UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
INSTITUTO DE OCEANOGRAFIA – IO
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA - PPGAq

ADITIVOS ALTERNATIVOS, SUSTENTÁVEIS E BIOATIVOS COM POTENCIAL APLICAÇÃO NA AQUACULTURA

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RIO GRANDE, RS

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Tese apresentada ao Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande – FURG, como requisito para a obtenção do título de Doutor.

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ATA DE DEFESA DA 88ª TESE DE DOUTORADO EM AQUICULTURA

No dia seis de novembro de dois mil e vinte e quatro, às nove horas, reuniu-se a Banca Examinadora de Tese de Doutorado em Aquicultura, de **ALAN CARVALHO DE SOUSA ARAÚJO**, orientado pelo Prof. Dr. José Maria Monsserat, composta pelos seguintes membros: Prof. Dr. José Maria Monsserat (Orientador – IO/FURG), Profa. Dra. Vilásia Guimarães Martins (Co-orientadora – EQA/FURG), Prof. Dr. Marcelo Borges Tesser (IO/FURG), Prof. Dra. Andressa Jantzen da Silva Lucas (UFPA), Prof. Dra. Meritaine da Rocha (EQA/FURG) e Prof. Dra. Ligia Uribe Gonçalves (INPA). Título da Tese: "**ADITIVOS ALTERNATIVOS, SUSTENTÁVEIS E BIOATIVOS COM POTENCIAL APLICAÇÃO NA AQUACULTURA**". Dando início à defesa, o Coordenador do PPGAq Prof. Dr. Ricardo Vieira Rodrigues, informou a todos que esta defesa será sob a forma de sigilo e todos os membros da banca e presentes à sessão deverão assinar o **Termo de Sigilo e Confidencialidade**. Em seguida passou a presidência da sessão ao Prof. Dr. José Maria Monsserat, que na qualidade de orientador, passou a palavra para o candidato apresentar a Tese. Após ampla discussão entre os membros da Banca e o candidato, a Banca se reuniu sob a presidência do Coordenador. Durante esse encontro ficou estabelecido que as sugestões dos membros da Banca Examinadora devem ser incorporadas na versão final da Tese, ficando a cargo do Orientador o cumprimento desta decisão. O candidato **ALAN CARVALHO DE SOUSA ARAÚJO** foi considerado **APROVADO**, devendo a versão definitiva da Tese ser entregue a Secretaria do PPGAq, no prazo estabelecido nas Normas Complementares do Programa. Este documento deverá ser mantido em sigilo e sob guarda da Secretaria do PPGAq. Nada mais havendo a tratar, foi lavrada a presente ata, que após lida e aprovada, será assinada pela Banca Examinadora, pelo candidato e pelo Coordenador do PPGAq.

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Prefácio

Se você pode sonhar, você pode fazer.

Walt Disney.

Dedicatória

A meus pais Antônio Alexandre e Amélia, meus irmãos Alex e Andressa pelas palavras de incentivo e força durante essa jornada.

Agradecimentos

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RESUMO GERAL

O presente estudo objetivou avaliar as propriedades bioativas de flocos microbianos do sistema BFT e hidrolisados proteicos de tenébrio gigante *Zophobas morio* para promover uma aquacultura mais sustentