean meal, canola meal and sunflower meal. When inactivated by heat treatment, antinutrients can reduce amino acid availability and protein digestibility by fish (NATIONAL RESEARCH COUNCIL - NRC, 2011; HARDY; BARROWS, 2002). However, there are other strategies to eliminate and/or minimize the effects of these components, such as selective breeding, genetic modification, or through supplementation (enzyme, mineral, etc.) (FRANCIS; MAKKAR; BECKER, 2001). As observed in recent studies, diets with a high inclusion of plant ingredients resulted in fish with better performance, immune system, digestibility, intestinal microbiological community and increased bioavailability of essential amino acids when supplemented with exogenous protease (GODA *et al*., 2020; HASSAAN *et al*., 2019; ZHENG *et al*., 2019). Thus, the hydrolysis of protein into individual amino acids and peptides during digestion is believed to be the main function of proteases (BOYD *et al*., 2020).

Exogenous proteases

Proteases perform various biological functions in homeostasis, apoptosis, signal transduction, reproduction and immunity. They account for more than 50% of global enzyme production, however, in animal production, they represent only 5% of the global feed enzyme market. Most studies include the protease within a cocktail, making it difficult to assess the enzyme's direct impacts on animal performance (BOYD *et al*., 2020).

Exogenous proteases are produced from bacteria (strains of *Bacillus* sp.), fungi (genus *Aspergillus* sp.) and yeasts (LI *et al*., 2013). Its stability in fish diets is mainly dependent on its pH specificity and thermolabile nature (KUMARI *et al*., 2013; ZHENG *et al*., 2019). Therefore, materials are used from mesophilic organisms and techniques such as amino acid modification and metal bonding to ensure enzymatic stability (LI *et al*., 2013; YEO; BAEK; PARK, 2001). However, the stability of the biomolecule in the bioconjugate material can perform the necessary functions efficiently (GOLE *et al*., 2001). Since the molecular recognition mechanism is characterized by the specificity of an enzyme in identifying and interacting with the exact substrate, through chemical affinity (ZHENG *et al*., 2019).

The food bolus substrate consists of the undigested fraction that serves as food for microbial fermentation in the intestine of animals, and its increased amount can cause digestive disorders. Exogenous protease in the diet can aid and compensate for the activity of digestive enzymes, so that macromolecular protein can be solubilized and hydrolyzed into low molecular peptides, peptones and various amino acids, available to be digested and/or absorbed, reducing the amount of substrate (SHI *et al*., 2016).

The level of protease inclusion in diets is influenced by many factors, mainly diet composition and digestive system. Few studies are related to the problems with the high inclusion of exogenous enzymes in the diet, but the unregulated use of these enzymes can cause damage to the intestinal mucosa and, consequently, induce negative effects on growth (KUMARI *et al*., 2013; LIU *et al*., 2016).

Nutritional implications of using exogenous proteases

The addition of exogenous protease to diets containing protein sources improves fish growth (GODA *et al*., 2019; HASSAAN *et al*., 2019; SALEH *et al*., 2021). Endogenous and exogenous enzymes can act in different ways on fish performance. Exogenous proteases can: (I) break down complex proteins and make amino acids and peptides available; (II) increase endogenous peptidase production; (III) be related to increased food intake; (IV) improve feed conversion; (V) reduce the effects caused by antinutrients present in plant protein sources; (VI) increase the protein efficiency rate, through protein consumption; (VII) decrease the use of fish meal.

Effects on growth

The growth of fish fed diets containing exogenous protease is related to higher feed intake and improved feed conversion and feed efficiency (Table 1) (GODA, *et al*., 2019; HASSAAN *et al*., 2019;

SHI *et al*., 2016). Enzymes can directly infer the rate of absorption of nutrients in the gastrointestinal tract (DEBNATH *et al*., 2005; GODA *et al*., 2012). After digestion, amino acids are absorbed and transported through the hepatic portal vein from the intestine to the liver and rapidly metabolized (PORTZ; FURUYA, 2012). The rate of absorption and concentration of free plasma amino acids varies with dietary ingredients. For channel catfish (*Ictalurus punctatus*) fed diets containing soybean meal and fish meal, free amino acid concentrations peaked at 12 hours after feeding (AMBARDEKAR; REIGH; WILLIAMS, 2009). For rainbow trout (*O. mykiss*) fed soybean meal and malt protein meal, the peak amino acid concentration was 21 hours after feeding (YAMAMOTO; UNUMA; AKIYAMA, 1998). However, proteases can hydrolyze protein complexes from lower quality protein sources and promote the use of nutrients from the release of amino acids and peptides, to stimulate food intake and improve feed efficiency as observed in Nile tilapia (*O. niloticus*) (RAGAA *et al*., 2017; SALEH *et al*., 2021), sea bass (*Dicentrarchus labrax* L.) (GODA *et al*., 2019), black carp (*Mylopharyngodon piceus*) (CHEN *et al*., 2009) and rainbow trout (*O. mykiss*) (DREW *et al*., 2005).

The mechanism that improves food consumption in fish fed diets supplemented with enzymes still needs to be studied and explained. Diets containing distiller dry grains in place of soybean meal and exogenous protease (1000 mg kg^{-1}) showed lower food intake compared to fish fed the control diet (without distiller's dry grains and exogenous protease) (GODA *et al*., 2019). In contrast, fish fed diets containing exogenous protease resulted in higher food intake (CHEN, 2009; KEMIGABO *et al*., 2019; RAGAA *et al*., 2017; SALEH *et al*., 2021). However, there are two possible explanations for the improvement in food consumption: (I) increased palatability of diets since diets containing exogenous proteases can make dietary amino acids available (HASSAAN *et al*., 2019) and release more free amino acids plasma levels and, consequently, stimulate food intake; (II) greater digestibility of nutrients, diets containing exogenous protease may result in a faster passage of ingested food through the digestive system, and accelerate the return of appetite, potentially promoting greater food intake (DEBNATH *et al*., 2005).

The biological and dietary characteristics of the species influence the growth response from a diet containing exogenous protease. The comfort temperature of the species affects the speed of nutrient digestion. Cold water fish such as rainbow trout (*O. mykiss*) result in longer digestion time compared to warm water fish such as channel catfish (*I. punctatus*) (AMBARDEKAR; REIGH; WILLIAMS, 2009). In addition, the slower digestion rate and absorption of plant ingredients may be related to the digestive processes and the delay in the evacuation of the system since omnivorous fish fed diets containing plant protein sources showed better protein utilization (LARSEN; DALSGAARD; PEDERSEN, 2012). On the other hand, carnivorous fish tend to evacuate the digestive system more quickly, thus having a lower ability to adapt the digestive system to dietary changes compared to omnivorous fish (PORTZ; FURUYA, 2012). Although exogenous proteases act on the use of nutrients and on performance parameters, the specific characteristics of the species must be considered during the formulation of diets.

Carnivorous fish demand diets with higher protein content and ingredients of high biological value compared to omnivorous and herbivorous fish (PORTZ; FURUYA, 2012). Fish feed represents more than half of the total production costs, and, among nutrients, protein is one of the most expensive (FAO, 2018). Studies focused on the addition of exogenous proteases in the diet of carnivores exert direct action on the appeals of sustainability. The use of the enzyme in the diet of carnivorous species results in improved performance parameters and greater digestibility of nutrients (DREW *et al*., 2005; FARHANGI; CARTER, 2007; GASCO *et al*., 2016; SOARES *et al*., 2008; YIGIT *et al*., 2018; LEE *et al*., 2020), greater retention of nitrogen and phosphorus (OGUNKOYA *et al*., 2006) and positive effects on health status (GODA, *et al*., 2019), in addition to allowing the use of lower cost vegetable protein sources compared to fish meal (DALSGAARD *et al*., 2012, 2016; FARHANGI; CARTER, 2007).

Table 1. Effects on growth of the addition of exogenous protease in diets with plant-based ingredients in fish feed.

Specie	Protease inclusion (mg kg^{-1})	Results	Reference
Prussian carp (*C. auratus gibelio*)	400[a]	↑ SGR ↓ FC ↑ PER	LIU *et al.* (2016)
Nile tilapia (*O. niloticus*)	250[b]	↑ WG ↑ SGR ↓ FC ↑ PER ↑ FI	SALEH *et al.* (2021)
Black carp (*M. piceus*)	1000, 2000 e 3000[c]	↑ WG ↓ FC ↑ FI	CHEN *et al.* (2009)
Peacock bass (*Cichla* sp.)	100[d]	↑ WP ↑ SGR ↓ FC	SOARES *et al.* (2008)
Nile tilapia (*O. niloticus*)	500[e]	↑ WG ↓ FC ↑ PER	HASSAAN *et al.* (2019)
Prussian carp (*C. auratus gibelio*)	150 e 175[f]	↑ WG ↓ FC ↑ PER	SHI *et al.* (2016)
Tilapia (*O. niloticus* × *O. aureus*)	175[g]	↑ WG ↓ FC	LI *et al.* (2015)
Nile tilápia (*O. niloticus*)	200 e 400[h]	↑ WG ↑ SGR ↓ FC ↑ PER ↑ FI	RAGAA *et al.* (2017)
Rainbow trout (*O. mykiss*)	250[i]	↓ FC	DREW *et al.* (2005)
Sea bass (*D. labrax* L.)	1000[j]	↑ WG ↑ SGR ↓ FC ↑ PER ↑ FI	GODA *et al.* (2019)

African catfish (*Clarias gariepinus*)	1100[k]	↑ WG ↑ FI ↓ FC	KEMIGABO *et al*. (2019)

[a]coated neutral protease (Kemin Industries Zhuhai Co., Ltd.); [b]protease (600.000 U g^{-1}, Novus Company, USA); [c]neutral protease (8000 U g^{-1}, Zhiwei); [d]fungal protease (Alltech Brazil); [e]protease (5000 U g^{-1}, Huvepharma, Antuérpia, Belgium); [f]alkaline protease (AG175™, JEFO Nutrition, Inc. Saint-Hyacinthe, Quebec, Canada); [g]alkaline protease (JEFO Nutrition, Inc. Saint-Hyacinthe, QC, Canada); [h]protease (Ronozyme ProAct™, DSM Nutrition Products, SP, Poland); [i]protease (Domestic poultry-250™; JEFO Nutrition, Inc. St.-Hyacinthe, QC); [j]protease (PROXYM ULTRA5®, Gloray Vet COMPANY); [k]protease (Kemin Industries (Zhuhai) Co. Ltd., China). Legend: Weight gain (WG); Specific growth rate (SGR); Feed conversion (FC); Protein efficiency rate (PER); Food intake (FI); Higher (↑); Lower (↓).

Diet composition influences the performance responses of fish fed exogenous proteases. A level of dietary protein that exceeds the need for maintenance and growth, results in negative effects on growth in fish fed diets containing exogenous protease (ADEOYE *et al*., 2016a; GODA, *et al*., 2019; SHI *et al*., 2016). On the other hand, the 2% reduction of crude protein in the diet containing fish meal, soybean meal and exogenous protease (200 and 400 mg kg^{-1}) in Nile tilapia (*O. niloticus*) feed maintained the performance parameters and improved the use of protein (RAGAA *et al*., 2017). Also, positive effects on the performance of the species in question were observed when fed with a diet containing 1% reduction of crude protein and exogenous protease (500 mg kg^{-1}) (SALEH *et al*., 2021). The prussian carp (*C. auratus gibelio*) showed a higher specific growth rate and lower feed conversion when fed a diet containing 2% reduction in crude protein and exogenous protease (300 mg kg^{-1}) (LIU *et al*., 2016). In summary, exogenous proteases work best in diets with less protein and when the composition contains lower quality ingredients.

Exogenous proteases may not result in effects on fish performance parameters (DALSGAARD *et al*., 2012; DREW *et al*., 2005; FARHANGI; CARTER, 2007; YIGIT *et al*., 2018). Part of these results is directly related to the formulation, composition, characteristics, and level of inclusion of the enzyme, quality of protein sources and processing of diets (GODA *et al*., 2019; LI, *et al*., 2015; SHI *et al*., 2016). Ingredients containing antinutrients interfere with protease activity (DREW *et al*., 2005; HASSAAN *et al*., 2019). Thus, an alternative is the removal of antinutrients and the inclusion of exogenous protease in the diet to assess the effect on fish growth.

Effects on nutrient digestibility

The presence of exogenous proteases in fish feed provides greater digestibility of nutrients, especially protein (Table 2) (FARHANGI; CARTER, 2007; RAGAA *et al*., 2017; SHI *et al*., 2016; KEMIGABO *et al*., 2019). Greater nutrient use is directly related to the release of amino acids and peptides in the digestive system. On the other hand, nutrient use can be impaired by the presence of antinutrients, as observed in sea bass (*D. labrax* L.) fed diets containing insect meal and exogenous protease (200 mg kg^{-1}) (GASCO *et al*., 2016) and in rainbow trout (*O. mykiss*) fed diets containing canola and pea seeds and exogenous protease (250 mg kg^{-1}).

Nutrient digestibility depends on the activity of endogenous and exogenous proteases in the digestive system. The pH of the gastrointestinal tract of fish has a direct influence on the activity of proteases (DABROWSKI; GLOGOWSKI, 1977). Acid proteases, such as pepsin, act according to their name, in an acidic environment, in the stomach of fish, while neutral and/or alkaline proteases, trypsin and chymotrypsin, act in neutral and/or alkaline portions of the intestine. In fish without a stomach, initial digestion is carried out by pancreatic alkaline trypsin (BALDISSEROTTO, 2013). Nile tilapia (*O. niloticus*) fed plant diets with the addition of exogenous enzymes (protease, β-glucanase and xylanase) increased the secretion of endogenous protease (trypsin) in the digestive system and greater protein retention (LIN; MAI; TAN, 2007). According to Francis; Makkar; Becker (2001), the presence of antinutrients (trypsin inhibitors) in the diet may not cause negative effects on performance, as these effects can be offset by the synthesis of endogenous proteases in the fish digestive system. In addition, the greater activity of endogenous enzymes in the gastrointestinal tract is related to the quality of the protein of the ingredients, since the silver catfish (*Rhamdia quelen*) fed with animal meal showed greater activity of endogenous proteases and greater weight gain, when compared to fish fed diets based on soybean meal and yeast (LAZZARI *et al*., 2010). However, it is observed that more concentrated ingredients that present proteins of high biological value and better nutritional quality require greater activity of endogenous proteases and tend to provide growth, consequently, the activity of exogenous proteases is lower, this is because these ingredients are highly digestible by fish.

Table 2. Effects on nutrient digestibility by the addition of exogenous protease in diets with plant ingredients in fish feed

Specie	Protease inclusion (mg kg^{-1})	Results	Reference
Nile tilapia (*O. niloticus*)	200 e 400[a]	↑ ADC CP	RAGAA *et al.* (2017)
Sea bass (*D. labrax* L.)	200[b]	↓ ADC DM; PC and ADF	GASCO *et al.* (2016)
Rainbow trout (*O. mykiss*)	250[c]	↑ ADC DM; GE; CP and fat	DREW *et al.* (2005)
Rainbow trout (*O. mykiss*)	300[d]	↓ ADC DM; GE and fat ↑ ACD CP	FARHANGI; CARTER (2007)
Black carp (*M. piceus*)	1000, 2000 e 3000[e]	↑ ADC CP	CHEN *et al.* (2009)
Nile tilapia (*O. niloticus*)	500[f]	↑ ADC CP, DE; and fat	HASSAAN *et al.* (2019)
Prussian carp (*C. auratus gibelio*)	150 e 175[g]	↑ ADC DM and CP	SHI *et al.* (2016)
Tilapia (*O. niloticus* × *O. aureus*)	175[h]	↑ ADC DM and CP	LI *et al.* (2015)
Truta arco íris (*O. mykiss*)	175[i]	↑ ADC DM and AA	LEE *et al.* (2020)
African catfish (*C. gariepinus*),	1100[j]	↑ ADC CP	KEMIGABO *et al*. (2019)

[a] protease (Ronozyme ProAct ™, DSM Nutrition Products, SP, Poland); [b]protease (Ronozyme ProAct™, DSM, Heerlen, Netherlands); [c]protease (Domestic poultry-250™; JEFO Nutrition, Inc., St. Hyacinthe, QC); [d]protease (Bio-Feed™ Pro, Novo Nordisk, Bagsvaerd, Denmark); [e]neutral protease (8000 U g^{-1}, Zhiwei); [f]protease (5000 U

g^{-1}, Huvepharma, Antwerp, Belgium); [g]alkaline protease (AG175™, JEFO Nutrition, Inc. Saint-Hyacinthe, Quebec, Canada); [h]alkaline protease (JEFO Nutrition, Inc. Saint-Hyacinthe, QC, Canada); [i]protease (JEFO Nutrition, Inc., Quebec, Canada); [j]protease (Kemin Industries (Zhuhai) Co. Ltd., China). Apparent digestibility coefficient (ADC); Crude protein (CP); Dry matter (DM); Acid detergent fiber (ADF); Gross energy (GE); Digestible energy (DE); Amino acids (AA); Higher (↑); Lower (↓).

The efficiency of exogenous protease activity is related to the development of the mucosa and intestinal structures of fish, as the increase in villi allows a greater capacity for nutrient absorption and, indirectly, stimulates the activity of endogenous enzymes and, consequently, promotes growth (ADEOYE *et al*., 2016a; GODA *et al*., 2019; KUMARI *et al*., 2013; ZHANG *et al*., 2012). Fish fed diets containing exogenous protease presented villi with greater height and surface area and goblet cells distributed along the villi, indicating a better morphological state of the intestine (ABD ELNABI *et al*., 2020; KUMARI *et al*., 2013; SALEH *et al*., 2021; WU *et al*., 2020). However, the hormone cholecystokinin inhibits stomach acid secretion and stimulates the secretion of endogenous enzymes by the pancreas into the intestinal lumen (BALDISSEROTTO, 2013). While the secretin hormone increases the release of pancreatic alkaline secretion. Possibly, the availability of nutrients and the improvement of the morphological state of the intestine induce the hormone cholecystokinin to secrete endogenous enzymes, since most of the digestion of food takes place in the intestine (CAHU *et al*., 2004; BALDISSEROTTO, 2013; HLOPHE-GININDZA *et al*., 2015).

Diet processing can influence nutrient digestibility and fish body composition, as observed in Prussian carp (*C. auratus gibelio*) (SHI *et al*., 2016) and tilapia (*O. niloticus* × *O. aureus*) (LI *et al*., 2015). In contrast, diets containing exogenous enzymes did not influence the body composition of tilapia (*O. niloticus*) (ADEOYE *et al*., 2016a), prussian carp *(C. auratus gibelio*) (LIU *et al*., 2016), black carp (*M. piceus*) (CHEN *et al*., 2009), pompano (*Trachinotus marginatus*) (SIMIÃO *et al*., 2018), rainbow trout (*O. mykiss*) (YIGIT *et al*., 2018) and sea bass (*D. labrax* L.) (GASCO *et al*., 2016). However, the digestibility of nutrients may be affected by both the processing of diets and exogenous proteases and interaction, as in the study by Shi *et al*. (2016). The authors observed greater protein and lipid retention in fish fed the extruded diet containing exogenous protease, suggesting better use of nutrients. Although, in the extrusion process, some proteins can be denatured and, consequently, facilitate the action of proteases during the digestion process (NRC, 2011), exogenous proteases must have thermal and pH stability to ensure enzymatic activity (LI *et al*., 2015).

Metabolic effects

Monitoring the metabolic effects and health status of fish is an important tool for evaluating fish performance (Table 3). The evaluation of the activity of the aminotransferase enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in plasma reflects the health status of the liver and its functions (ZHAI; LU; CHEN, 2014). Diets containing low-quality ingredients can result in changes in the health status of fish. Ragaa *et al*. (2017) in Nile tilapia (*O. niloticus*) did not observe any influence of exogenous protease supplementation on plasma ALT and AST activity. Hassaan *et al*.

(2019) observed that Nile tilapia (*O. niloticus*) fed diets containing cottonseed meal and exogenous protease had lower plasma ALT and AST activity, indicating lower activity of amino acid metabolism and greater immune response of fish, with increased total protein and albumin. The authors suggest that the immune response is due to the presence of gossypol, an anti-nutritional factor in cotton (FRANCIS; MAKKAR; BECKER, 2001). In addition, the lower activity of aminotransaminase enzymes may be due to the use of vegetables in diets and the absence of some essential amino acid, as suggested by Abd Elnabi *et al*. (2020).

Diet processing and the inclusion of exogenous protease did not influence the plasma concentration of total proteins and albumin in prussian carp (*C. auratus gibelio*) (SHI *et al*., 2016). The sea bass (*D. labrax*) when fed diets containing dry distiller grains and exogenous protease showed an increase in the plasma concentration of total proteins, in the number of red and white blood cells, in the concentration of hemoglobin and in the hematocrit rate, the authors attributed to these results the greater demand of oxygen for its dissociation (GODA *et al*., 2019). On the other hand, in the study by Adeoye *et al*. (2016b) no hematological changes were observed in fish fed a combination of exogenous enzymes (protease, carbohydrase and phytase). Currently, the use of diets enriched with plant protein sources and lower quality ingredients, supplemented with protease has not had a negative effect on fish health, however, studies related to the mechanism of action of exogenous proteases in relation to blood parameters need to be developed (GODA *et al*., 2020; HASSAAN *et al*., 2019; SHI *et al*., 2016).

Table 3. Metabolic effects of the addition of exogenous protease in diets with plant ingredients in fish feed.

Specie	Protease inclusion (mg kg^{-1})	Results	Reference
Nile tilapia (*O. niloticus*)	250[a]	Blood: ↑ number of white blood cells ↑ hematocrit rate ↓ cholesterol content	SALEH *et al.* (2021)
Nile tilapia (*O. niloticus*)	500[b]	Blood: ↑ concentration of hemoglobin ↑ hematocrit rate Plasma: ↑ concentration of total proteins ↑ concentration of albumin ↓ ALT and AST activity	HASSAAN *et al.* (2019)
Sea bass (*D. labrax* L.)	1000[c]	Blood: ↑ number of red blood cells ↑ number of white blood cells ↑ concentration of hemoglobin ↑ hematocrit rate Plasma: ↑ concentration of total proteins ↓ concentration of albumin ↑ cholesterol content Liver: ↓ ALT and AST activity ↓ concentration of AP	GODA *et al.* (2019)

Prussian carp (*C. auratus gibelio*)	400[d]	Serum: ↑ concentration of AP Hepatopancreas: ↓ AST activity	LIU *et al.* (2016)
Nile tilapia (*O. niloticus*)	500[e]	Blood: ↑ number of red blood cells ↑ number of white blood cells ↑ concentration of hemoglobin ↑ hematocrit rate Plasma: ↓ ALT and AST activity ↑ concentration of total proteins and albumin	HASSAAN *et al.* (2021)

[a]protease (600.000 U g^{-1}, Novus Company, USA); [b]protease (5000 U g^{-1}, Huvepharma, Antwerp, Belgium); [c]protease (PROXYM ULTRA5®, Gloray Vet COMPANY); [d]coated neutral protease (Kemin Industries Zhuhai Co., Ltd.); [e]SunHY Biology Co. Ltd., China. Legend: Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Alkaline phosphatase (AP); Higher (↑); Lower (↓).

The aminotransferase enzymes, ALT and AST, are biomarkers of amino acid metabolism and their activity increases when there are deamination and transamination processes (NELSON; COX, 2004). The highest AST activity was observed in the hepatopancreas of prussian carp (*C. auratus gibelio*) fed with lower levels of protein and without exogenous protease in the diet, possibly due to the processes of amino acid deamination (LIU *et al*., 2016). Kumari *et al*. (2013) indicated a greater use of dietary protein with greater activity of aminotransferase enzymes in tissues, liver and muscle, of fish fed with nanoencapsulated trypsin. On the other hand, sea bass (*D. labrax* L.) fed diets containing the highest content of dry distillery grains (50%) and exogenous protease showed lower AST and ALT and alkaline phosphatase activity in the liver and greater growth (GODA, *et al*., 2019). However, it is observed that exogenous proteases do not have direct effects on protein metabolism, however, they can increase the availability of nutrients for the action of aminotransferase enzymes.

Synergistic effects

Exogenous proteases together with other exogenous enzymes result in improved growth (ALI ZAMINI *et al*., 2014; LIN; MAI; TAN, 2007; SIMIÃO *et al*., 2018), nutrient digestibility (HLOPHE-GININDZA *et al*., 2015) and alteration of the intestinal microbiota of fish (ADEOYE *et al*., 2016a, 2016b; HASSAAN *et al*., 2021). However, the addition of enzyme complexes or blends allows enzymes to act simultaneously, each one in its specific substrate, thus allowing greater availability of nutrients in the digestive system of fish (GOMES *et al*., 2019; LIN; MAI; TAN, 2007). Furthermore, the combination of acids and exogenous proteases in the diet showed positive results in growth, nutrient digestibility and villus height (HASSAAN *et al*., 2020; HUAN *et al*., 2018). On the other hand, to ensure beneficial effects, processing techniques and diet composition must be considered, as well as species and other factors that can inactivate and/or reduce enzymatic activity.

The combination of exogenous proteases and probiotics has positive effects on fish performance (Table 4). However, when the level of enzyme inclusion in the diet is not enough, the functions

performed by probiotics can be compromised and not achieve satisfactory results. Thus, the detection of alterations resulting from supplementation is not possible as observed in Channa argus (DAI *et al*., 2018). On the other hand, enzymes favor the action of probiotics, since they release substrate for probiotic activity, and the combination of both improves fish performance (ADEOYE *et al*., 2016a, 2016b; HASSAAN *et al*., 2021).

In diets developed for fish, soybean meal and corn ingredients supply most of the metabolic energy required (NRC, 2011). However, antinutrients can make it difficult to use this energy and other nutrients. On the other hand, the presence of probiotics in the digestive tract can produce metabolic products during fermentation, such as lipopeptide and peptidase. These metabolites can alter the activity of digestive enzymes and even the pH of the digestive tract (MURA; BAUER, 1978). It was possible to observe that the combination of exogenous enzymes and probiotics alters the intestinal microbiota without deleterious effects on the intestinal health of fish (ADEOYE *et al*., 2016b; HASSAAN *et al*., 2021; JIANG *et al*., 2014). The reason for such effects may be that exogenous enzymes alter the environment of the intestine, such alterations of pH or intestinal substrates caused by enzymatic decomposition since positive effects of the combination of exogenous enzymes and probiotics were not observed by Dai *et al*. (2018). However, the mechanism of action of this combination in terms of improving fish performance is not yet known.

Table 4. Synergistic effects of protease and probiotic mixture composition in fish diets

Specie	Composition	Results	Reference
Nile tilapia (*O. niloticus*)	1.85×10^5 *B. pumilus* (CFU kg^{-1}) + 500 mg protease kg-1	Performance: ↑ WG; SGR; FI; PER ↓ FC Intestine histology: ↑ number of GC; mucosal thickness and enterocyte height Immunology: ↑ phagocytic activity ↑ concentration of IgM and Lys	(HASSAAN *et al*., 2021)
Snake head (*C. argus*)	1×10^6 *B. amyloliquefaciens* (CFU g^{-1}) + 300 mg protease kg^{-1}	Performance: = WG; SGR; FI; PER; FC Serum: = albumin, total proteins and Lys ↑ concentration of AP Digestive enzymes: ↑ pepsin in the stomach ↑ trypsin and amylase in the liver	(DAI *et al.*, 2018)
Nile tilapia (*O. niloticus*)	20 mg kg^{-1} (*B. subtilis, B. licheniformis* e *B. pumilus*) + 30 mg protease kg^{-1}	Performance: ↑ SGR and PER ↓ FC Serum: = Lys Intestine histology: ↑ number of GC; MV and EAS	(ADEOYE *et al*., 2016a, 2016b)

Legend: Weight gain (WG); Specific growth rate (SGR); Feed conversion (FC); Protein efficiency rate (PER); Food intake (FI); Goblet cells (GC); Immunoglobulin (IgM); Alkaline phosphatase (AP); Lysozyme (Lys); Microvilli (MV); Enterocyte absorption surface (EAS). Same as control treatment (=); Higher (↑); Lower (↓).

Bottlenecks and opportunities for the use of exogenous proteases

The use of exogenous enzymes in animal production is an established nutritional strategy. Biotechnology companies tend to develop new enzymes and optimize existing methods to provide better rates of productivity and feed efficiency. Additionally, its use is increasingly recurrent because of the high cost of ingredients, variability in the composition and quality of animal flours, which increase the production cost and limit the profitability of fingerling production.

As previously described, exogenous proteases have many positive effects on growth, nutrient digestibility, health status, dietary quality, economics, and environment in fish production. However, knowledge of sources and characterization; protease inclusion levels, mechanisms of action and substrate: protease ratio are important aspects to ensure greater efficiency of exogenous proteases in fish feeding.

Sources and characteristics of proteases

The exogenous protease encapsulation technique can guarantee the stability and delivery of exogenous enzymes. Nanoencapsulation can prevent exogenous protease effects from being restricted in the digestive system by protease inhibitors and possible hydrolysis caused by digestive proteases (ZHENG *et al*., 2019). Chitosan, as a thermostable material, can immobilize many enzymes and help protect biomolecules from adverse effects. Exogenous trypsin, when nano encapsulated in chitosan to strengthen its efficacy and mimic proteolytic activity in the gastrointestinal tract via controlled release, improved growth and nutrient digestibility in *Labeo rohita* fed for 45 days (KUMARI *et al*., 2013). On the other hand, pepsin immobilized in colloidal gold nanoparticles had its activity investigated on 3D supports and its stability was observed compared to free enzyme, suggesting the authors that the enzyme has biocatalytic activity in material (GOLE *et al*., 2001). In another study, the protease was coated in a fluidized bed to increase its heat stability, it was possible to observe positive effects on the growth of prussian carp (*C. auratus gibelio*) (LIU *et al*., 2016).

Exogenous protease effects are responsive to gastric conditions that cause protein denaturation and degradation. Next, after being exposed to Nile tilapia (*O. niloticus*) gastrointestinal pH conditions, the protease activity of alginate capsules without and with bentonite at pH 3 did not undergo denaturation, but was affected at pH 2.5; 2 and 1.5 (RODRIGUEZ *et al*., 2018). Bovine trypsin supplemented in the diet of common carp (*C. carpio*) resulted in increased proteolytic activity and this increase in activity was correlated with the proportion of exogenous trypsin (DABROWSKI; GLOGOWSKI, 1977).

Fish feed is processed by compression granulation (pelleting) and/or extrusion (NRC, 2011). During these processes, favorable conditions for enzymatic hydrolysis (temperature and humidity) may

occur, in two ways: (I) inactivation of the exogenous enzyme, as they are thermosensitive; (II) enzymatic activation and, consequently, greater availability of nutrients from the diet (HASSAAN *et al*., 2019). However, enzyme stability prevents enzymatic hydrolysis during these processes (LI *et al*., 2015). In the study by Shi *et al*. (2016) protease inactivation was observed in the extrusion process, where only 37.65% of the proteolytic activity was maintained after this process; while during the pelleting of the feed the rate of retention of the protease activity was 77.98% and 79.30% (LI *et al*., 2015; SHI *et al*., 2016), where both cases showed high thermal stability of the protease compared to the extruded feed. In Nile tilapia (*O. niloticus*) fed a diet containing shrimp enzyme microcapsules, alkaline protease activity was 27% higher than in fish fed a control diet without enzyme immobilization, indicating that alginate-bentonite capsules are good vehicles for enzyme delivery. On the other hand, the high temperature of extrusion processing causes loss of microencapsulated enzymes, making it difficult to include them in diets (RODRIGUEZ *et al*., 2018). Thus, additional studies involving growth bioassays are needed to demonstrate the action and efficiency of enzymes subjected to different compositions and feed processing techniques.

Exogenous enzymes can change the physical quality of pellets, making them softer, and consequently, increase feed consumption and improve fish growth. In the same study, the forms of incorporation of enzymes in the feed did not differ (extruded feed without enzyme; feed with enzyme added before extrusion; feed with enzyme added in a vacuum coating machine after extrusion), however, the addition of enzyme after extrusion improved extrusion digestibility of nutrients in Atlantic salmon (*Salmo salar*) (JACOBSEN *et al*., 2018). The addition of exogenous protease in post-extrusion vaccum coater in diets containing soybean meal resulted in better digestibility of nutrients by rainbow trout (*O. mykiss*), and no positive effect was observed when supplementing the enzymes together (protease, β-glucanase and xylanase) in the diet containing soybean meal (DALSGAARD *et al*., 2012). For the same species and similar enzyme application, Dalsgaard *et al*. (2016) observed that exogenous enzymes can reduce the anti-nutritional effects of plant ingredients and improve nutrient digestibility and fish growth. The incorporation of post-extrusion enzymes with the use of vacuum coater is an important strategy in fish feeding, since in addition to improving pellet quality, it also increases the availability of nutrients and their use by the fish.

Protease levels in fish feed

Black carp (*M. piceus*) fed diets containing levels of 1, 2 and 3 g kg^{-1} of exogenous protease, low inclusion of fish meal (150 g kg^{-1}) and high inclusion of soybean meal (260 g kg^{-1}) resulted in increased weight gain and improved apparent protein digestibility coefficient (CHEN *et al*., 2009). However, fish showed no change in weight gain when fed with levels above 1 g kg^{-1} of protease. Goda *et al*. (2019) tested the inclusion of 1 g kg^{-1} of exogenous protease in the diet containing high fish meal inclusion (300 g kg^{-1}) and low distillery grain content (187.5 g kg^{-1}) and observed better growth and feed efficiency, higher plasma total protein concentration, lower alkaline phosphatase concentration, lower

ALT and AST activity in the liver, greater villus length and height and increase in the number of goblet cells in the intestine of sea bass (*D. labrax* L.). The high content of distillery grains in substitution of soybean meal increase of 24% in the final weight of the fish, and in the economic evaluation, a lower cost of feed/kg of weight was observed with the replacement. On the other hand, in the study by Yigit *et al*. (2018) no greater growth, nutrient digestibility and change in body composition were observed in rainbow trout (*O. mykiss*) fed a diet containing low inclusion of fish meal (310 g kg^{-1}), high inclusion of soybean meal (440 g kg^{-1}) and two levels of protease (1 e 2 g kg^{-1}). Levels above 1 g kg^{-1} of exogenous protease in the diet did not result in increased weight gain or positive effects on apparent nutrient digestibility (CHEN *et al*., 2009; GODA *et al*., 2019; YIGIT *et al*., 2018).

The extruded diet (at 110±5ºC) containing low inclusion of fish meal (30 g kg^{-1}) and high inclusion of soybean meal (260 g kg^{-1}) showed lower exogenous protease activity when compared to a diet which contains 90 g kg^{-1} of fish meal and 160 g kg^{-1} of soybean meal. In the diet with the highest nutritional quality (90 g kg^{-1} of fish meal and 160 g kg^{-1} of soybean meal) supplementation with 125, 150 and 170 mg kg^{-1} of exogenous protease did not influence the performance of the prussian carp (*C. auratus gibelio*). However, fish fed a pelleted diet containing low inclusion of fish meal (30 g kg^{-1}) and high inclusion of soybean meal (260 g kg^{-1}) without supplementation showed lower growth; on the other hand, when supplemented with protease, this same diet resulted in an average increase of 11% in fish growth and better digestibility of nutrients (SHI *et al*., 2016). In tilapia (*O. niloticus* × *O. aureus*) fed a pelleted diet containing low inclusion of fish meal (30 g kg^{-1}) and high inclusion of soybean meal (300 g kg^{-1}) and 175 mg kg^{-1} of protease showed an average increase of

12% in final weight due to improved feed efficiency and apparent digestibility of nutrients when compared to the control group without exogenous protease in the diet. However, there was no difference in the final weight of fish fed the pelleted diet containing 90 g kg^{-1} of fish meal and 260 g kg^{-1} of soybean meal with enzyme supplementation in relation to the diet without protease. Furthermore, no greater growth was observed in tilapia fed the extruded diet containing 30 g kg^{-1} or 90 g kg^{-1} of fish meal and exogenous protease compared to fish fed the pelleted diet containing 90 g kg^{-1} of fish meal and protease (LI *et al*., 2015). Both studies showed negative effects of exogenous protease if added to extruded diets, but benefits were reported with feeding pelleted diets, and the effects can be attributed to the thermosensitive characteristic of the proteases.

Ragaa *et al*. (2017) observed in Nile tilapia (*O. niloticus*) an average increase of 13% in final weight, greater weight gain and apparent protein digestibility coefficient when fed a diet containing 400 mg kg^{-1} of exogenous protease, low inclusion of fish meal (80 g kg^{-1}) and high of soybean meal (361.1 g kg^{-1}). In addition, a 2% reduction in the protein level (70 g kg^{-1} of fish meal and 350.50 g kg^{-1} of soybean meal) with 200 and 400 mg kg^{-1} of protease in the fish diet, resulted in an improvement in apparent protein digestibility and a 7% increase in final weight compared to fish fed diets without enzyme. Nile tilapia (*O. niloticus*) that received diets containing high inclusion of fish meal (150 g kg^{-1}) and low inclusion of cottonseed meal (120 g kg^{-1}) and another containing low inclusion of fish meal

(110 g kg^{-1}) and high cottonseed meal (160 g kg^{-1}), both containing 500 mg kg^{-1} of exogenous protease, resulted in an approximate 24% increase in final weight, better availability of amino acids and of the apparent digestibility coefficient of dry matter, protein and lipids (HASSAAN *et al*., 2019). Improvement in the apparent digestibility coefficient of protein and lipid was also observed in prussian carp (*C. auratus gibelio*) fed a diet containing low inclusion of fish meal (60 g kg^{-1}) and high inclusion of soybean meal (180 g kg-1) and 400 mg kg^{-1} of exogenous protease, promoting with this formulation an economy of 20 g kg^{-1} of protein in the diet (LIU *et al*., 2016).

Rainbow trout (*O. mykiss*) fed a diet containing low (280 g kg^{-1}) and high (350 g kg^{-1}) inclusion of fish meal and 175 mg kg^{-1} of protease showed better performance (ZHANG *et al*., 2012). In the study by Dalsgaard *et al*. (2012), in the extruded diet with 228 mg kg^{-1} of exogenous liquid protease, containing low inclusion of fish meal (201.7 g kg^{-1}) and high inclusion of soybean meal (343.8 g kg^{-1}), there was an improvement in the apparent digestibility of nutrients and in the growth of rainbow trout (*O. mykiss*), but there was no improvement in the digestibility of nutrients in the diets with sunflower meal (246 g kg^{-1}) and canola meal (263.5 g kg^{-1}) supplemented with protease. Dalsgaard *et al*. (2016) observed that exogenous protease supplemented under the same conditions as the previous study resulted in higher apparent digestibility of nutrients in rainbow trout (*O. mykiss*) diets. Although the supplementation of 250 mg kg^{-1} of exogenous protease to the canola diet (240 g kg^{-1}) resulted in improved feed efficiency, the flaxseed diet (240 g kg^{-1}) did not affect the digestibility of nutrients in rainbow trout (*O. mykiss*) (DREW *et al*., 2005). Thus, it is observed that the activity of exogenous enzymes is influenced by alternative sources of protein in fish feed and, consequently, can cause adverse effects on the use of nutrients and growth.

Positive effects on intestinal villi growth and health were observed in *L. rohita* fed chitosan encapsulated trypsin in the diet than unencapsulated trypsin (KUMARI *et al*., 2013). The increase in the specific growth rate and in the protein efficiency rate and lower feed conversion showed that even after feed processing, trypsin nanoencapsulated in chitosan remained stable and active, indicating a positive correlation between feed intake and digestive enzyme activity. In tilapia (*O. mossambicus*) it was observed greater growth, improvement in apparent protein digestibility and intestinal enzyme activities when fed with a diet containing 500 mg kg^{-1} of exogenous protease, 77.1 g kg^{-1} of fish meal and 75 g kg^{-1} of kikuyu leaf bran (HLOPHE-GININDZA *et al*., 2015). Atlantic salmon (*Salmo salar* L.) fed a diet containing 339 g kg^{-1} of soybean meal and a mixture of proteolytic enzymes and carbohydrates (1 mg kg^{-1}) showed greater growth compared to fish fed the diet based on fish meal and/or with soybean meal without the addition of enzymes (CARTER *et al*., 1994). Common carp (*Cyprinus carpio* L.) fed a diet containing supplemental protease and 100 g kg^{-1} of fish meal showed better weight gain compared to the group that received diets containing 150 g kg^{-1} and 200 g kg^{-1} of fish meal (LENG *et al*., 2008). In contrast, the use of exogenous enzymes (300 mg kg^{-1} protease, 75 mg kg^{-1} phytase and 250 mg kg^{-1} xylanase) and probiotics as individual supplements did not show positive effects on Nile tilapia (*O. niloticus*) performance parameters (ADEOYE *et al*., 2016b), and the antinutrients, non-starch

polysaccharides and trypsin inhibitors, are substrates for the xylanase and protease enzymes, respectively. Possibly, the differences between the results of growth and nutrient digestibility are related to other factors, such as the interaction of enzymes with the composition of diets and their substrates, digestive system, species and the stability of proteases in feed processing.

Protease: substrate interaction

Enzymes have degrees of specificity in relation to their different substrates, for example, digestive trypsin can hydrolyze a peptide bond in which the amino group is made up of basic amino acids such as lysine, arginine and histidine. In the study by Dalsgaard *et al*. (2012) it was observed that the addition of protease in diets containing soybean meal, sunflower meal and canola meal showed better nutrient digestibility with soybean meal than in other sources for rainbow trout (*O. mykiss*). Possibly, the presence of phytic acid in these ingredients prevented the enzyme's action in improving the use of nutrients (FRANCIS; MAKKAR; BECKER, 2001). Thus, an alternative is to evaluate the activity of exogenous proteases used in fish diets according to the enzyme-substrate specificity of each ingredient.

Sea bass (*D. labrax* L.) fed a pelleted diet containing 200 mg kg^{-1} of protease, high inclusion of fish meal (445.5 g kg^{-1}) and low inclusion of *Tenebio molitor* larvae meal (247.5 g kg^{-1}), showed no improvement in nutrient digestibility (GASCO *et al*., 2016). In the study by Henry *et al*. (2018) no change was observed in the immunological parameters of fish fed diets containing exogenous enzymes and *T. molitor* larvae meal (247.5 g kg^{-1}). However, the trypsin inhibition activity was reduced in the presence of proteases, leading the authors to believe that the enzymes are not recommended in diets containing *T. molitor* larvae meal, since they could have inhibited some protein responsible for immune functions and reduce the apparent digestibility of dietary protein.

In the study by Chen *et al*. (2009) there was an increase in the apparent digestibility of the protein, but not in the dry matter, so that the authors believed that the exogenous protease reached its substrate due to the increased availability of protein. However, not all enzymes are equally effective in digesting their substrates (LIN; MAI; TAN, 2007) and the effects of enzymes can be diminished in diets that contain highly digestible, high-density ingredients (SHI *et al*., 2016). Therefore, the enzyme: substrate relationship demands information more focused on the bioavailability of amino acids, on the interaction with endogenous enzymes during digestion and on the prior quality of the protein sources that make up the diets.

Final considerations

Supplementation of exogenous proteases in plant-based diet results in positive effects on fish growth, nutrient digestibility, and metabolism, and indirectly brings social, economic and environmental benefits. Although there are many studies with carnivorous species, due to the need for diets concentrated in protein, the use of exogenous proteases for omnivorous species also improves the growth and digestibility of nutrients from plant sources. Based on the results indicated, it is clear the importance

of using exogenous proteases in fish feeding, either individually or in combination. Feeding fish represents a high cost in the production system, and the increase in fish weight gain provided by the use of protease may offset its additional cost in the diet.

There are contradictory results attributed to the levels of enzyme inclusion, the composition of the diets and the cultivation conditions and, consequently, it compares studies difficult. In addition, some issues related to the use of protease have not yet been elucidated: (I) protease and its effect on improving food consumption; (II) protease effect on hematological parameters; (III) mechanism of action of the protease to the specific substrate; (IV) dynamics involved between protease: probiotic: intestinal environment.

Future research is needed to understand the mechanism of action of proteases in plant protein sources, in addition to evaluations regarding the thermal stability of proteases, new enzymatic coating techniques and forms of incorporation into the feed, to optimize and guarantee economic production and sustainable

Conflict of interest

The authors declare that the research was conducted in the absence of any potential conflicts of interest.

Ethical statements

The authors confirm that the ethical guidelines adopted by the journal were followed by this work, and all authors agree with the submission, content and transfer of the publication rights of the article to the journal. They also declare that the work has not been previously published nor is it being considered for publication in another journal.

The authors assume full responsibility for the originality of the article and may incur on them, any charges arising from claims, by third parties, in relation to the authorship of the article.

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MANUSCRITO II

Protease improves performance, GHR gene expression, nutrient deposition, hematological and biochemical indicators of Nile tilapia (*Oreochromis niloticus*)*

*Manuscrito submetido ao periódico Animal Feed Science and Technology.

Protease in a feather meal-based diet improves performance, GHR gene expression, nutrient deposition, hematological and biochemical indicators of Nile tilapia (*Oreochromis niloticus*)

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Abbreviations: AA, amino acids; WGR, weight gain relative; FE, feed efficiency; PER, protein efficiency ratio; FI, feed intake; FCR, feed conversion ratio; CP, crude protein; CL, crude lipid; BPR, body protein retention; BLR, body lipid retention; BPD, body protein deposition; BPD, body lipid deposition; RBCs, red blood cells; MCV, mean cell volume; TP, total proteins; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GHR, growth hormone receptor; IGF-I, Insulin-like growth factor-I

Abstract: Feather meal has a high protein content and can be an alternative ingredient in aquatic feed. In this context, exogenous proteases help in the hydrolysis of proteins and can improve nutrient absorption. This study was carried out with the objective of evaluating the effects and estimating the optimal level of protease in diets for Nile tilapia, on performance, digestive indexes, nutrient deposition, hematological and biochemical parameters, and expression of genes linked to growth. Five treatments: control (without protease); 14,550; 23,700; 29,250; 45,000 U/kg (named: control, 194, 316, 390 and 600 mg/kg, respectively) were included in an extruded diet to fish (initial weight: 5.69 ± 0.27 g) fed for 49 days. Nile tilapia fed the 390 mg/kg diet had higher performance compared to the control diet but did not differ from other exogenous protease levels. Therefore, the greatest relative weight gain obtained with the inclusion of protease was estimated at 440 mg/kg. Crude lipid content was higher at the 600 mg/kg level but did not differ from the 390 mg/kg group. Protein and lipid retention were higher at the 390 mg/kg level compared to the control group. There was a positive linear effect on the number of red blood cells and negative regarding mean corpuscular volume. Lower concentration of amino acids was observed at higher levels of protease. The protease had a positive linear effect on the concentration of total proteins and amino acids and a negative effect on the ammonia content in the liver and muscle. In liver, alanine aminotransferase activity showed a positive linear effect. The protease increased growth hormone receptor gene expression at the level of 390 mg/kg. In conclusion, exogenous protease has a positive effect on growth, feed efficiency, gene expression, and protein retention and deposition, without negative effects on hematological and physiological indicators of Nile tilapia. The estimated optimal inclusion level was 440 mg/kg of protease (33,000 U/kg) on relative weight gain.

Keywords: Amino acids; Aminotransferases, Enzymes, Fish nutrition, Red blood cells.

1. Introduction

Feeding fish has been one of the major challenges in the activity, largely due to the need for a higher protein nutritional content in the feed. For a long time, fishmeal has been used in the formulation of fish diets, due to its high protein content and amino acid balance, as well as being a palatable ingredient (Furuya, 2010). However, it is a limited and finite resource, and its excessive use has been criticized by environmental organizations (FAO, 2022; Hardy, 2010). In 2020, Brazil totaled 3.6 million tons of meal of animal origin, more than 17% corresponding to the production of fish waste and feathers (ABRA, 2020). Feather meal, from poultry processing, emerges as a viable alternative to compose aquafeeds due to its abundance and high protein content, ranging between 80 to 90% crude protein. Furthermore, it is also rich in essential amino acids such as cystine, threonine and arginine (Lee et al., 2020). Soybean meal is the main vegetable protein source used in animal nutrition, but it is limited in methionine (Furuya, 2010). The combination of protein sources can ensure nutritional balance (Abimorad *et al*., 2014). However, it is believed that the inclusion of protease can improve the use of proteins and amino acids (AA) from protein by-products in the diet.

Exogenous protease can complement the activity of endogenous enzymes and break down complex proteins into free AA and oligopeptides, aiding in the assimilation of nutrients. Recent studies show that reducing fishmeal in the diet using protease improved performance and nutrient utilization by fish (Hassaan *et al*., 2019; Shi *et al*., 2016). Greater protein retention was observed in tilapia (*Oreochromis niloticus* × *O. aureus*) fed diets based on meat and bone meal and the inclusion of protease and organic acid (Huan *et al*., 2018). In rainbow trout *(Oncorhynchus mykiss*), an improvement in AA digestibility was observed in different protein ingredients containing protease (Lee *et al*., 2020). Increasing protease levels in diets containing poultry by-product meal resulted in positive effects on the performance, digestive indexes, and body composition of Carp rohu (*Labeo rohita*) up to the level of 600 mg/kg (Maryam *et al*.,

2022). Positive effects of protease were also observed in increasing the expression of growth regulatory genes in Nile tilapia (*O. niloticus*) and Grass carp (*Ctenopharyngodon idella*) species (Feng *et al*., 2023; Hassaan *et al*., 2019).

Nile tilapia (*O. niloticus*) is an omnivorous freshwater species that presents good growth and rusticity (El-Sayed, 2006). Its production is growing rapidly, so much so that it ranks third among the most produced species worldwide, and Brazil is the fourth largest producer in the world (FAO, 2022). As already mentioned, the benefits of using exogenous proteases are many and it is believed that the use of feather meal can be improved by hydrolysis with protease. Thus, the objective was to evaluate the effects of protease in a feather meal-based diet on performance, digestive indexes, body composition, nutrient deposition, biochemical and hematological parameters, and expression of genes linked to Nile tilapia growth.

2. Material and methods

2.1. Fish and experimental conditions

The experimental procedures were conducted at the Fish Farming Laboratory, Universidade Federal de Santa Maria, *Campus* Palmeira das Missões, Rio Grande do Sul, Brazil. The Nile tilapias were adapted to the experimental conditions for two weeks and fed the experimental control diet. Subsequently, the initial biometry was performed with 300 juveniles, with an initial weight of 5.69 ± 0.27 g, distributed in 20 tanks (220 L, 15 fish each). The experiment lasted 49 days.

The experiment was carried out in a water recirculation system that contained water inlet and outlet in the tanks, individual aeration, a decanter, two mechanical and biological filters, and a water reservoir with a capacity of 2,000 L, equipped with a heating system. The water flow was approximately 1.7 L/min/tank. The fish were fed three times a day (08:00 am, 1:30, and 6:00 pm) until they reached apparent satiety, identified by the feeding behavior of the

fish. The amount of feed ingested was measured daily and the tanks were cleaned twice a day (10:00 am and 4:00 pm).

The temperature and dissolved oxygen (DO) content of the water were checked daily with YSI ProODO™ technology (YSI Inc. Ohio, USA). The pH (YSI™, pH100), total alkalinity (by neutralization titration), total hardness (by complexation titration), unionized ammonia and nitrite (*kit* Alfakit™) were measured weekly. Water quality parameters were maintained as needed for Nile tilapia (De León-Ramírez *et al*., 2022; El-Sayed, 2006).

The water samples were collected at the entrance of the decanter and the data obtained were as follows: temperature: 25.76 ± 1.03°C; oxygen: 6.51 ± 0.75 mg/L; pH: 7.58 ± 0.19; unionized ammonia: 0.10 ± 0.01 mg/L; nitrite: 0.32 ± 0.12 mg/L; alkalinity: 42.07 ± 7.25 mg $CaCO_3$/L; and hardness: 50.19 ± 9.95 mg $CaCO_3$/L.

2.2. Dietary protease

A diet containing 35% crude protein (CP) and 3,000 kcal/kg digestive energy was formulated (Table 1) to meet the nutritional requirements of Nile tilapia (Furuya, 2010). Liquid protease was added to the diet at four levels: 14,550; 23,700; 29,250; 45,000 units (PROT) *per* kg of diet and one without protease (named 194; 316; 390; 600 mg/kg and control, respectively). RONOZYME™ ProAct is a serine protease (EC 3.4.21) produced from *Bacillus licheniformis* (DSM 19670) and contained 75,000 PROT/g, obtained from DSM Nutritional Products Ltd. (Mszczonow, Poland). One PROT unit is defined as the amount of enzyme that releases 1 µmol of *p*-nitroaniline from 1 µM of substrate (Suc-Ala-Ala-Pro-Phe- *p*-nitroaniline) *per* minute at pH 9.0 and temperature 37ºC. The experimental design was completely randomized, replicating four tanks for each of the five treatments.

The ingredients were grounded, sieved (diameter of 500 µm), and mixed. Then, oils and distilled water (approximately 180 mL/kg of diet) were added and mixed again until complete

homogenization. The mixture was extruded in a single-screw extruder (Inbramaq, model Labor PQ 30, São Paulo, Brazil) with a diameter of 2.0 mm. Then, the pellets were dried for 24 h in an oven with forced air circulation at 55°C. Subsequently, the enzyme was added by spraying with a hand pump and the diets were stored at -20°C throughout the experimental period. The formulation and composition of the diets are shown in Table 1.

2.3 Growth performance

All fish were anesthetized with an eugenol solution (50 mg/L) (Vidal *et al*., 2008), at the beginning and end of the experiment, to evaluate performance. Fish growth parameters were calculated using initial weight (IW) and final weight (FW) (g), total length (TL) (cm), feed intake (FI, g/fish) as follows: daily weight gain (g): DWG = (FW-IW)/days; relative weight gain relative (%): RWG = (FW-IW/IW)×100; survival (%): S = (final fish number×100)/initial fish number; apparent feed conversion ratio: FCR = FI/(FW-IW); feed efficiency (g): FE = (FW-IW)/FI (dry matter) and protein efficiency ratio: PER = (FW-IW)/protein intake (g).

For subsequent evaluations and analyses, the fish were euthanized with an overdose of eugenol (250 mg/L), and posterior spinal cord sectioning for tissue collection to calculate the following parameters: condition factor: CF = (FW/TL^3)×100; hepatosomatic index (%): HSI = (liver weight/FW)×100; celomatic fat index (%): CFI = (abdominal cavity fat weight/FW)×100; digestive somatic index (%): DSI = (digestive tract weight/FW)×100; intestinal quotient: IQ = intestine length/total fish length and carcass yield (%): CY = (gutted weight with head and gills/FW)×100 were calculated.

2.4 Whole-body proximate composition and body nutrient deposition

The composition of the diets (Table 1) and the whole-body proximate composition were determined following the methodologies described by the Association of Official Analytical

Chemists (AOAC, 1995). Crude lipid (CL) was extracted with methanol/chloroform and quantified by the procedure of Bligh and Dyer (1959). Neutral detergent fiber was determined using the method described by Van Soest (1967), using a solution with sodium lauryl sulfate. Nitrogen-free extract was calculated according to Bureau, Kaushik, and Cho (2002) and digestive energy according to Jobling (1983).

Whole fish samples collected at the end of the experiment were homogenized to determine the final composition. The same procedure was adopted to determine the initial composition by sampling 10 fish. The dry matter (DM) content was determined by drying the samples at 105°C until reaching constant weight (method 934.01). The mineral matter (MM) content was determined by incineration at 550°C for 4 h (method 968.08). The CP content was determined using the adapted micro-Kjeldahl method (954.01). Subsequently, body protein retention (BPR), lipid retention (BLR), protein deposition (BPD), and lipid deposition (BLD) were calculated as described by Shi *et al.* (2016) and Durigon *et al.* (2023).

2.5 Hematological and biochemical parameters

Two fish *per* tank (eight *per* treatment) were randomly selected for blood sample collection, which was performed by puncturing the caudal vein with heparin as an anticoagulant. Blood samples were divided into two portions. The first portion was used for counting red blood cells (RBC), hematocrit (Ht), and hemoglobin (Hb). The RBC count was performed after dilution of 10 µL of blood in a formaldehyde citrate solution and the count was performed in a Neubauer chamber (Loptik Labor™) with the aid of an optical microscope (Bioval™). Ht was determined by the microhematocrit technique, the microcapillary was filled with approximately 10 µL (5 cm) of blood and centrifuged at 11,680×*g* for 15 min, after which the cell column was measured by the Ht ruler (Benfer™). The Hb was determined using a commercial colorimetric *kit* (Labtest™), which consists of homogenizing 20 µL of blood in a

color reagent containing potassium cyanide and, subsequently, the absorbance at 540 nm was determined using a spectrophotometer (Bioespectro™). The hematimetric indexes were determined: mean corpuscular volume (MCV) = hematocrit×10/number of RBC, mean corpuscular Hb (MCH) = Hb×10/number of RBC] and mean corpuscular Hb concentration (MCHC) = Hb×100/Ht.

The second portion of the blood sample was centrifuged at 1,248×g, 4ºC, for 10 min to obtain plasma. The glucose, albumin, and total proteins (TP) levels were determined with commercial colorimetric *kits* Labtest™. The total globulin was calculated by subtracting the total albumin from the TP. Total ammonia was determined by the sodium salicylate technique (Verdouw *et al*., 1978). The absorbance reading of all analyzes was determined using a spectrophotometer (Bio Spectro™).

2.6. Biochemical parameters in tissues

At the end of the experiment, muscle, and liver samples were collected. A homogenized tissue sample was heated with KOH at 100°C for 20 min. Subsequently, TP content was estimated using bovine serum albumin as a standard (Bradford, 1976). Glycogen content was determined as described by Krisman (1962), ethyl alcohol was used for glycogen precipitation and estimated with iodine-iodide. A tissue sample (50 mg) was homogenized in 20 mM potassium phosphate buffer, pH 7.5, and centrifuged at 1,248×gfor 10 min, and the supernatant was used for quantification of AA (Spies, 1957). Total ammonia was determined with sodium salicylate using the technique by Verdouw *et al*. (1978). The activity of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined with a colorimetric *kit* following the method described by Reitman and Frankel (1957).

175 *2.7. Gene expression*

176 Total ribonucleic acid (RNA) from liver and muscle (100 mg) was extracted using
177 TRIzol™ (Invitrogen™) according to the manufacturer protocol. The quality of RNA was
178 assessed using the Nanodrop™ ND-1000 spectrophotometer. The RNA was treated with DNase
179 enzyme (Turbo DNAse I *kit*, RNase-free), final volume of 11 μL, and reverse transcribed into
180 complementary deoxyribonucleic acid (cDNA) using a reverse transcriptase enzymatic reagent
181 *kit* (SuperScript™ IV) according to the manufacturer instructions, final volume 20 μL. Real-
182 time polymerase chain reaction (PCR) assays were performed on a real-time PCR system
183 (QuantStudio™) using SYBR Quantitative PCR SuperMix (PowerUp™ SYBR™ Green
184 Master Mix) for each reaction. Gene-specific primers for growth hormone receptor = GHR
185 (forward: TCTTGTATTTGGGACTGTGGG; reverse: CGATGCCTTTGATTTTGGGTG)
186 (GenBank code AY973232.1) and Insulin-like growth factor-I = IGF-I (forward:
187 CGATGTGCTGTATCTCCTGTAG; reverse: CTCGCTCTCCACAGACAAAC) (GenBank
188 code EU272149.1) were applied. The 18 s rRNA gene (GenBank code JF698683) varied
189 according to the treatments and it was decided not to use it as a gene for normalizing gene
190 expression. The β-actin gene (forward: CCACCTTCAACTCCATCATGAA; reverse:
191 GCAATGCCAGGGTACATGGT) (GenBank code KC195970) was used as an internal
192 control. Two controls were performed for the analyzed genes, non-reverse transcription and a
193 negative control. The real-time PCR began with 2 min at 95ºC, followed by 40 cycles of 15 s
194 at 95ºC, 1 min at 60ºC. At the end of each reaction, the specific amplification of each gene was
195 confirmed by melting curve analysis. All reactions were performed to a final volume of 10 μL
196 and in triplicate. The expression level of GHR and IGF-I were calculated by the $2^{-\Delta\Delta CT}$ method
197 (Livak and Schmittgen, 2001).

198

199

2.8. Statistical analysis

Results were expressed as average ± standard deviation. Significant differences between all treatments at $p < 0.05$ were indicated by one-way analysis of variance (ANOVA) and Tukey's mean comparison test using R™ statistical software version 4.3.0. The optimum dose of a dietary protease was determined using polynomial regression analysis (Yossa; Verdegem, 2015).

3. Results

3.1 Growth performance

As shown in Table 2, the FW, DWG, RWG, FE, and PER of fish were significantly higher at the dietary protease level of 390 mg/kg compared to the control group but did not differ from the other levels. However, no significant changes were observed in other parameters (CT, S, FI, CF and digestive indexes), including FCR. Regression analysis showed that the optimal level of protease inclusion to achieve the highest RWG is 440 mg/kg (Figure 1).

3.2. Whole-body proximate composition and body nutrient deposition

Dry matter, MM, and CP had no significant effect of protease. The CL was higher at levels of 390 and 600 mg/kg of exogenous protease than at the level of 316 mg/kg. In general, fish fed 390 mg/kg dietary protease had higher protein and lipid retention than the control group but not increased the level of 600 mg/kg (Table 3). According to the significant quadratic effect, the maximum level of protease inclusion to achieve the highest protein and lipid depositions is 370 mg/kg and 550 mg/kg, respectively (Figure 2A and B).

225 *3.3. Hematological parameters*

226 The number of red blood cells and MCV parameters had a positive and negative linear
227 effect, respectively, with the inclusion of protease in diet (Table 4). In the other parameters (Hb,
228 Ht, MCH and MCHC) there was no effect of the inclusion of exogenous enzyme.

229

230 *3.4. Plasma and tissue biochemical parameters*

231 Protease had no effect on glucose, TP, albumin, and globulin (Table 5). The
232 concentration of AA had a negative linear effect.

233 In the liver, all parameters had a significant linear effect, except ALT enzyme activity
234 (Table 5). Total protein concentration was higher at the 600 mg/kg level but did not differ from
235 the 390 mg/kg level. Glycogen was lower at the 600 mg/kg level than control group. Higher
236 AST enzyme activity was observed levels 316, 390 and 600 mg/kg of exogenous protease in
237 relation to the control diet but did not differ from the 194 mg/kg level. Amino acids and
238 ammonia concentration had a positive and negative linear effect, respectively, with the
239 inclusion of protease in diet (Figure 3A).

240 In muscle, the protease had a linear effect on the concentrations of TP, AA and ammonia
241 (Table 5; Figure 3B). The inclusion of protease resulted in lower activity of the AST enzyme
242 compared to the control group. Regression analysis determined that the optimal protease
243 inclusion level for lowest AST activity is 430 mg/kg. The protease had no effect on glycogen
244 and ALT enzyme activity.

245

246 *3.5. Gene expression*

247 As shown in Table 6, the expression of the GHR gene in the liver of fish was
248 significantly higher at the dietary protease level of 390 mg/kg compared to the control group
249 but did not differ from the other levels (194, 316 and 600 mg/kg). Regression analysis showed

that the maximum dose of protease inclusion to achieve the highest expression of the GHR gene is 245 mg/kg. The IGF-I gene was not influenced by exogenous protease. In muscle, the inclusion of protease did not influence the expression of the Nile tilapia GHR and IGF

4. Discussion

4.1. Growth performance

By-product meals from slaughter processing are sources of protein that do not compete with human nutrition and can be a suitable strategy for use in fish feeding. In the present study, the results demonstrated that fish fed the control diet (without protease) had lower performance compared to the level of 390 mg/kg. The presence of less digestible components (bones, feathers and phytic acid) and/or the absence of protease may justify the lower performance, including FE and PER. Likewise, for Rainbow trout (*O. mykiss*) the absence of the enzyme in feather meal resulted in lower digestibility of DM, gross energy and AA. On the other hand, the protease improved the digestibility of at least one AA from each protein ingredient tested (Lee *et al.,* 2020). Furthermore, better digestion of dietary proteins, indicated by PER, suggests a lower excretion of nitrogenous compounds into the aquatic environment and reinforces the sustainability role of the protease (Saleh *et al.* 2021). This result favors the nutrition sector, which will be able to formulate more strategic diets to seek maximum FE and better fish performance.

Protease levels significantly affected performance, metabolism, and expression of the GHR gene linked to Nile tilapia growth in this study. There was a positive relationship between dietary protease and fish growth up to the level of 390 mg/kg. The inclusion of protease significantly improves protein digestion by hydrolyzing complex proteins into free AA and oligopeptides, thus facilitating the transport, absorption, and complete utilization (Feng *et al*., 2023; Goda *et al*., 2019; Hassaan *et al*., 2019; Liu *et al*., 2018; Shi *et al*., 2016). Regarding food consumption, it was expected that there would be similar responses to WGD and WGR, but no

effect of protease on FI and FCR was observed. Presumably, the regulation of consumption is not directly related to the inclusion of protease. Feng *et al.* (2023) did not observe an effect of protease on the FCR of Grass carp (*Ctenopharyngodon idella*). Shi *et al.* (2016) pointed out that the protease in the extruded diet did not improve the FCR of Gibel carp (*Carassius auratus*). Recent studies reported that the FCR of tilapia (*Oreochromis* spp.) weighing 30 to 70 g ranged from 1.21 to 1.71 (Hassaan *et al.*, 2019; 2021; Huan *et al.*, 2018; Saleh *et al.*, 2021; Soltan *et al.*, 2023). In this study, the FCR was 1.02-1.09 for Nile tilapia, these results can be attributed to extrusion processing and adequate fish feeding management.

In the present study, based on the quadratic polynomial regression model, the level that presented maximum WGR is 440 mg/kg, equivalent to an enzymatic activity of 33,000 U/kg. Similarly, studies containing protease in the diet of Nile tilapia (*O. niloticus*) (Wu *et al.*, 2020); Grass carp (*C. idella*) (Feng *et al.*, 2023) and Rohu carp (*L. rohita*) (Maryam *et al.*, 2022) showed a better level of weight gain, at levels 5.8 U/kg; 7,393 U/kg and 259,80 U/kg, respectively. In contrast, other studies demonstrated that protease did not improve fish performance at levels of 175 mg/kg (Gasco *et al*., 2016) and 200 mg/kg (Huan *et al*., 2018). Possible, negative results were related to the incompatibility between the quality of ingredients in diets (Gasco *et al.*, 2016; Huan *et al.*, 2018). From this, it is suggested that a lack of understanding of the true effect of the protease on the substrate implies nutritional imbalances and/or lack of enzymatic activity, as reviewed by Schneider and Lazzari (2022). It is worth mentioning that the results obtained in this study were using protease in liquid form, applied by spraying with a manual pump in a diet after the extrusion process, as the protease may lose efficiency during this processing (Li *et al*., 2015; Shi *et al.*, 2016).

4.2. Whole-body proximate composition and body nutrient deposition

In this study, it was surprising that the protease had no effect on CP, on the other hand, there was a positive effect on BPR and BPD, especially at levels 316 and 390 mg/kg. Similarly, the inclusion of exogenous protease (300 e 600 mg/kg) did not affect the CP content of Gibel carp (*C. auratus* G.) (Liu *et al.,* 2018). In contrast, Maryam *et al.* (2022) showed that the inclusion of protease up to the level of 450 mg/kg for Carp rohu (*L. rohita*) influenced the body composition. In African catfish (*Clarias gariepinus* B.), increasing dietary protease (750 U/kg to 1,250 U/kg) improved body protein retention (Kemigabo *et al.,* 2019). The mechanism of action of proteases on intact proteins allows part of this product to be used for protein synthesis (Cowieson and Roos, 2016; Wilson, 2002). In summary, mixed diets (of animal and vegetable origin) better meet the nutritional needs of the species and are therefore commonly used. However, for a more effective enzyme recommendation for the species, it is necessary to evaluate the action of this protease in each protein ingredient in the diet.

The increase in the content of CL and BLR as well as BLD in the present study indicates that the lipid metabolism of fish was also affected by the exogenous protease. In contrast, Adeoye *et al.* (2016) and Hassaan *et al.* (2019) found no differences in the CL content of Nile tilapia (*O. niloticus*) fed 200 and 175 mg/kg of protease, respectively. Similarly, Shi *et al.* (2016) did not observe any influence of protease levels on CL content and BLR in the extruded diet. In this study, the highest inclusions of proteases (390 and 600 mg/kg) in the extruded feed resulted in a higher CL content. The protease increases the energy availability of the diet (Cowieson and Roos, 2016; Soltan *et al.,* 2023) and stimulates the protein-sparing effect, causing the fish to use lipids as metabolic fuels for growth (Mingarro *et al.,* 2002).

4.3. Hematological parameters

Hematological assessment is linked to physiological changes in fish health (Witeska *et al*., 2022). The increase in RBC indicates a greater supply of oxygen to the cells (Gopalraaj *et al*., 2023) and associated with the increase in body weight may be an adequate process of erythropoiesis (Ahmed *et al*., 2020). In the present study, the protease linearly influenced the number of RBC and MCV, since Nile tilapia fed with the highest level of protease (600 mg/kg) showed an increase in the number of RBC and a decrease in MCV. This result suggests a compensatory effect of the animal, since growing fish have a high need for oxygen and smaller RBC. Similarly, Maryam *et al.* (2022) observed an increase in blood indicators including the number of RBC an of rohu (*L. rohita*) with protease supplementation at the level of 450 mg/kg in a diet containing ingredients of animal and vegetable origin and concluded that improved health status. These results are consistent with studies that observed improvement in hematological parameters with protease in fish mixed diets (Goda *et al*., 2019; Hassan *et al*., 2019; Magouz *et al.*, 2022; Soltan *et al*., 2023). In contrast, the inclusion of protease (500 mg/kg) did not influence the health status of Nile tilapia (*O. niloticus*) (Saleh *et al.*, 2021). Although there is high variability in hematological responses in fish (Ahmed *et al.*, 2020), the action of proteases in diets: mixed (Feng *et al.*, 2022; Gasco *et al.*, 2016; Liu *et al.*, 2018) and based of plants (Adeyoe *et al.*, 2016; Hassaan *et al.*, 2021; Wu *et al.*, 2020) has been little explored and new answers are needed to determine the mechanism of action of the enzyme on blood indicators.

4.4. Plasma and tissue biochemical parameters

In the present study, glucose, TP, albumin, and globulin levels were not influenced by the inclusion of protease. Similar results were observed in Gibel carp (*C. auratus*) (Liu *et al*., 2018), Carp rohu (*L. rohita*) (Maryam *et al*., 2022), and Nile tilapia (*O. niloticus*) (Saleh *et al*.,

2021). The liver is the main organ that synthesizes protein and dietary metabolites. Exogenous protease can improve the nutritional value of protein sources. In the diet of Grass carp (*C. idella*), an increase in aminotransferases activities in the hepatopancreas and a reduction in plasma ammonia concentration were observed (Feng *et al*., 2023). Aminotransferase enzymes are responsible for the synthesis of AA and the presence of the AST enzyme is greater in the mitochondria of hepatocytes (Wilson, 2002). The decrease in deamination of AA is signaled by the reduction in ammonia content. In the present study, the protease linearly increased the concentration of proteins and AA in tissues (liver and muscle) and the activity of the AST enzyme in the liver and decreased the ammonia content. These results associated with BPD and FE suggest an improvement in the use of dietary protein by fish.

In muscle, AST enzyme activity did not differ between dietary protease levels; lower enzyme activity was estimated at 430 mg/kg of protease inclusion. The protease resulted in a decrease in plasma AA concentration and relative weight gain increased and reached a maximum at the level of 440 mg/kg and decreased thereafter. In contrast, rohu (*L. rohita*) and European seabass (*Dicentrarchus labrax*) had no effect of protease on the activity of aminotransferase enzymes was observed (Goda *et al.,* 2019; Maryam *et al.,* 2022). It was recently discovered that protease can promote the transfer of AA through transporters regulated by the TOR signaling pathway (Feng *et al.,* 2023). Although the efficiency of exogenous protease activity depends on the protein ingredients in the diet, there was a notable improvement in the digestibility of at least one AA from each ingredient without and with protease (175 mg/kg) in Rainbow trout (*O. mykiss*) (Lee *et al.,* 2022). Differences between species, diet composition and type and level of protease make it difficult to interpret results obtained in muscle. However, plasma AA were transported to tissues for metabolization and protein synthesis.

376 *4.5. Gene expression*

377 The nutritional conditions of fish can be identified by the somatotropic axis, GHR and
378 IGF-I, which helps regulate growth (Gómez-Requeni *et al*., 2004; Perez-Sanchez and Le Bail,
379 1999; Petro-Sakuma *et al*., 2020). In the present study, the highest expression of the GHR gene
380 in liver was observed in Nile tilapia fed a 390 mg/kg diet, and the highest growth had the same
381 result. The highest expression of this gene would have been regulated by the endocrine
382 metabolism of somatolactin (SL) and not by growth hormone (GH) (Mingarro *et al*., 2002;
383 Pierce *et al*., 2012). SL is responsible for energy homeostasis, in a phase of accelerated growth,
384 fish need a greater supply of cellular energy (Pierce *et al*., 2012). Although endocrine
385 metabolism has not been evaluated in this study, it is suggested that the protease was able to
386 stimulate protein synthesis through the metabolization of AA in the liver. This can be
387 confirmed, since the expression of the IGF-I gene in the liver was not affected by the inclusion
388 of the protease. In other study with Nile tilapia (*O. niloticus*), there was an effect of protease on
389 the expression of the GH and IGF genes on feed intake but there was a negative correlation
390 between these genes (GH and IGF). Supposedly, the increase in the expression of GH and
391 decreased IGF were linked to low feed intake, and not to the effect of exogenous protease
392 (Hassaan *et al*., 2019). Lower GHR gene expression and lower growth were observed in fish
393 fed diets containing soy saponin (Tian *et al.,* 2018); fumonisin B_1 and B_2 (Silva *et al.,* 2019)
394 and inclusion of vegetable sources (50%) replacing fish meal (Gómez-Requeni *et al.,* 2004).
395 These results are possibly related to amino acid imbalance and the presence of antinutrients in
396 fish diets. In the present study, the protease had no significant effect on the expression of GHR
397 and IGF-I genes in Nile tilapia muscle. It is possible that the mobilization of lipids for the
398 biosynthesis of muscle proteins would be accompanied by an increase in the expression of these
399 genes (GHR and IGF-I). Since SL and GH metabolism plays an important role in the regulation

and signaling of these genes and fish can use lipids as metabolic fuels for growth (Cowieson and Roos, 2016; Gómez-Requeni *et al*., 2004; Mingarro *et al*., 2002).

5. Conclusion

The inclusion of serine protease (Ronozyme™ ProAct) has a positive effect on performance, feed efficiency, growth hormone receptor gene expression, hematological parameters, and protein metabolism. Based on regression analysis, the optimal protease level is 440 mg/kg (33,000 U/kg) for maximum relative weight gain.

Ethics approval

The experimental procedures were approved by the Ethics in Animal Use Committee of the Universidade Federal de Santa Maria with protocol number 4351200721.

Declaration of Interest

The authors declare that they have no competing interests.

Contribution

Thamara Luísa Staudt Schneider: project administration; Formal analysis, Writing.

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Bruno Bianchi Loureiro: validation.

Nilce Coelho Peixoto: methodology, formal analysis, supervision.

Daniel Angelo Sganzerla Graichen: methodology, validation, supervision.

Rafael Lazzari: project administration, formal analysis, supervision, writing.

Acknowledgment

The authors are grateful to DSM™; Puro Trato™ Animal Nutrition (Santo Augusto, RS, Brazil), Mais Frango™ Ltda. (Miraguaí, RS, Brazil) and Mig-PLUS™ Agroindustrial Ltda. (Casca, RS, Brazil) for the donation of ingredients. Financial support for this work was provided by the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Financing Code 001 and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [312849/2020-6].

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Table 1

Diet formulation and composition with protease

Ingredient (g/kg)	Protease (mg/kg)				
	0*	194	316	390	600
Fish meal (66% CP) [1]	100	100	100	100	100
Feather meal (72% CP)	120	120	120	120	120
Soybean meal	360	360	360	360	360
Ground corn	260	260	260	260	260
Wheat bran	112.8	112.8	112.8	112.8	112.8
Soybean oil and canola oil (1:1)	20	20	20	20	20
Vitamins and minerals[2]	15	15	15	15	15
Ascorbic acid	5	5	5	5	5
Salt	5	5	5	5	5
Methionine	2	2	2	2	2
Butylated hydroxytoluene	0.2	0.2	0.2	0.2	0.2
Analyzed composition (g/kg)					
Dry matter	932.8±2.6	923.5±2.5	926.0±1.5	929.3±2.3	931.2±0.9
Mineral matter	76.5±0.3	72.7±2.0	70.8±1.7	73.0±1.6	77.0±2.6
Crude protein (CP)	359.6±2.3	360.4±1.2	358.9±9.7	361.1±0.4	359.9±7.8
Crude lipid	84.7±1.1	89.4±3.4	79.9±2.3	79.6±2.3	83.6±1.2
Neutral detergent fiber	63.20±6.6	63.20±6.6	63.20±6.6	63.20±6.6	63.20±6.6
NFE [3]	416.0	414.3	427.2	423.1	416.3
DE (kcal/kg) [4]	3,095	3,135	3,074	3,073	3,088
Relative activity of protease (prot/kg) [5]	0	14,550	23,700	29,250	45,000

* Control, without protease

[1] Fish waste meal

[2] Composition (kg/product): folic acid 370 mg; pantothenic acid 3,900 mg; biotin 40 mg; cobalt: 58 mg; copper 740 mg; choline 75 g; iron 7,500 mg; inositol 10 g; iodine 43 mg; manganese 7,800 mg; niacin 8,800 mg; selenium 38 mg; vitamin A 780,000 IU; vitamin B1 1,400 mg; vitamin B12 1,900 mcg; vitamin B2 1,450 mg; vitamin B6 1,400 mg; vitamin C 19.5 g; vitamin D3 160,000 IU; vitamin E 14,800 IU; vitamin K3 475 mg; zinc 1,400 mg

[3] NFE: Nitrogen-free extracts = [1000 − (crude protein + crude lipid + mineral matter + neutral detergent fiber)]

[4] DE: Digestible energy = [(crude protein x 5.64 x 0.75) + (crude lipid x 9.44 x 0.90) + (NFE x 4.11 x 0.50)] x 10

[5] Analyzed by BIOPRACT™ GmbH (Berlin, Germany)

Table 2

Growth, digestive indexes, and carcass yield of Nile tilapia (*Oreochromis niloticus*) fed with protease for 49 days

Parameter	Protease (mg/kg)				
	0*	194	316	390	600
Final weight (g)[1]	52.76±2.18[b]	55.30±3.98[ab]	57.81±3.95[ab]	63.52±4.84[a]	57.58±1.89[ab]
Total length (cm)	14.54±0.50	14.60±0.32	14.72±0.21	14.97±0.40	14.84±0.11
Daily weight gain (g)[2]	1.00±0.13[b]	1.01±0.08[ab]	1.06±0.08[ab]	1.18±0.10[a]	1.06±0.03[ab]
Survival (%)	88.33±19.15	86.67±16.33	93.33±0.00	95.00±6.38	83.33±8.61
Apparent feed conversion ratio	1.06±0.08	1.08±0.12	1.03±0.04	1.02±0.11	1.09±0.13
Feed efficiency (g)[3]	0.96±0.12[b]	1.11±0.06[ab]	1.20±0.10[ab]	1.26±0.12[a]	1.07±0.09[ab]
Protein efficiency ratio (%)[4]	2.65±0.32[b]	2.84±0.15[ab]	3.07±0.27[ab]	3.25±0.31[a]	2.85±0.24[ab]
Feed intake (g/fish)	51.52±8.56	52.07±5.70	47.63±4.96	48.63±3.80	52.24±3.88
Condition factor	1.72±0.05	1.78±0.07	1.81±0.06	1.89±0.04	1.76±0.03
Digestive indexes					
Hepatosomatic (%)	2.85±0.61	2.78±0.36	2.73±0.62	2.88±0.36	2.64±0.64
Celomic fat (%)	2.32±0.91	1.74±0.75	1.73±0.81	1.82±1.03	1.58±0.80
Digestive somatic (%)	5.39±1.05	4.82±1.06	5.05±0.77	4.72±0.62	5.08±0.54
Intestinal quotient	6.90±1.18	6.62±0.70	6.71±0.69	6.38±0.31	7.16±0.44
Carcass yield (%)	84.82±0.83	86.20±1.03	85.71±0.89	85.83±1.35	85.86±0.87

Values are expressed as the mean ± standard deviation; parameter n=4; digestive indexes n=5. Means with different letters on the line differ significantly ($p < 0.05$).

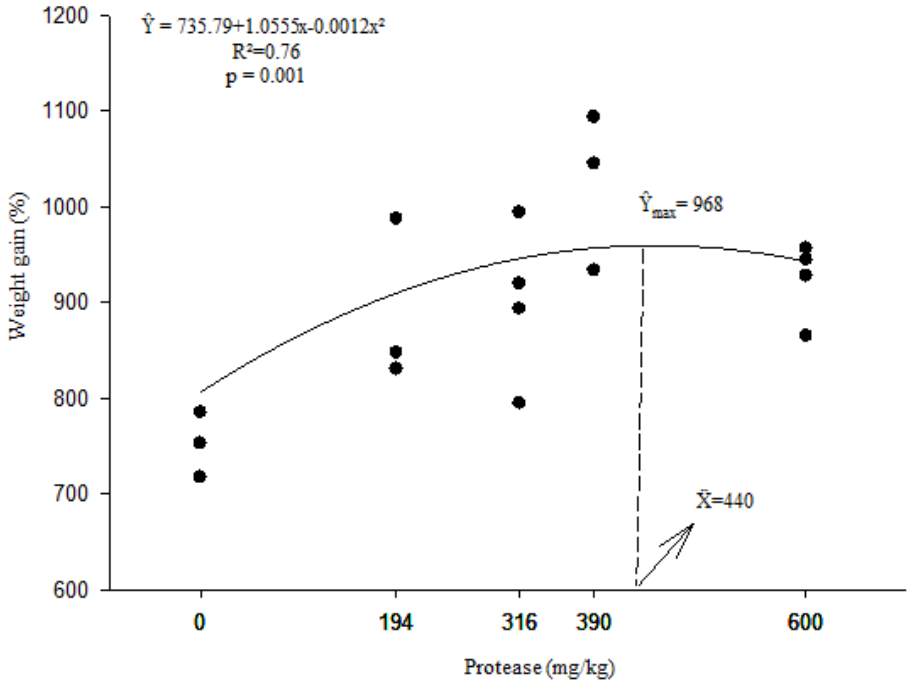
* Control, without protease.

[1] Quadratic effect: $\hat{Y} = 52.36+0.0442x-0.0001x^2$, $R^2=0.84$, p=0.0029

[2] Quadratic effect: $\hat{Y}= 0.95 + 0.0009x-0.000001x^2$, $R^2=0.82$, p=0.0020

[3] Quadratic effect: $\hat{Y} = 0.97+0.0014x -0.000002x^2$, $R^2=0.86$, p=0.0142

[4] Quadratic effect: $\hat{Y} = 2.60+0.0027x-0.000004x^2$, $R^2=0.76$, p=0.0493



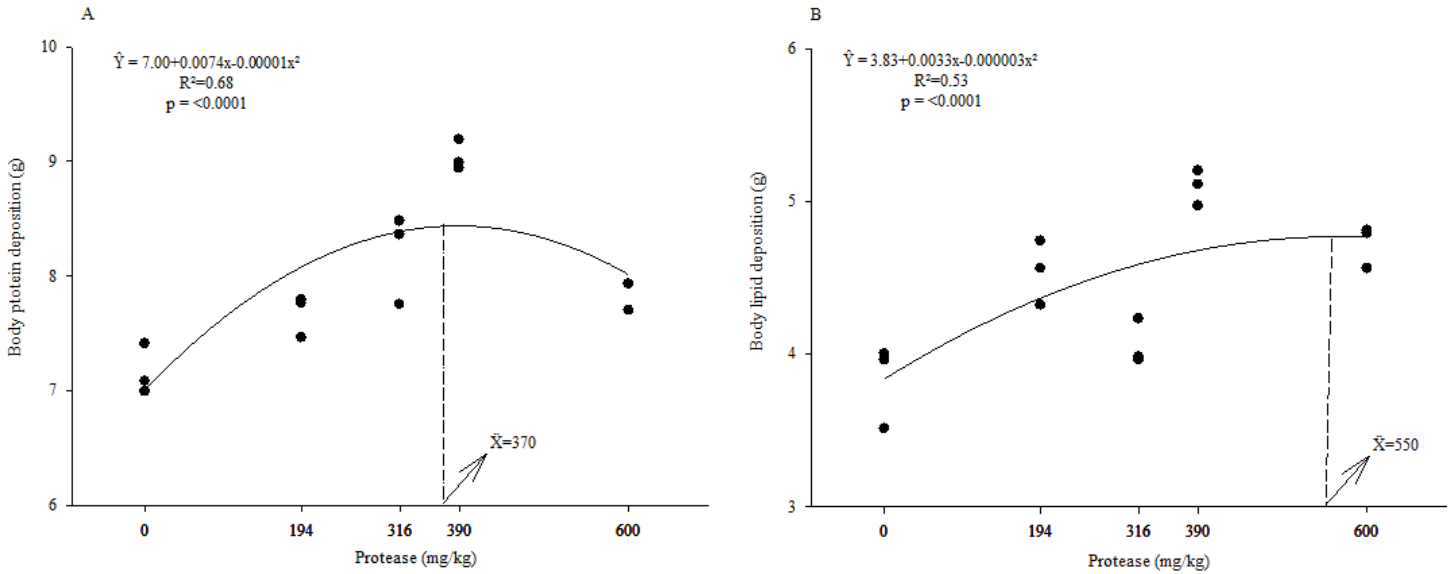
626
627 Figure 1. Relative weight gain in Nile tilapia (*Oreochromis niloticus*) fed with different levels of protease for 49 days, n=4.

628 Table 3

629 Proximate composition and nutrient deposition of Nile tilapia (*Oreochromis niloticus*) fed with protease for 49 days (% dry matter)
630

Parameter	Inicial	Protease (mg/kg)				
		0*	194	316	390	600
Dry matter (%)	23.55±0.31	27.26±0.22	26.48±0.82	26.52±0.67	26.59±0.22	27.73±0.98
Mineral matter (%)	3.42±0.25	3.20±0.11	3.02±0.29	2.79±0.16	3.10±0.06	3.20±0.07
Crude protein (%)	14.82±0.92	14.72±0.62	14.96±0.22	15.18±0.16	15.31±0.60	15.18±0.16
Crude lipid (%)[1]	8.94±0.46	8.49±0.32^{bc}	8.23±0.42^{cb}	8.04±0.20^{c}	9.12±0.30^{ab}	9.24±0.21^{a}
Protein retention (%)[2]	-	38.85±3.27^{b}	42.47±1.88^{ab}	46.70±4.08^{a}	49.90±4.81^{a}	43.36±3.12^{ab}
Lipid retention (%)[3]	-	91.33±14.60^{b}	94.04±6.23^{b}	110.75±12.13^{ab}	133.64±5.03^{a}	110.37±6.51^{ab}

631 Values are expressed as the mean ± standard deviation; n=3. Means with different letters on the line differ significantly ($p < 0.05$).
632 * Control, without protease
633 [1] Quadratic effect: $\hat{Y} = 8.42-0.0016x+0.00001x^2$, $R^2=0.60$, p=0.0034
634 [2] Quadratic effect: $\hat{Y} = 37.97+0.0485x-0.0001x^2$, $R^2=0.79$, p=0.0559
635 [3] Quadratic effect: $\hat{Y} = 86.56+0.1326x-0.0001x^2$, $R^2=0.55$, p=0.0001



636
637
638 Figure 2. Body protein deposition (A) and body lipid deposition (B) in Nile tilapia (*Oreochromis niloticus*) fed with protease for 49 days, n=3.

Table 4

Hematological parameters of Nile tilapia (*Oreochromis niloticus*) fed with protease for 49 days

Parameter	Protease (mg/kg)				
	0*	194	316	390	600
Red blood cells ($x10^6$/µL)[1]	0.86±0.04[b]	1.02±0.06[ab]	0.96±0.14[ab]	1.24±0.18[ab]	1.30±0.34[a]
Hemoglobin (g/dL)	7.25±0.52	7.37±0.72	7.37±0.51	7.52±0.35	7.18±0.43
Hematocrit (%)	31.71±3.31	33.92±3.62	33.92±3.62	33.92±0.92	33.04±3.05
MCH (pg)	84.06±2.70	72.72±8.37	78.94±17.62	61.79±13.34	58.52±16.45
MCV (fL)[2]	367.48±26.90[a]	333.21±23.64[abc]	358.22±42.64[ab]	276.74±42.03[bc]	265.22±58.46[c]
MCHC (g/dL)	22.93±1.06	22.05±4.27	21.90±2.69	22.18±1.28	21.84±1.86[b]

Values are expressed as the mean ± standard deviation, n=5. Means with different letters on the line differ significantly ($p < 0.05$).

*Control, without protease.

MCH: mean corpuscular hemoglobin

MCV: mean cell volume

MCHC: mean corpuscular hemoglobin concentration

[1] Linear effect: $\hat{Y} = 0.8499+0.0008x$, $R^2=0.80$, p=0.0211

[2] Linear effect: $\hat{Y} = 373.3216-0.1772x$, $R^2=0.71$, p=0.0074

Table 5

Biochemical parameters of Nile tilapia (*Oreochromis niloticus*) fed with protease for 49 days

Parameter	Protease (mg/kg)				
	0*	194	316	390	600
Plasma					
Glucose (mg/dL)	56.55±9.06	58.72±5.57	59.76±10.73	63.43±8.14	63.524±9.68
Amino acids (µmol/dL)[1]	782.55±124.44^{a}	773.23±102.46^{a}	743.63±39.54^{a}	612.61±96.21^{ab}	477.35±64.05^{b}
Total proteins (g/dL)	3.38±0.18	3.34±0.39	3.57±0.20	3.60±0.23	3.69±0.23
Albumin (g/dL)	1.22±0.18	1.11±0.22	1.23±0.12	1.27±0.15	1.19±0.19
Globulin (g/dL)	2.22±0.23	2.20±0.26	2.19±0.25	2.14±0.18	2.35±0.20
Liver					
Total proteins (mg/g)[2]	19.23±1.40^{b}	20.40±2.00^{b}	19.74±2.34^{b}	22.15±1.36^{ab}	24.28±3.22^{a}
Glycogen (mg/g)[3]	25.29±8.69^{a}	22.29±5.40^{ab}	20.86±4.98^{ab}	19.93±4.82^{ab}	13.76±5.20^{b}
ALT (U/g)	526.68±30.14	503.71±67.22	492.10±63.30	463.48±31.42	534.83±58.44
AST (U/g)[4]	450.85±14.49^{b}	460.79±20.26^{ab}	529.01±71.93^{a}	526.40±32.32^{a}	522.87±34.67^{a}
Muscle					
Total proteins (mg/g)[5]	14.64±0.48^{d}	14.92±0.40^{cd}	16.26±1.34^{c}	17.76±0.80^{b}	20.58±0.93^{a}
Glycogen (mg/g)	1.83±0.41	1.67±0.28	1.63±0.29	1.65±0.33	1.59±0.36
ALT (U/g)	43.25±5.89	34.44±2.22	37.80±9.56	36.63±9.80	37.08±2.64
AST (U/g)[6]	253.88±15.92^{a}	204.06±18.86^{b}	208.89±27.90^{b}	200.38±26.61^{b}	210.25±20.51^{b}

Values are expressed as the mean ± standard deviation, n=6. Means with different letters on the line differ significantly ($p < 0.05$).

* Control, without protease.

ALT: alanine aminotransferase

AST: aspartate aminotransferase

[1] Linear effect: $\hat{Y} = 837.94-0.5335x$, $R^2=0.82$, $p<0.0001$

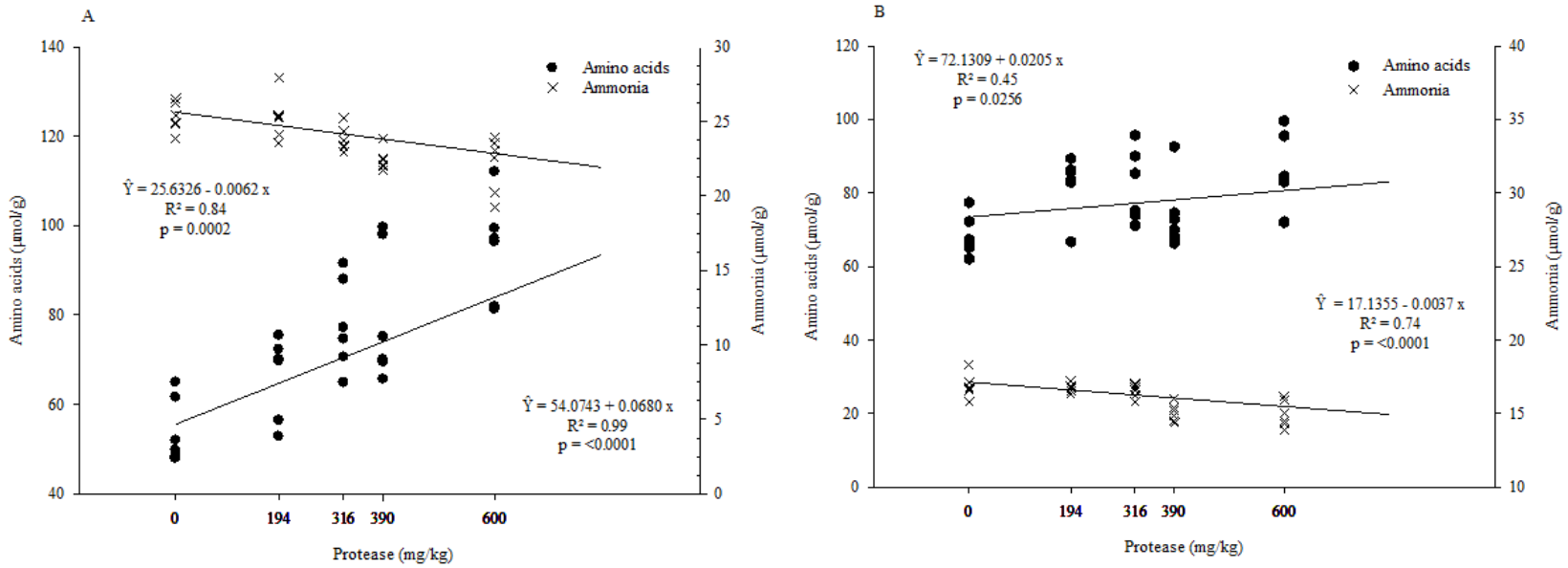
[2] Linear effect: $\hat{Y} = 18.6611+0.0083x$, $R^2=0.81$, $p=0.0028$

[3] Linear effect: $\hat{Y} = 25.9813-0.0185x$, $R^2=0.95$, $p=0.0373$

[4] Linear effect: $\hat{Y} = 454.9929+0.1433x$, $R^2=0.68$, $p=0.0028$

[5] Linear effect: $\hat{Y} = 13.74+0.0103x$, $R^2=0.90$, $p<0.0001$

[6] Quadratic effect: $\hat{Y} = 251.3796-0.2584x-0.0003x^2$, $R^2=0.92$, $p=0.0022$



664
665 Figure 3. Amino acids and ammonia in liver (A) and muscle (B) in Nile tilapia (*Oreochromis niloticus*) fed with protease for 49 days, n=6.

Table 6

Gene expression of growth hormone receptor and insulin like growth factor I of Nile tilapia (*Oreochromis niloticus*) fed with protease for 49 days

Parameter	Protease (mg/kg)				
	0[a]	194	316	390	600
Liver					
GHR[1]	0.57±0.15[b]	0.89±0.39[ab]	1.25±0.52[ab]	1.83±0.46[a]	1.05±0.22[ab]
IGF-I	0.69±0.34	0.79±0.20	0.56±0.17	0.83±0.54	1.12±0.42
Muscle					
GHR	1.24±0.58	0.61±0.03	0.92±0.19	1.31±0.69	1.44±0.60
IGF-I	0.85±0.45	0.68±0.06	0.72±0.22	0.61±0.11	0.64±0.19

Values are expressed as the mean ± standard deviation, n=4. Means with different letters on the line differ significantly ($p < 0.05$).

[a] Control, without protease.

[1] Quadratic effect: $\hat{Y} = 0.45+0.0049x-0.00001x^2$, $R^2=0.67$, p=0.0036

MANUSCRITO III

Protease in Nile tilapia diets: growth, chemical composition, nutrient retention, digestibility, digestive enzymes, intestinal morphometry, and blood-biochemical responses*

*Manuscrito será submetido ao periódico Aquaculture.

Protease in Nile tilapia diets: growth, chemical composition, nutrient retention, digestibility, digestive enzymes, intestinal morphometry, and blood-biochemical responses

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Abstract:

A study was conducted to evaluate the effects of protease included in diets containing soybean meal (SM) for Nile tilapia on performance, digestibility, digestive enzyme, intestinal morphometry, and biochemical responses. Three diets (36% crude protein) were formulated with SM replacing fish waste meal and two levels of exogenous protease (0 or 440 mg/kg) were evaluated, called: SM1 (SM1-0 and SM1-440), SM3 (SM3-0 and SM3-440) and SM6 (SM6-0 and SM6-440). Fish (11.60±0.32 g) were randomly allocated to 18 tanks (220 L) for 49 days. The results demonstrated greater weight gain and better feed conversion rate in fish fed the SM1 group (SM1-0 and SM1-440 diets) compared to the SM6 group. The SM6-440 diet had a higher somatic digestive index compared to the SM1-440 diet. There was an increase in the hepatosomatic index and intestinal quotient of fish that received the SM6-440 diet in relation to SM6-0. There was greater apparent protein digestibility with the SM6-440 diet than with the SM6-0 diet. On the other hand, diets SM1-440 and SM3-440 presented lower apparent lipid digestibility compared to diets SM1-0 and SM3-0. The SM3-0 diet resulted in greater activity of digestive enzymes (trypsin and chymotrypsin) than the SM1-0 and SM6-0 diets. There was a reduction in trypsin activity in diets SM1-440 and SM3-440 compared to diets without protease (SM1-0 and SM3-0). In the SM6 group there was an increase in the height and width of the villi and in the number of goblet cells in relation to the SM1 group. The SM3 group resulted in higher concentrations of total protein and ammonia in the liver than other groups. In general, there was a decrease in the amino acid content and the activity of aminotransferase enzymes with the inclusion of protease in the diets. It is concluded that the SM3-0 diet has better nutritional value and the inclusion of protease (SM3-440) allows increasing the inclusion of SM in the diet without a negative effect on the growth and metabolism of Nile tilapia.

Keywords: aquaculture, histology, protein metabolism, *Oreochromis niloticus*.

1. Introduction

The global production of Nile tilapia (*Oreochromis niloticus*) is significant, representing 9% of global fish production and was classified as the third most produced species in 2020 (Food and Agriculture Organization- FAO, 2022). In Brazil, this species registered an increase of 5,28% in 2023 compared to 2022, reaching 65.30% of the national production of farmed fish, in 2023 (Associação Brasileira da Piscicultura- PEIXE BR, 2024).

The growth of fish farming drives the sustainable use of animal by-products from slaughterhouses to replace fish meal (FM), reducing production costs. The use of by-products such as meat and bone meal, fish waste, offal, and feathers present the challenge of avoiding excess proteins, considering the associated environmental problems (Abdel-Tawwab et al., 2010; Cardoso et al., 2021; Huan et al., 2018; Lee et al., 2020; Maryam et al., 2022).

Soybean meal (SM), a plant protein source widely used in feed due to its availability and good amino acid (AA) profile, faces challenges related to antinutritional factors. These factors can negatively impact the activity of endogenous digestive enzymes, resulting in poor protein digestion and absorption (Kumar et al., 2016; Yaghoubi et al., 2016). Furthermore, the presence of undigested feed can introduce harmful substances into the fish body presenting potential health risks (Abdel-Tawwab et al., 2010; Wu et al., 2020).

The inclusion of protease in tilapia diets has been the subject of several studies, which have highlighted benefits such as better growth and nutrient utilization (Hassaan et al., 2019; Magouz et al., 2022; Soltan et al., 2023), reduced dependence on fishmeal and optimization of pelletizing and extrusion processes (Li et al., 2015; 2018), as well as improvements in digestibility (Huan et al., 2018; Ragaa et al., 2017) and water quality (Saleh et al., 2021). Other studies have investigated the immunological, histological, and genetic effects of plant-based diets (Abd Elnabi et al., 2020; Adeoye et al., 2016; Hassaan et al., 2020; Wu et al., 2020).

Given the possibility of increasing the availability of proteins of plant origin through the inclusion of exogenous protease, it is necessary to carry out studies that explore by-products of animal origin and evaluate the increase in plant protein. Although extruded diets are widely used in fish feeding (Li et al., 2015; Shi et al., 2016), the high temperature during the process requires the inclusion of the enzyme after extrusion (Daslgaard et al., 2012; 2016; Schneider et al., *in submission*). Therefore, it is essential to consider not only the composition of diets but also feed production processes when evaluating the effectiveness of exogenous protease inclusion.

Based on our previous research results, this study was carried out to evaluate the effect of diets containing increasing levels of soybean meal, exogenous protease and their interaction on growth, feed utilization, chemical composition, nutrient retention and deposition, digestibility, enzymes digestive activity, intestinal morphometry and blood and biochemical responses of Nile tilapia.

2. Materials and methods

The present study was approved by the Ethics Committee on the Use of Animals (Authorization nº 4351200721) at the Universidade Federal de Santa Maria (UFSM).

2.1. Experimental diets

In total, six isonitrogenous (36% crude protein) and isocaloric (18 MJ/kg gross energy) experimental diets were formulated to meet the nutritional needs of Nile tilapia (*O. niloticus*) according to Abdel-Tawwab et al. (2010). The inclusion of soybean meal (SM) was based on protein content, totaling three experimental groups (SM1; SM3; SM6). Protease was included in the diets at levels of 0 and 440 mg/kg (Schneider et al., *in submission*). The treatments were named as follows: SM1-0; SM1-440; SM3-0; SM3-440; SM6-0; SM6-440 (Table 1). The

enzyme is a serine protease (EC 3.4.21) and contains 75,000 PROT units/g (supplied by DSM Nutritional Products Ltd., Mszczonow, Poland). One PROT unit is defined as the amount of enzyme that releases 1 µmol of p-nitroaniline from 1 µM of substrate (Suc-Ala-Ala-Pro-Phe p-nitroaniline) per minute at pH 9.0 and temperature 37ºC.

The ground ingredients were mixed with oils and distilled water. The mixture was extruded in a single-screw extruder (Inbramaq, model Labor PQ 30, São Paulo, Brazil). Each diet contained 1 g/kg of chromium oxide (Cr_2O_3) as a biological marker for digestibility measurements. Then, the pellets (2.0 mm diameter) were dried in an oven with forced air recirculation at 55°C for 24 h. Subsequently, the enzyme was added by spraying with a manual pump and the diets were stored at -20°C throughout the experimental period. The composition of the diets and analysis of enzyme recovery are presented in Table 1.

2.2. Experimental conditions and fish feeding management

The experiment was carried out at the Fish Farming Laboratory, Universidade Federal de Santa Maria, Campus Palmeira das Missões, Rio Grande do Sul, Brazil. Nile tilapia, *O. niloticus*, were obtained from AquaViva Commercial Fish Farm, Victor Graeff, Brazil. The fish were acclimated to the experimental conditions for two weeks. After that, 360 fish (all male juvenile, average initial weight of 11.60±0.32 g) were randomly stocked in 18 tanks (220 L) with a density of 20 fish per tank (1 g/L) with three replicate tanks for each of the six treatments. For 49 days, the fish were manually fed until satiety (identified through the fish's feeding behavior), three times a day (08:00 am, 1:30 and 6:00 pm), and feed intake was measured at the end of each day. Feces were collected daily by siphoning (10:00 am and 4:00 pm). The fecal material was dried in an air recirculation oven (55ºC, 72 h), grounded and stored (-20ºC) to determine digestibility.

Water temperature and oxygen were recorded daily with YSI ProODO™ technology (YSI Inc. Ohio, USA), pH (YSI™, pH100), total alkalinity (by neutralization titration), total hardness (by complexation titration), unionized ammonia and nitrite (Alfakit™ colorimetric *kit*) were measured weekly. During the experiment, water quality parameters was evaluated: temperature: 25.94±1.00°C; dissolved oxygen: 5.71±0.57 mg/L; pH: 7.01±0.45; unionized ammonia: 0.03±0.02 mg NH_3/L; nitrite: 0.46±0.09 mg NO_2^-/L; alkalinity: 34.62±10.34 mg $CaCO_3$/L and hardness: 85.64±4.45 mg $CaCO_3$/L. Quality was maintained within normal limits for tilapia growth (De León-Ramírez et al., 2022; El-Sayed, 2006).

2.3. Chemical analysis

Chemical composition was performed on experimental diets and fish samples (initial: 10 fish) and final (three fish per tank) according to the methods of the of Official Analytical Chemists (AOAC, 1995). Dry matter was determined by oven drying at 105°C until constant weight was reached (method 934.01). The mineral matter content was estimated after incineration of the samples in a muffle furnace at 550°C for 4 h (method 968.08). Crude protein (N × 6.25) was determined by the micro-Kjeldahl method after acid digestion (method 954.01). The crude lipid was determined by the chloroform and methanol extraction method (Bligh and Dyer, 1959). The neutral detergent fiber content of the experimental diets was determined by the method described by Van Soest (1967). Nitrogen-free extracts (NFE) and gross energy were calculated by Bureau et al. (2002) and Brett (1973), respectively.

2.4. Performance and feed utilization indexes

All fish were anesthetized with an eugenol solution (50 mg/L) (Vidal *et al*., 2008). Fish performance was determined based on: initial (IW) and final (FW) weight, total length (TL), weight gain (WG), relative weight gain (RWG), survival (S), feed efficiency (FE), protein

efficiency ratio (PER), apparent feed conversion ratio (FCR) and feed intake (FI) according to the following equations:

Weight gain (g): WG = (FW − IW);

Relative weight gain (g): RWG = [(WG/IW) × 100];

Survival (%): S = (final fish number×100)/initial fish number);

Feed efficiency (g): FE = [WG/FI (dry matter)];

Protein efficiency ratio: PER = [WG/protein intake (dry matter)];

Apparent feed conversion ratio: FCR = (FI/WG).

2.5. Somatic indexes

At the end of the experiment, nine fish per treatment (three fish per repetition) were euthanized by spinal cord sectioning to determine the weight: carcass, digestive tract, liver and visceral fat, according to the following equations:

Condition factor: $CF = (FW/TL^3) \times 100$;

Carcass yield (%): CY = [(gutted weight with head and gills)/(FW) × 100];

Hepatosomatic index (%): HSI = [(liver weight/FW) × 100];

Digestive somatic index (%): DSI = [(digestive tract weight/FW) × 100];

Celomic fat index (%): CFI = [(abdominal cavity fat weight/FW) × 100];

Intestinal quotient: IQ = (digestive tract length/TL).

2.6. Nutrient retention and deposition

From the analysis of the initial and final chemical composition of the whole fish, the retention and body deposition of nutrients were calculated using the formulas:

Protein retention (g): PR = 100 × [(FW×(%)CPBf)-(IW×(%)CPBi]/(protein intake)];

Lipid retention (g): LR = 100 × [(FW×(%)CLBf)-(IW×(%)CLBi]/(lipid intake)];

Body protein deposition (g): BPD = [FW×(%)CPBf/100)]-[IW×(%)CPBi/100)];

Body lipid deposition (g): BLD = [FW×(%)CLBf/100)]-[IW×(%)CLBi/100)].

Where: initial (IW) and final (FW) body weight, initial (CPBi) and final (CPBf) body protein, and initial (CLBi) and final (CLBf) body lipid.

2.7. Apparent digestibility coefficients

Nutrient digestibility was estimated by analyzing the chemical composition in feed and fecal samples (AOAC, 1995; Bligh and Dyer, 1959). Chromium oxide was quantified using 1.5-diphenylcarbazide (Bremer Neto et al., 2005). The following equation determined the apparent digestibility coefficient (ADC) of the experimental diets as proposed by the Nutritional Requirement of Fish - NRC (1993):

ADC = [100-(Cr_2O_3% in diet/Cr_2O_3% in feces) × (% nutrients in feces/% nutrients in diet)] × 100.

2.8. Digestive enzymes activity

After seven weeks of experiment, tissues were collected from nine fish per treatment (three fish per replicate) after fasting for 12 hours to determine the activity of pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1). The digestive tracts of each fish sampled was separated into the stomach and total intestine and weighed. Each section was homogenized in buffer solution (10 mM phosphate/20 mM Tris, pH 7.5, in 50% [v/v] glycerol). After centrifugation (1200 g/10 min), the supernatant was used as the enzyme source. Acid protease activity was measured in the stomach homogenate using casein as a substrate according to the methods described by Hidalgo et al. (1999), and one unit of enzyme was defined as the amount of enzyme needed to catalyze the formation of 1 µg of tyrosine/min/mg protein.

To evaluate trypsin activity, α-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) (Sigma-Aldrich™, St. Louis, MO, USA) was used as substrate. The intestinal extracts were incubated for 2 min in a 2 mL Tris/$CaCl_2$ buffer solution, pH 8.1. To evaluate chymotrypsin activity, the substrate used was benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma-Aldrich™, St. Louis, MO, USA). The extracts were incubated for 2 min in a 2 mL Tris/CaCl2 buffer solution, pH 7.8. One unit of enzyme was defined as the amount of enzyme needed to hydrolyze 1 μmol of substrate (TAME or BTEE)/min/mg protein. Trypsin and chymotrypsin activity readings were taken in a spectrophotometer at 247 and 256 nm, respectively, according to Hummel (1959). All samples were assayed in duplicate, and the readings were normalized using blank solutions. The protein content of the crude extracts was determined following Lowry et al. (1951), with bovine serum albumin as the standard.

2.9. Intestinal morphometry

At the end of the experiment, six fish were randomly selected from each treatment (two fish per repetition) for morphometric analysis. The samples were placed in a 10% formaldehyde solution for 24 h and then preserved in 70% alcohol. After routine histological processing, samples were embedded in paraffin and blocks were cut using a microtome (Thermo Scientific™, HM 355S, Germany). Transverse sections (5 μm) were stained with periodic acid-Schiff and hematoxylin-eosin. The histological slides were observed using the Axio Scope model A1 microscope (ZEISS™, Germany), photomicrographed with an Axiocam camera and analyzed using the ImageJ software (1.54d). In the anterior intestine, the height, width and number of goblet cells of the villi were evaluated (64 villi per treatment, in total 384 villi were measured).

2.10. Blood sampling, erythrocyte, and biochemical analysis

At the end of the experiment, blood samples were collected by caudal puncture from nine fish per treatment (three fish per repetition) using a syringe containing heparin. Each sample was divided into two portions: the first portion was used for erythrocyte analysis. The blood parameters evaluated were: number of erythrocytes, hematocrit and hemoglobin. Subsequently, the blood indexes were calculated: mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean concentration of corpuscular hemoglobin (MCHC). The second portion was used to obtain plasma, with centrifugation at 1,248×*g*, 4ºC, for 10 min. In plasma, the concentration of total proteins, albumin, and glucose was analyzed using commercial Labtest™ colorimetric *kits*. Serum globulin was calculated by subtracting albumin values from total protein. The quantification of AA was determined using the methodology of Spies (1957).

Liver and muscle collection (nine fish per treatment) was carried out at the end of the experiment. Tissue samples were heated in 6 M potassium hydroxide solution at 100°C for 20 min to analyze total protein (Bradford, 1976) and glycogen (Krisman, 1962) content. Homogenization in 20 mM potassium phosphate buffer solution, pH 7.5, centrifuged at 1,248×g for 10 min was performed to evaluate the quantification of AA (Spies, 1957) and the activity of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a colorimetric *kit* (Reitman and Frankel, 1957). To determine the total ammonia content (Verdouw et al., 1978) homogenization was carried out in trichloroacetic acid buffer solution (10%).

2.11. Statistical Analysis

All data were analyzed using R™ software, version 4.3.0 and graphs were created using SigmaPlot™ software version 14.5. The data were subjected to Shapiro-Wilk normality analysis. Two-way ANOVA was used to analyze the individual effects of diets and exogenous protease

and the interaction between them. The Tukey test was applied if there was a significant difference ($p < 0.05$) and the results are presented as means with mean standard error (SEM).

3. Results

3.1 Growth performance

SM1 group presented greater final weight, WG, weight gain relative, feed efficiency, and protein efficiency ratio compared to the SM6 group. No differences were found in survival and feed intake between the different diets. There was no effect of the inclusion of protease and significant interaction on the performance and use of the feed by Nile tilapia (Table 2).

3.2 Somatic indexes

There were significant differences among diets and interaction on the digestive somatic index. The DSI was significantly lower in fish fed the SM1-440 diet compared to the SM6-440 diet. The inclusion of protease significantly increased the HSI and intestinal quotient. There were no effects of diets, exogenous protease, and significant interaction on FC, CY, and celomic fat index (Table 3).

3.3. Chemical composition and nutrients retention and deposition

No differences were observed for diets, exogenous protease or interaction on dry matter, mineral matter, CP, CL, retention and deposition of proteins and lipids (Table 4).

3.4. Nutrient digestibility

The apparent digestibility coefficient of crude protein was higher in fish from the SM6 group compared to the other groups, and higher with the inclusion of 440 mg/kg of protease (SM6-440) compared to the SM6-0 diet (Figure 1A). There was an effect of the interaction on

the ADC of crude protein. The highest ADC of crude lipid was in fish fed the SM6-440 diet compared to the SM1-440 and SM3-440 diets. There was a lower ADC of crude lipid in fish that received diets SM1-440 and SM3-440 compared to diets SM1-0 and SM3-0 (Figure 1B).

3.5. Digestive enzymes

In the SM1-440 diets, higher pepsin activity was observed compared to the SM6-440 diets but did not differ from the SM3-440 diet. There was an effect of the interaction on the activity of pepsin and trypsin. In the SM3-0 diet there was greater trypsin and chymotrypsin activity in the intestine compared to the SM1-0 and SM6-0 diets. Fish that were fed the SM6-440 diet had higher trypsin activity compared to the SM1-440 diet but did not differ from the SM3-440 diet. There was a decrease in trypsin activity in fish that received diets SM1-440 and SM3-440 compared to diets SM1-0 and SM3-0 (Figure 2).

3.6. Intestinal morphometry

The experimental diets influenced intestinal morphometry (Table 5). The SM1-0 and SM3-0 diets had lower villus height and width and number of goblet cells compared to the SM6-0 diet. The inclusion of protease resulted in greater villus height compared to groups without exogenous protease (Figure 3). There was a significant interaction in the height and width of the villi. In general, the SM6-0 and SM6-440 diets had greater villus height and width and number of goblet cells compared to the SM1-0 and SM1-440 diets.

3.7. Erythrocyte parameters

There was no difference among diets, exogenous protease and their interaction on hematological parameters (erythrocytes, hematocrit, and hemoglobin) and calculated blood indices (MCV, HCM, and CHCM) (Table 6).

3.8. Biochemical parameters in plasma and tissues

In general, the SM1 group had lower concentrations of AA, total proteins, and globulin compared to the SM6 group. The SM3-440 diet had higher concentration of albumin and globulin compared to the SM1-440 diet but did not differ from the SM6-440 diet (Table 7).

In the liver, the diets influenced the concentration of total proteins, ammonia, and glycogen in tilapia, especially in the SM3 group, which generally presented higher concentrations of these elements compared to the other groups. An effect of the interaction on the concentration of total proteins and glycogen was observed. The inclusion of protease reduced concentrations of AA and ALT and AST enzymatic activities compared to group without protease (Figure 4).

There was a significant interaction in the concentration of total proteins in muscle tissue, higher concentrations in the SM1-0 and SM3-440 diets compared to the others. In general, the SM1 group had a lower concentration of AA and a higher ammonia content compared to the SM6 group. The concentration of AA was higher in the SM1-440 and SM3-440 diets compared to the SM6-440 diet. There was a significant effect of the inclusion of the protease and a significant interaction on the concentration of glycogen, a higher concentration was observed in the SM1-440 and SM3-0 diets compared to the others diets (Table 8).

4. Discussion

4.1. Growth performance

Final weight, TL, WG, and WGR indicated that different dietary compositions directly influenced performance, with a positive relationship between growth and specific ingredients used, as evidenced in the SM1 group with greater inclusion of protein by-products. While substitution with animal waste is considered a sustainable practice (Huan et al., 2018; Maryam et al., 2022), the presence of non-digestible components limits inclusion. As observed in this study, the SM6 group (SM6-0 and SM6-440 diets) with greater SM inclusion had lower

performance. Possibly, the presence of antinutritional factors, lower activity of digestive enzymes, and imbalance of AA of diets are factors that can result in lower growth (Kumar et al., 2016; Yaghoubi et al., 2018, 2016; Zheng et al., 2022).

The review by Cowieson and Roos (2016) highlights that exogenous protease, in addition to its proteolytic function, can influence nutrient digestibility, affecting endogenous secretion and intestinal health. However, studies indicate that the inclusion of protease in Nile tilapia and Rainbow trout (*Oncorhynchus mykiss*) diets, even at different SM concentrations, did not improve performance parameters (Adeoye et al., 2016; Yigit et al., 2018). In contrast, the inclusion of protease can alleviate the negative effects of including plant-based protein sources in diets (Hassaan et al., 2019; Saleh et al., 2021; Soltan et al., 2023). However, it is important to note that exogenous protease efficacy is related to the nutritional composition of diets, as demonstrated in a study with fishmeal-free diets for tilapia (*O. niloticus*×*O. aureus*) (Li et al., 2018). Therefore, caution is recommended when increasing the inclusion of SM and exogenous protease in tilapia diets, as this may have adverse effects on the performance, digestive enzyme activity and intestinal health of omnivorous fish (Lin and Luo, 2011; Liu et al., 2018; Wu et al., 2020).

Although there was no significant variation in feed intake (FI) between groups of tilapias fed with different diets, the SM1 group, which received a diet containing exogenous enzyme (SM1-440), had better results in the protein efficiency ratio (PER). This means that, even consuming a similar amount of feed, tilapia fed the SM1-440 diet were able to utilize dietary nutrients more efficiently than those fed the SM6-440 diet. This greater efficiency in the use of nutrients by the SM1 group was reflected in a better performance in terms of apparent feed conversion ratio (FCR), indicating a more adequate use of nutrients compared to other previous studies that reported higher FCR for tilapia fed with different types of diets (Li et al., 2018; Hassaan et al., 2019; Saleh et al., 2021). Under the specific conditions of this study, the

inclusion of protease did not demonstrate an effect on the performance parameters evaluated, indicating a limited adaptation with nutritional efficiency.

4.2. Somatic indexes

In the present study, there was an increase in DSI in the SM6-440 diet compared to the SM1-440 diet, suggesting an adaptation of the digestive tract to improve nutrient digestibility. The enzyme influenced the liver health and intestinal status of fish, with higher rates of HSI observed in diets with 440 mg/kg of protease when compared to diets without the exogenous enzyme. There was a higher IQ in fish that received the SM6-440 diet compared to the diet without protease (SM6-0), indicating a possible increase in intestinal development. The enzyme ability to hydrolyze proteins can lead to liver overload and increased intestinal development (Feng et al., 2023; Wu et al., 2020). Furthermore, protease has beneficial effects on nutrient digestibility (Cowieson and Roos, 2016; Lee et al., 2020), and the combination with other enzymes has been shown to improve the digestive tract of Nile tilapia (Soltan et al., 2023). These results, associated with digestibility values and intestinal morphometry, suggest that the inclusion of protease had a positive effect in diets with higher amounts of soybean meal (SM6-440 diet).

4.3. Nutrient digestibility

The protein quality of by-products in diets depends on their composition and availability of AA, which is why determining digestibility has been an important assessment tool (Lee et al., 2020; Shomorin et al., 2019). The inclusion of protease has improved nutrient digestibility in diets with low or free fishmeal content for African catfish (*Clarias gariepinus*) (Kemigabo et al., 2019); tilapia (*O. niloticus* × *O. aureus*) (Li et al., 2018, 2015) and Gibel carp (*Carassius auratus* G.) (Shi et al., 2016). In this study, the SM6 diet without protease (SM6-0) had an improvement in the ADC

of crude protein compared to the other diets (SM1-0 and SM3-0). Furthermore, the inclusion of protease (SM6-440 diet) had a positive effect on the ADC of crude protein compared to the SM6-0 diet, indicating a beneficial role of this enzyme in optimizing protein digestion.

In relation to the ADC of crude lipid, the SM6 diet with protease (SM6-440) presented the highest results. This suggests a possible synergistic response between the specific composition of the diet and the action of the protease in improving lipid digestibility (Cowieson and Roos, 2016). However, in this study, it is surprising that the inclusion of protease had a reducing effect on ADC of crude lipid in the SM1-440 and SM3-440 diets. Liu et al. (2018) had that the digestibility of protein and lipid was significantly improved but the inclusion of 600 mg/kg of protease resulted in a lower ADC of crude lipid compared to the control diet, suggesting negative feedback between the synthesis and secretion of digestive enzymes. In the present study, the lower digestibility of lipids may be due to the lower activity of digestive trypsin in the intestine when including 440 mg/kg of protease since lipase activity was not evaluated in the digestive tract.

4.4. Digestive enzymes

Fish growth is related to digestive capacity and improved nutrient utilization, which can be reflected by the activities of digestive enzymes. In the present study, pepsin activity decreased in fish with inclusion of vegetable protein, soybean meal (SM). Exogenous proteases can complement endogenous proteolytic activity to increase the availability of AA and peptides to be better absorbed, reducing nutritional barriers and improving feed utilization rate and promoting growth (Cowieson and Roos, 2016; Shi et al., 2016; Soltan et al., 2023). In addition to the growth-promoting action, the exogenous protease was significantly affected by the compound ingredients of the diet, in this case, the SM1 group (SM1-0 and SM1-440 diets) containing more by-products of animal origin, resulted in increased activity in the stomach

through the action of the endogenous enzyme pepsin in comparison in the SM6 group (SM6-0 and SM6-440 diets).

In the present study, diets influenced the activity of digestive enzymes in the intestine, the SM3-0 diet had greater trypsin and chymotrypsin activity than the SM1-0 and SM6-0 diets. This discovery may be related to the adaptability of digestive physiology by the species (Lin and Luo, 2011). Diets SM1-0 and SM3-0 significantly influenced the higher trypsin activity of fish compared to diets SM1-440 and SM3-440. In contrast, the protease improved the activity of digestive enzymes in the intestine in diets with greater inclusion of plant protein (Feng et al., 2023; Hassaan et al., 2020, 2019). This result associated with performance results may be related to the limited action of the protease on the diets.

4.5. Intestinal morphometry

In this study, the improvement in nutrient digestibility by the protease can be attributed to the better intestinal structure (Hassaan et al., 2020; Liu et al., 2018), evidenced by less development of villi and number of goblet cells in the SM1-0 and SM3-0 diets in compared to the SM6-0 diet. Villus development may be affected by the presence of indigestible materials in animal by-products (Huan et al., 2018; Maryam et al., 2022). While the greater development may be related to the gastrointestinal adaptation of tilapia to plant proteins (Lin and Luo, 2011), this effect is attenuated by the inclusion of protease, as observed in the SM6-440 diet. Exogenous protease has also been shown to improve intestinal morphology and villus development, increasing nutrient digestion and absorption capacity, as evidenced by previous studies (Abd Elnabi et al., 2020; Feng et al., 2023; Liu et al., 2018; Saleh et al., 2021; Wu et al., 2020).

399 *4.6. Erythrocyte parameters*

400 Blood indexes are important for monitoring fish health. In a previous study, high
401 inclusion of plant protein resulted in indicators of anemia (Yaghoubi et al., 2016) but in this
402 study, there was no influence of diets or exogenous protease on erythrocyte parameters.
403 Previous studies in Nile tilapia and Gibel carp (*C. auratus* G.) did not observe significant
404 differences with protease in diets (Adeoye et al., 2016; Liu et al., 2018; Saleh et al., 2021). Diets
405 with lower quality ingredients containing protease did not negatively affect fish health (Hassaan
406 et al., 2019; Shi et al., 2016; Soltan et al., 2023). Blood parameters are within the reference
407 ranges for Nile tilapia, indicating good health of the fish under the experimental conditions
408 (Dal'Bó et al., 2015).

409

410 *4.7. Plasma biochemical parameters*

411 In the present study, plasma glucose was not affected by experimental conditions,
412 similar to previous studies with Gibel carp (*C. auratus* G.) (Liu et al., 2018) and Nile tilapia
413 (Saleh et al., 2021; Schneider et al., *in submission*). In general, the SM6 group (SM6-0 and
414 SM6-440 diets) had a higher concentration of total proteins and AA than the SM1 group (SM1-
415 0 and SM1-440 diets), with no difference concerning the SM3 group (SM3-0 and SM3-440
416 diets). This suggests an increase in anabolic processes due to the increase in the availability of
417 total proteins and AA (Hassaan et al., 2019; Soltan et al., 2023). Plasma albumin, responsible
418 for the storage and transport of AA, correlates with their availability (Whicher and Spence,
419 1987). Previous studies have shown that elevated albumin levels are associated with stress
420 conditions and immunostimulatory responses in different diets (Goda et al., 2019; Hassaan et
421 al., 2019; Soltan et al., 2023). In the present study, the SM3-440 diet presented a higher
422 concentration of albumin and globulin compared to the SM1-440 diet, with no difference to the

SM6-440 diet. This may indicate a stronger innate response in fish, due to the greater availability of plasma proteins and AA.

4.8. Biochemical parameters in tissues

The experimental diets in this study significantly affected the levels of total proteins, ammonia, and glycogen in Nile tilapia liver, with a significant interaction in total proteins and glycogen. Increased levels of proteins and AA are associated with protein metabolism and can negatively influence growth if there is an imbalance in the diet (Zheng et al., 2022). In general, the SM3 group (SM3-0 and SM3-440 diets) presented higher concentrations of total proteins and ammonia than the other groups. This result may suggest an imbalance of AA in the diet, affecting protein synthesis and growth. Previous studies have indicated that diets deficient in essential AA can cause liver damage due to reduced activity of aminotransferase enzymes (Kumar et al., 2016; Yaghoubi et al., 2018). Fish fed diets containing protease had lower concentration of AA and activity of aminotransferase enzymes (ALT and AST) compared to those fed diets without protease. This suggests a possible limited adaptation to the efficiency of AA utilization, highlighting the importance of ensuring an adequate supply of AA in the diet to optimize fish growth and performance.

In the present study, the total concentration of proteins in the muscle was influenced exclusively by their interaction. Notably, diets SM1-440 and SM3-0 exhibited higher total proteins concentration compared to other diets. These results associated with the PER indicate improved use of AA, which may explain the greater growth of fish. In general, the SM3 group (SM3-0 and SM3-440 diets) exhibited a higher concentration of AA and glycogen and a lower ammonia content compared to the SM1 group (SM1-0 and SM1-440 diets). Possibly these results associated with the lack of effect on the activity of aminotransferase enzymes (ALT and AST) in the muscle and the increased activity of digestive enzymes (trypsin and chymotrypsin)

in the intestine, suggest a better nutritional and health status with the SM3-440 diet. New studies on the effect of including exogenous enzymes in diets on tissue biochemical parameters need to be carried out to better explain the mechanism of action in fish, since their effects vary with the composition of the diet, as observed in studies with Gibel carp (*C. auratus*) (Shi et al., 2016) and tilapia (*O. niloticus x O. aureus*) (Li et al., 2015).

5. Conclusions

It is concluded that the SM1-0 diet resulted in better performance and protein efficiency rate and the SM6-0 diet had a positive effect on increasing digestive indices and nutrient utilization. The SM3-0 diet presented better nutritional and physiological balance and with the inclusion of protease (SM3-440) it allows the inclusion of soybean meal in the diet to be increased without a negative effect on the growth and metabolism of Nile tilapia. Furthermore, the exogenous enzyme had positive effects on digestive enzyme activity and intestinal morphology.

Declaration of Interest

The authors declare that they have no competing interests.

Contribution

Thamara Luísa Staudt Schneider: project administration; formal analysis, writing.

Roberta Cristina Scheid: methodology, formal analysis.

Giulia Guedes Gianello: methodology, formal analysis.

Alexandra Pretto: methodology, validation.

Nilce Coelho Peixoto: methodology, formal analysis, supervision.

Rômulo Batista Rodrigues: methodology, validation, supervision.

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Acknowledgments

The authors are grateful to DSM™; Puro Trato™ Animal Nutrition (Santo Augusto, RS, Brazil), Mais Frango™ Ltda. (Miraguaí, RS, Brazil) and Mig-PLUS™ 437 Agroindustrial Ltda. (Casca, RS, Brazil) for the donation of ingredients. Financial support for this work was provided by the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Financing Code 001 and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [312849/2020-6].

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679 Table 1

680 Composition of the experimental diets of Nile tilapia

681

Ingredient (%)	Diet					
	SM1		SM3		SM6	
	SM1-0	SM1-440	SM3-0	SM3-440	SM6-0	SM6-440
Fish waste meal [66% crude protein (CP)]	15	15	10	10	5	5
Feather meal (72% CP)	8.5	8.5	8.5	8.5	8.5	8.5
Poultry by-product meal (57% CP)	4	4	4	4	4	4
Soybean meal	32	32	40	40	47	47
Ground corn	28	28	28	28	28	28
Wheat bran	7.13	7.13	4.13	4.13	2.13	2.13
Soybean and canola oil (1:1)	2	2	2	2	2	2
Vitamin and mineral[a]	1.5	1.5	1.5	1.5	1.5	1.5
Ascorbic acid	0.05	0.05	0.05	0.05	0.05	0.05
Salt	0.5	0.5	0.5	0.5	0.5	0.5
Methionine	0.2	0.2	0.2	0.2	0.2	0.2
Amido	1	1	1	1	1	1
Antioxidant[b]	0.02	0.02	0.02	0.02	0.02	0.02
Chromium oxide	0.1	0.1	0.1	0.1	0.1	0.1
Analyzed composition (%)						
Dry matter	94.24±0.13	92.03±1.38	93.52±0.09	94.02±0.25	93.27±0.14	93.84±0.24
Mineral matter	8.62±0.06	7.75±0.52	7.43±0.40	7.33±0.34	6.85±0.07	6.67±0.11
Crude protein	36.69±0.61	35.21±0.73	35.87±0.31	36.73±0.07	35.79±0.44	35.38±0.49
Lysine[c]	1.97	1.97	1.98	1.98	1.98	1.98
Methionine[c]	0.74	0.74	0.70	0.70	0.66	0.66
Crude lipid	7.34±0.34	6.71±0.16	7.12±0.19	7.56±0.34	6.76±0.25	6.54±0.33
Neutral detergent fiber	14.33±0.92	14.33±0.92	10.42±1.74	10.42±1.74	11.08±0.94	11.08±0.94
NFE[d]	33.02	36.00	39.16	37.96	39.52	40.33
Gross energy (MJ/kg) [e]	17.23	17.15	18.01	18.18	17.91	17.86
Relative activity of protease (prot/kg) [f]	0	27,390	0	25,930	0	21,370

682

683 [a] Composition (kg/product): folic acid 370 mg; pantothenic acid 3,900 mg; biotin 40 mg; cobalt: 58 mg; copper 740 mg; choline 75 g; iron 7,500 mg; inositol 10 g; iodine 43 mg;
684 manganese 7,800 mg; niacin 8,800 mg; selenium 38 mg; vitamin A 780,000 IU; vitamin B1 1,400 mg; vitamin B12 1,900 mcg; vitamin B2 1,450 mg; vitamin B6 1,400 mg; vitamin
685 C 19.5 g; vitamin D3 160,000 IU; vitamin E 14,800 IU; vitamin K3 475 mg; zinc 1,400 mg; [b] Butylated hydroxytoluene; [c] Calculated based on the aminogram of the ingredients; [d]
686 Nitrogen-free extracts = [100 − (crude protein + lipids + mineral matter + crude content)]; [e] Gross energy calculated using gross calorific values of 23.63; 39.52; and 17.15 kJ/g for
687 protein, fat and carbohydrate (Brett, 1973); [f] Analyzed by BIOPRACT® GmbH (Berlin. Germany).

Table 2

Growth and feed utilization of Nile tilapia fed experimental diets for 49 days

Group	Protease (mg/kg)	FW (g)	TL (cm)	WG (g)	WGR (%)	S (%)	FCR	FE (g)	PER (%)	FI (g/fish)
SM1	0	91.28^{A}	16.71^{A}	79.64^{A}	684.06^{A}	98	1.06^{B}	1.03^{A}	2.64	82.35
	440	93.02^{A}	16.45	81.48^{A}	706.23^{A}	93	1.03^{B}	1.06^{A}	2.78^{A}	83.42
SM3	0	86.53^{AB}	16.11^{AB}	75.14^{AB}	659.32^{AB}	93	1.10^{AB}	0.97^{AB}	2.53	82.90
	440	88.27^{AB}	16.00	76.64^{AB}	659.12^{B}	98	1.13^{AB}	1.01^{AB}	2.59^{AB}	80.56
SM6	0	83.65^{B}	15.76^{B}	72.19^{B}	629.87^{B}	98	1.18^{A}	0.92^{B}	2.41	83.77
	440	85.30^{B}	16.02	73.37^{B}	613.94^{C}	97	1.16^{A}	0.93^{B}	2.46^{B}	84.28
Mean standard error		1.80	0.22	1.69	10.79	2.04	0.04	0.02	0.06	2.75
Two-way ANOVA										
Diet		0.0036	0.0198	0.0019	<0.001	0.6513	0.0188	0.0015	0.0030	0.7129
Protease		0.2659	0.8402	0.2914	0.8234	0.7446	0.8350	0.1939	0.1263	0.9113
Diet + protease		0.9997	0.4993	0.9797	0.2471	0.0816	0.7107	0.7505	0.7622	0.8043

Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate differences without and with protease within the same diet, according to Tukey's test ($p<0.05$), n=3. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg). FW: final weight; TL: total length; WG: weight gain; WGR: weight gain relative; S: survival; FCR: apparent feed conversion ratio; FE: feed efficiency; PER: protein efficiency ratio; FI: feed intake.

Table 3

Condition factor, digestive indexes, and carcass yield of Nile tilapia fed experimental diets for 49 days

Group	Protease (mg/kg)	CF	CY (%)	HSI (%)	DSI (%)	CFI (%)	IQ
SM1	0	1.96	84.95	1.84^{b}	6.56	1.89	6.04
	440	2.10	85.29	2.19^{a}	5.49^{B}	1.78	6.32
SM3	0	2.07	84.84	2.00^{b}	5.61	1.72	6.24
	440	2.16	85.45	2.25^{a}	6.66^{AB}	1.35	6.36
SM6	0	2.14	84.82	1.72^{b}	6.69	1.61	6.15^{b}
	440	2.08	84.35	2.12^{a}	7.58^{A}	1.24	7.11^{a}
Mean standard error		0.10	0.92	0.08	0.35	0.22	0.20
Two-way ANOVA							
Diet		0.6264	0.7884	0.0600	0.0055	0.1697	0.0717
Protease		0.5192	0.8363	<0.001	0.3181	0.1168	0.0079
Diet + protease		0.6210	0.8344	0.6016	0.0075	0.7959	0.0877

Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate differences without and with protease within the same diet, according to Tukey's test ($p<0.05$), n=6. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg). CF: condition factor; CY: carcass yield; HSI: hepatosomatic index; DSI: digestive somatic index; CFI: celomic fat index; IQ: intestinal quotient.

Table 4

Chemical composition, retention, and deposition of nutrients in Nile tilapia fed experimental diets for 49 days

Group	Protease (mg/kg)	Chemical composition				Retention		Body deposition	
		Dry matter (%)	Mineral matter (%)	Crude protein (%)	Crude lipid (%)	Protein (%)	Lipid (%)	Protein (g)	Lipid (g)
SM1	0	30.55	4.21	15.87	4.10	42.56	48.77	12.82	2.94
	440	30.08	3.94	14.06	4.79	38.95	65.80	11.46	3.67
SM3	0	30.19	3.76	14.29	4.74	36.13	56.23	10.73	3.33
	440	30.12	3.59	14.73	4.25	38.41	48.54	11.36	2.96
SM6	0	30.64	3.62	16.38	3.78	40.24	41.93	12.07	2.36
	440	30.28	3.69	15.93	3.99	40.05	46.87	11.86	2.58
Mean standard error		0.19	0.20	0.73	0.34	2.59	5.48	0.72	0.31
Two-way ANOVA									
Diet		0.7122	0.0985	0.1067	0.1727	0.3860	0.0990	0.3018	0.0544
Protease		0.4249	0.4641	0.3313	0.6226	0.8155	0.3082	0.6065	0.4518
Diet + protease		0.3308	0.6802	0.3360	0.2502	0.5386	0.1194	0.4165	0.2478

Significance level ($p<0.05$) according to Tukey's test, n=3. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg).

Table 5

Intestinal morphometry and number of goblet cells of Nile tilapia fed experimental diets for 49 days

Group	Protease (mg/kg)	Intestine		
		Villus height (µm)	Villus width (µm)	Goblet cells number (units/villus)
SM1	0	185.35^{Cb}	79.18^{B}	83^{B}
	440	274.25^{Ba}	77.54^{B}	78
SM3	0	210.46^{Bb}	77.27^{Bb}	76^{B}
	440	269.76^{Ba}	87.02^{Aa}	90
SM6	0	254.54^{Ab}	91.15^{Aa}	102^{A}
	440	295.81^{Aa}	80.24^{ABb}	95
Mean standard error		5.08	2.84	5.22
Two-way ANOVA				
Diet		<0.001	0.0369	0.0020
Protease		<0.001	0.6867	0.9115
Diet + protease		<0.001	0.0015	0.1010

Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate differences without and with protease within the same diet, according to Tukey's test ($p<0.05$), n=44. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg).

Table 6

Erythrocyte parameters of Nile tilapia fed experimental diets for 49 days

Group	Protease (mg/kg)	Erythrocytes (x10^6/µL)	Hematocrit (%)	Hemoglobin (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
SM1	0	1.16	34.30	7.69	306.96	66.42	22.47
	440	0.97	35.95	7.84	385.20	77.10	21.59
SM3	0	1.14	37.30	8.08	350.09	78.72	21.92
	440	1.03	34.74	8.02	338.41	81.17	22.28
SM6	0	1.02	34.83	7.19	338.78	76.20	21.93
	440	1.10	33.96	7.48	292.09	56.92	21.23
Mean standard error		0.10	1.00	0.32	25.91	5.66	1.01
Two-way ANOVA							
Diet		0.9597	0.2764	0.1267	0.5016	0.1137	0.8549
Protease		0.3357	0.4829	0.6446	0.7680	0.6892	0.6298
Diet + protease		0.3998	0.1353	0.8721	0.0873	0.0792	0.8120

Significance level ($p<0.05$) according to Tukey's test, n=6. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg). MCV: mean cell volume = (hematocrit×10/number of erythrocytes); MCH: mean corpuscular hemoglobin = (hemoglobin×10/number of erythrocytes); MCHC: mean corpuscular hemoglobin concentration = (hemoglobin×100/hematocrit).

722 Table 7

723 Biochemical parameters in the plasma of Nile tilapia fed experimental diets for 49 days

724

Group	Protease (mg/kg)	Glucose (mg/dL)	Total proteins (g/dL)	Amino acids (µmol/dL)	Albumin (g/dL)	Globulin (g/dL)
SM1	0	59.97	3.55^{B}	1944.66^{B}	1.18	2.28^{B}
	440	50.85	3.41^{B}	1908.65^{B}	1.05^{B}	2.27^{B}
SM3	0	59.24	3.70^{AB}	2027.64^{B}	1.32	2.46^{AB}
	440	54.62	3.90^{A}	2407.34^{AB}	1.36^{A}	2.63^{A}
SM6	0	49.92	4.06^{A}	2819.65^{A}	1.18	2.71^{A}
	440	53.24	4.02^{A}	2454.31^{A}	1.19^{AB}	2.51^{AB}
Mean standard error		4.97	0.11	143.85	0.07	0.07
Two-way ANOVA						
Diet		0.5515	<0.001	<0.001	0.0168	<0.001
Protease		0.4037	0.9433	0.9517	0.6397	0.7962
Diet + protease		0.4638	0.3144	0.0572	0.4064	0.0525

725

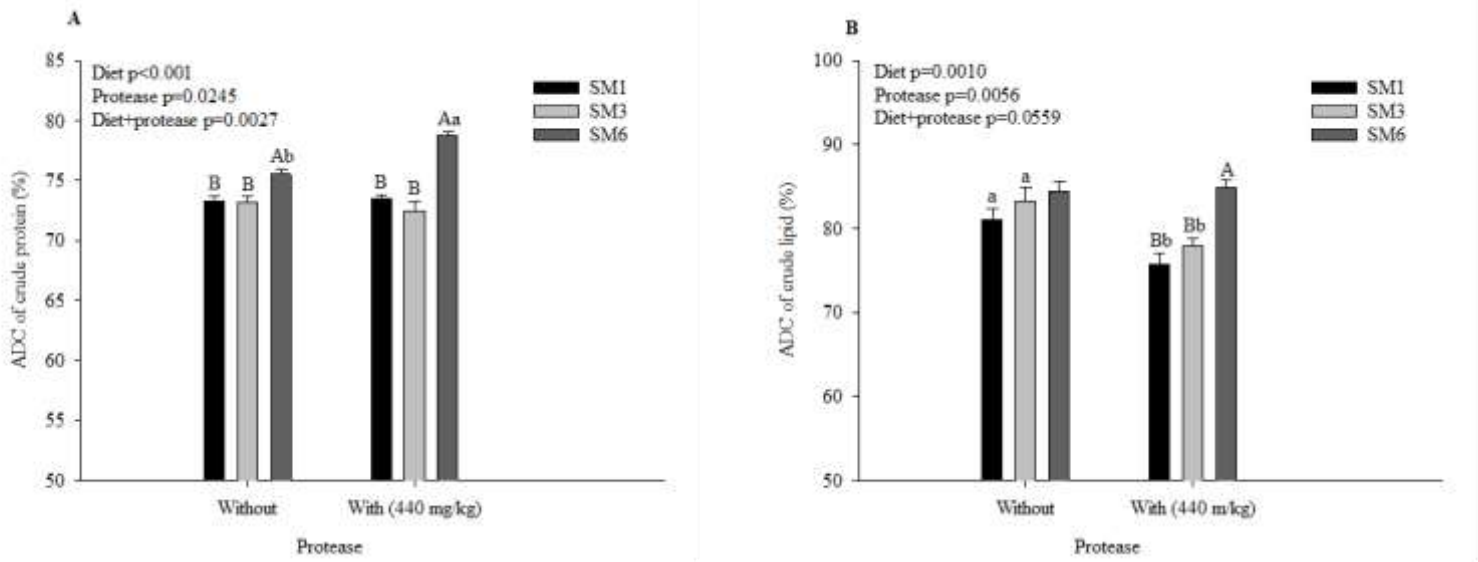
726 Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate differences without and with protease within the same diet, according
727 to Tukey's test ($p<0.05$), n=4. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg).

Table 8

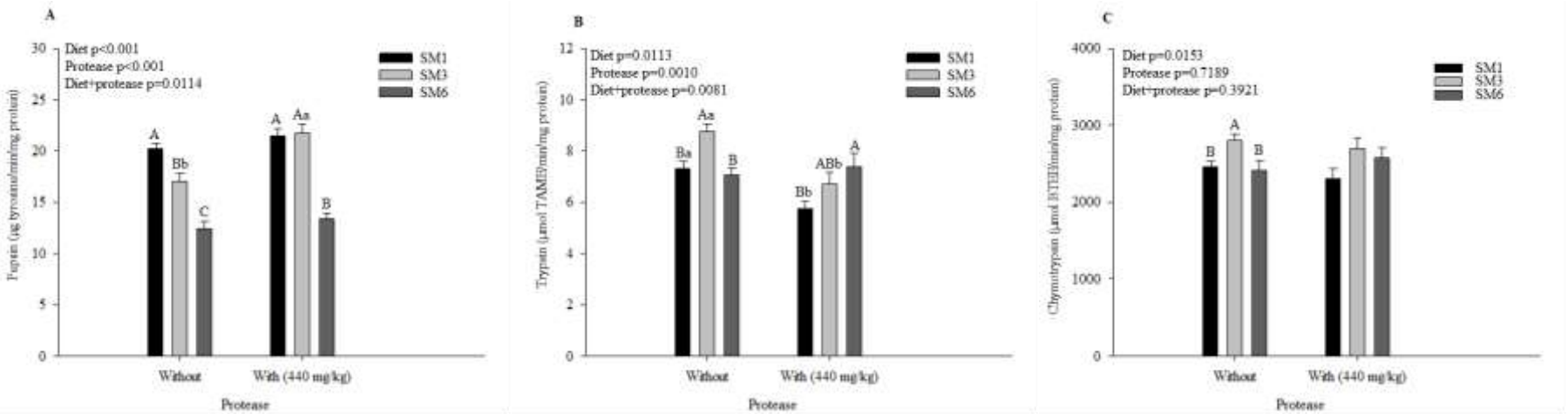
Biochemical parameters of Nile tilapia muscle fed experimental diets for 49 days

Group	Protease (mg/kg)	Total proteins (mg/g)	Amino acids (µmol/g)	Ammonia (µmol/g)	ALT (U/g)	AST (U/g)	Glycogen (mg/g)
SM1	0	29.49	37.86^{Cb}	13.79^{A}	5.06	87.64	24.15^{b}
	440	36.46	53.70^{Ba}	12.64^{A}	6.15	84.60	26.12^{a}
SM3	0	36.93	52.12^{Bb}	11.57^{B}	5.12	74.58	26.81^{a}
	440	27.09	78.21^{Aa}	12.94^{A}	3.51	73.54	21.07^{b}
SM6	0	30.79	73.61^{Aa}	11.47^{B}	4.17	98.05	25.22
	440	29.96	62.74^{Bb}	11.57^{B}	5.03	75.03	24.66
Mean standard error		1.12	3.38	0.29	0.67	10.03	0.56
Two-way ANOVA							
Diet		0.0813	<0.001	<0.001	0.1501	0.3810	0.0868
Protease		0.1898	<0.001	0.6027	0.8421	0.2792	0.0034
Diet + protease		<0.001	<0.001	<0.001	0.1021	0.4890	<0.001

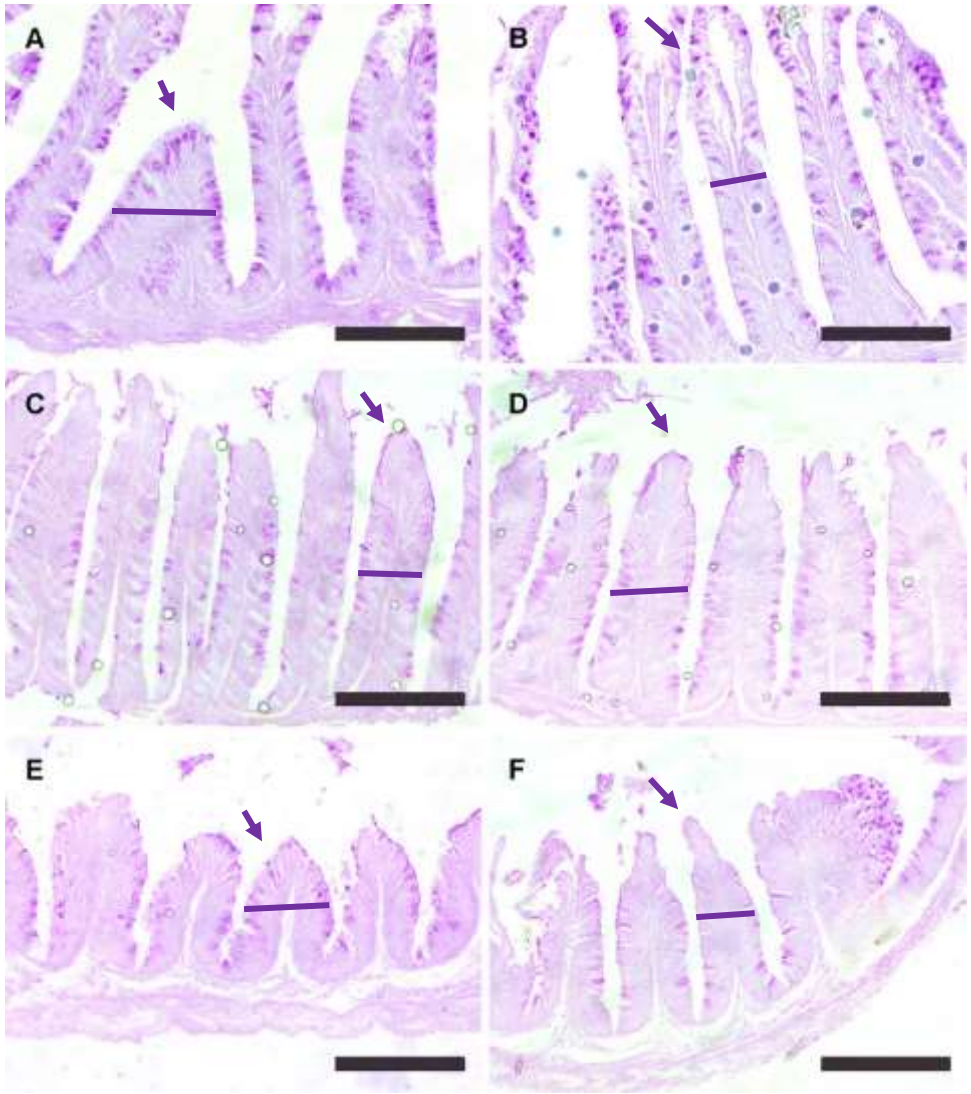
Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate differences without and with protease within the same diet, according to Tukey's test ($p<0.05$), n=6. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg). ALT: alanine aminotransferase; AST: aspartate aminotransferase.



735
736 Figure 1. Apparent digestibility coefficient (ADC) of Nile tilapia fed experimental diets for 49 days. **A.** ADC of crude protein; **B**. ADC of crude lipid. SM: soybean meal, SM1;
737 SM3 and SM6 groups. Without and with protease (440 mg/kg). Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate
738 differences without and with protease within the same diet, according to Tukey's test ($p<0.05$), n=3.



739
740 Figure 2. Digestive enzyme activity of Nile tilapia fed experimental diets for 49 days. **A.** Pepsin; **B**. Trypsin; **C.** Chymotrypsin. SM: soybean meal, SM1; SM3 and SM6 groups.
741 Without and with protease (440 mg/kg). Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate differences without and with
742 protease within the same diet, according to Tukey's test ($p<0.05$), n=7. TAME: α-*p*-tosyl-L-arginine methyl ester hydrochloride. BTEE: benzoyl-L-tyrosine ethyl ester.



743
744 Figure 3. Height (arrow) and width (line) of intestinal villi of Nile tilapia fed experimental diets for 49 days. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with
745 protease (440 mg/kg). **A**. SM1-0; **B**. SM1-440; **C**. SM3-0; **D**. SM3-440; **E**. SM6-0; **F**. SM6-440. Scale bars = 300 μm

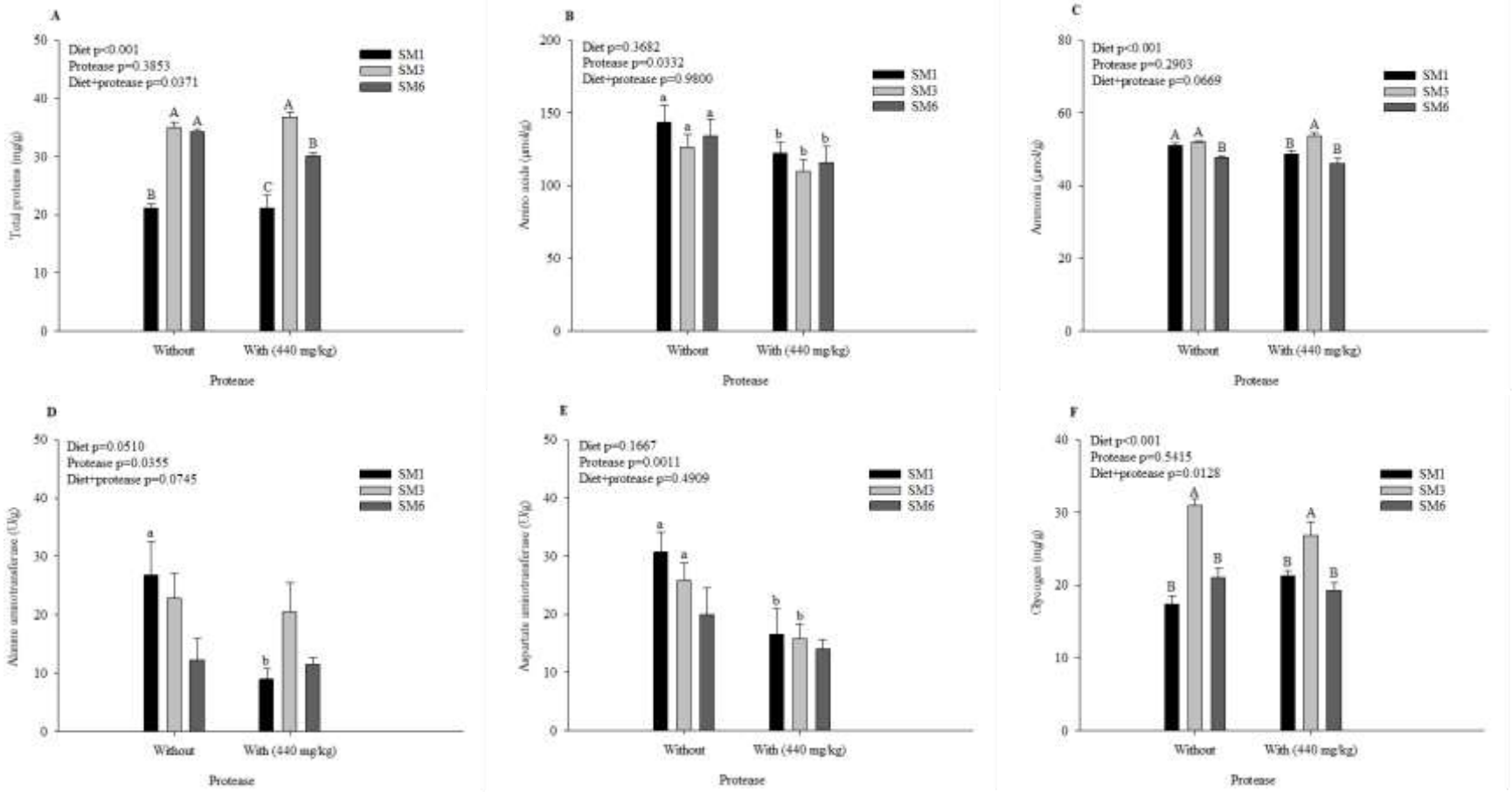


Figure 4. Biochemical parameters of Nile tilapia liver fed with experimental diets for 49 days. **A.** Total proteins; **B**. Amino acids; **C.** Ammonia; **D.** Alanine aminotransferase; **E.** Aspartate aminotransferase; **F.** Glycogen. SM: soybean meal, SM1; SM3 and SM6 groups. Without and with protease (440 mg/kg). Capital letters indicate differences between diets with the same protease inclusion and lowercase letters indicate differences without and with protease within the same diet, according to Tukey's test ($p<0.05$), n=6.

5 DISCUSSÃO GERAL

A busca pelo melhoramento de formulações de rações é uma prática comum na aquicultura, visando aumentar a rentabilidade e promover dietas mais sustentáveis, ao mesmo tempo em que se busca melhorar o desempenho e a saúde dos peixes. O uso de enzimas exógenas na produção animal é uma estratégia estabelecida, com o objetivo de melhorar a produtividade e a eficiência alimentar. Esse método tem sido aplicado devido aos altos custos e à variabilidade na qualidade dos ingredientes. No caso das proteases exógenas, elas têm efeitos positivos sobre o crescimento, digestibilidade de nutrientes, saúde, qualidade da dieta, aspectos econômicos e ambientais na produção de peixes. No entanto, a eficácia das proteases também está ligada à composição das dietas, forma de inclusão e aos hábitos alimentares das espécies. Além disso, novas pesquisas são importantes para compreender melhor o mecanismo de ação de proteases exógenas e garantir uma produção sustentável (Manuscrito I).

A protease em dietas extrusadas formuladas com subprodutos pode potencializar a qualidade do ingrediente inferior (mais acessível) e maximizar os ganhos produtivos. Para isso, é importante a determinação do nível de inclusão da enzima e a avaliação de recuperação de sua atividade. Embora as espécies onívoras, como a tilápia, exijam menos proteína e aminoácidos em comparação as carnívoras, a busca por dietas completas com uso de ingredientes mais sustentáveis em comparação a farinha de peixe, tem se tornado cada vez mais comum. A inclusão da serina protease (Ronozyme™ ProAct), aplicada após o processo de extrusão da dieta, teve um impacto positivo no crescimento, eficiência alimentar, expressão do GHR, parâmetros hematológicos e metabolismo dos peixes (Manuscrito II). Com a presença da protease na dieta, o peso final das tilápias aumentou em média 11% em comparação com a dieta controle. Isso se deve, em grande parte, ao aumento na eficiência proteica, retenção proteica e taxas de deposição, possivelmente devido à melhoria no metabolismo proteico com a enzima. Esses resultados destacam a importância da relação entre o nível de enzima e a composição da dieta. Os peixes alimentados com 390 mg/kg de protease na dieta tiveram melhor desempenho e expressão do hormônio receptor do crescimento (*GHR, sigla em inglês*) no fígado do que com a dieta controle, mas não foram diferentes dos peixes alimentados com as demais dietas (194; 316 e 600 mg/kg). A partir da análise de regressão polinomial foi

identificado o máximo ganho em peso relativo com a inclusão de 440 mg/kg de protease exógena. A ação da protease na metabolização de aminoácidos no fígado, associada à melhoria do metabolismo proteico, resultou em um aumento na expressão do GHR. O efeito compensatório sugerido nos parâmetros hematológicos visou atender ao rápido crescimento das tilápias. A inclusão ideal de protease resultou em melhores condições fisiológicas, sem alterar os índices digestivos e a conversão alimentar.

A crescente inclusão de fontes proteicas vegetais na alimentação de peixes é uma prática comum e bem estabelecida. Isso se deve à disponibilidade dessas fontes e à estabilidade de sua composição. Por outro lado, a inclusão de farinha de subprodutos de origem animal na formulação auxilia no equilíbrio de aminoácidos, diluição de custo e é uma prática sustentável na produção animal em substituição a farinha de peixe. A ação da protease sobre essas fontes pode resultar em uma melhoria significativa na composição bromatológica das dietas, otimizando a liberação de nutrientes essenciais, principalmente, aminoácidos.

No Manuscrito III, foi observado que as dietas com maior proporção de farelo de soja (grupo SM6) apresentaram crescimento e utilização de nutrientes menores em comparação com o grupo SM1. Aparentemente, a qualidade proteica das dietas atenuou a inclusão de protease, uma vez que, não houve alteração significativa nos parâmetros de desempenho das tilápias. A inclusão de protease exógena teve efeitos positivos nos índices digestivos, digestibilidade de proteínas e lipídios, atividade de enzimas digestivas e morfometria intestinal. Isso indica que a enzima teve efeito benéfico sobre a saúde dos órgãos digestivos, principalmente o fígado e o intestino dos peixes alimentados com a dieta SM6-440. Além disso, houve melhoria na digestibilidade aparente da proteína e um aumento na altura e largura das vilosidades intestinais e no número de células caliciformes nos peixes que receberam a dieta SM6-440. Isso pode ser atribuído ao aumento da atividade endógena da tripsina na presença da protease no grupo SM6 (SM6-440) em comparação com a dieta SM1-440.

Os peixes alimentados com dietas contendo protease (SM1-440; SM3-440 e SM6-440) mostraram menor concentração de aminoácidos e atividade das enzimas aminotransferases (ALT e AST) no fígado em comparação aos alimentados com dietas sem protease (SM1; SM3 e SM6). Por outro lado, houve efeito de interação e observou-se no grupo SM3 maiores concentrações de proteína total e amônia no

fígado independentemente da inclusão de protease. Isso sugere uma adaptação metabólica limitada à eficiência da utilização de aminoácidos, destacando a importância do balanceamento adequado desses nutrientes na dieta para otimizar o crescimento dos peixes.

A tilápia do Nilo é uma espécie conhecida por seu rápido crescimento e boa conversão alimentar, conforme evidenciado nos Manuscritos I e II, alcançou um peso médio diário de 1,16 e 1,34 g, respectivamente. Em ambos os estudos, a inclusão de protease não afetou significativamente o consumo alimentar e a conversão alimentar. No entanto, muitos fatores podem ter influenciado esses resultados, destacando a necessidade de mais pesquisas para avaliar os mecanismos de ação e a viabilidade econômica dessa estratégia em dietas para a espécie. Além disso, é importante explorar as avaliações nutrigenômicas, que analisam a expressão de genes relacionados com o metabolismo de nutrientes e a síntese proteica, para fornecer informações para intervenções dietéticas. Contudo, a incorporação da enzima por meio de pulverização sobre as dietas é uma prática aceitável tanto para empresas quanto para piscicultores. Essa abordagem visa consolidar a aplicação de enzimas, como a protease, em rações para peixes na aquicultura nacional, seguindo a tendência observada com outras enzimas em outras produções com animais.

6 CONCLUSÕES

O estudo revela que a inclusão de protease em dieta extrusada contendo farinha de penas como ingrediente alternativo teve efeitos positivos no crescimento e nas respostas hematológicas e bioquímicas de tilápias. Além disso, observou-se uma associação positiva entre o maior crescimento das tilápias e o aumento da expressão no fígado do hormônio receptor de crescimento (*GHR, sigla em inglês*). Os resultados indicam que o nível adequado de protease em dietas para tilápias é de 440 mg/kg (equivalente a 33.000 U/kg), com base no ganho em peso relativo.

Dietas contendo maior inclusão de farelo de soja (grupo SM6) resultou em inferior desempenho e utilização de nutrientes, por outro lado, o grupo SM1 teve melhores resultados. A inclusão de protease não tem efeitos sobre esses parâmetros, mas resulta em efeito positivo sobre índices digestivos, digestibilidade de nutrientes atividade de enzimas digestivas e morfometria intestinal, geralmente, na dieta contendo maior farelo de soja (SM6-440) em comparação a dieta SM1-440. Em resumo, o grupo SM3 demonstra um melhor equilíbrio nutricional e fisiológico, sugerindo que a inclusão de protease (SM3-440) permite a incorporação na formulação de 400 g/kg de farelo de soja sem efeitos negativos no crescimento da tilápia. Não houve efeito da protease exógena sobre os parâmetros hematológicos. Dieta com maior proporção de proteína oriunda de origem animal parece atenuar o efeito da protease sobre os parâmetros avaliados, como o desempenho, atividade das enzimas digestivas e o aproveitamento dos nutrientes.

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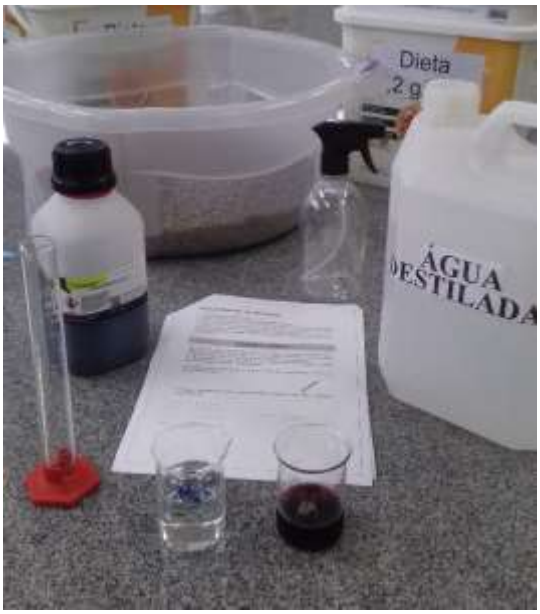
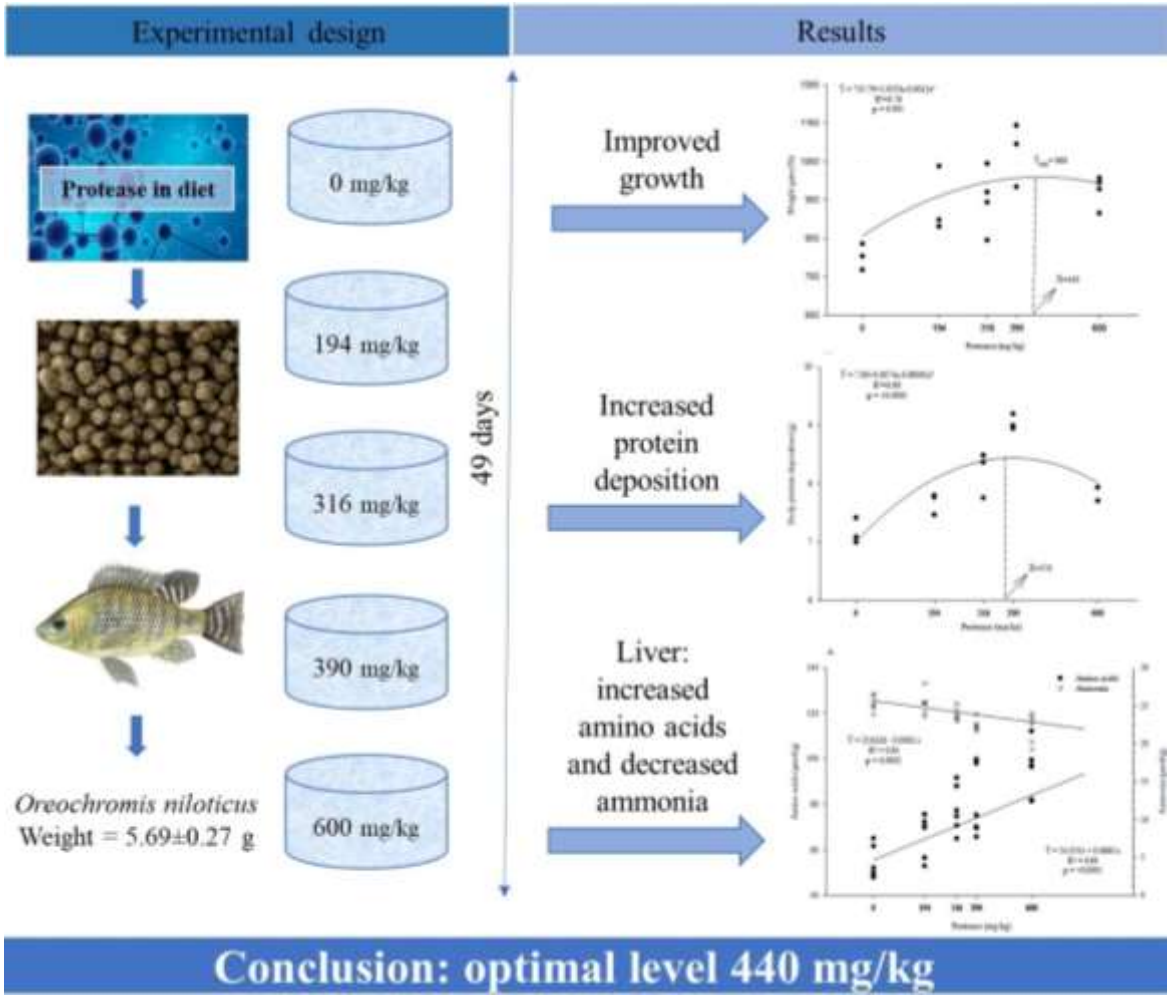
APÊNDICE A – APLICAÇÃO DA PROTEASE EXÓGENA

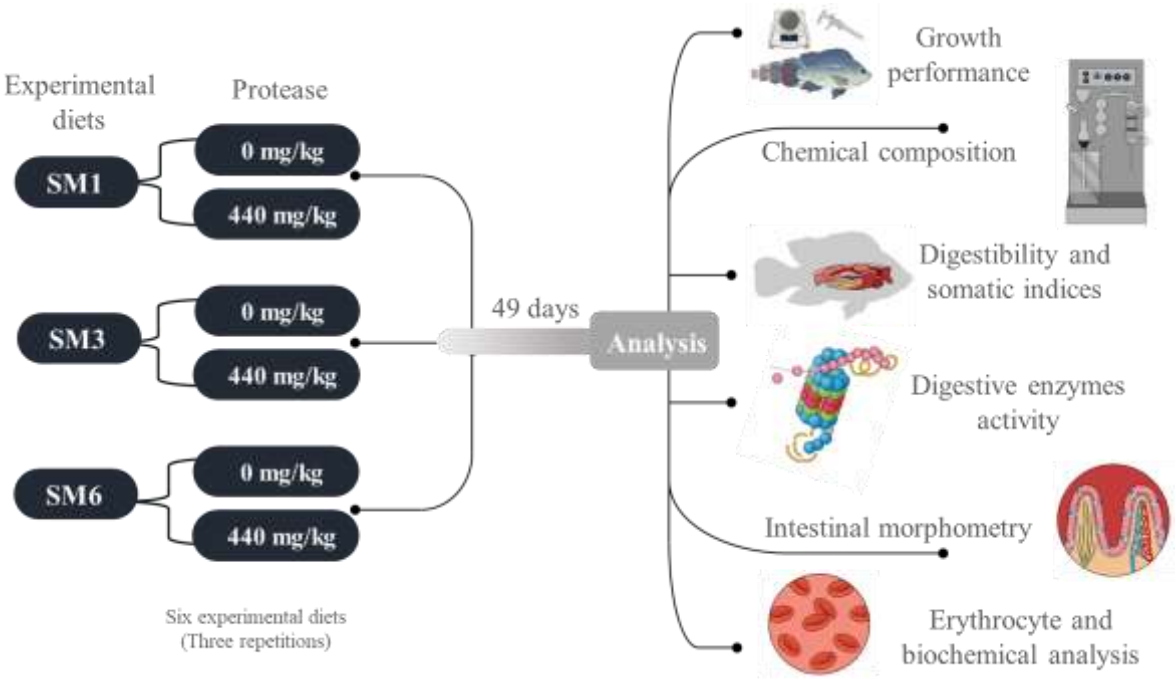


APÊNDICE B - RESUMO GRÁFICO DO MANUSCRITO II

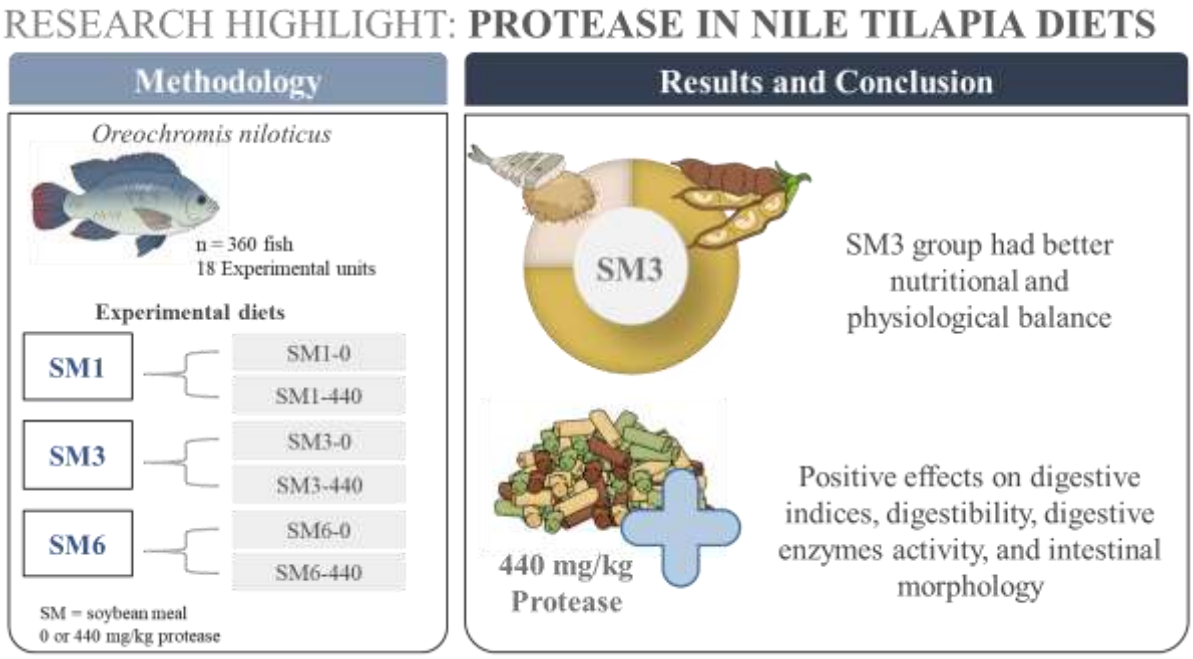


APÊNDICE C – EXEMPLAR TILÁPIA DO NILO - MANUSCRITO III

APÊNDICE D – METODOLOGIA DO MANUSCRITO III



APÊNDICE E – RESUMO GRÁFICO DO MANUSCRITO III



ANEXO A - CERTIFICADO DE APROVAÇÃO DA CEUA - UFSM

Universidade Federal de Santa Maria

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Protease exógena em dietas para tilápia do Nilo (Oreochromis niloticus)", protocolada sob o CEUA nº 4351200721 (ID 003552), sob a responsabilidade de **Rafael Lazzari** *e equipe; Thamara Luísa Staudt Schneider; Luciana F. Christofari; Luiza Hermes; Andressa Pelizari; Mara Rubia Schmidt; Saionara Pereira Xavier; Giulia Guedes Gianello* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 05/10/2021.

We certify that the proposal "Exogenous protease in diets for Nile tilapia (Oreochromis niloticus)", utilizing 750 Fishes (750 males), protocol number CEUA 4351200721 (ID 003552), under the responsibility of **Rafael Lazzari** *and team; Thamara Luísa Staudt Schneider; Luciana F. Christofari; Luiza Hermes; Andressa Pelizari; Mara Rubia Schmidt; Saionara Pereira Xavier; Giulia Guedes Gianello* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 10/05/2021.

ANEXO B - NORMAS DE PERIÓDICOS

Manuscrito I
Pesquisa Agropecuária Gaúcha
http://revistapag.agricultura.rs.gov.br/ojs/index.php/revistapag/diretrizes

Manuscrito II
Animal Feed Science and Technology
https://www.sciencedirect.com/journal/animal-feed-science-and-technology/publish/guide-for-authors

Manuscrito III
Aquaculture
https://www.sciencedirect.com/journal/aquaculture/publish/guide-for-authors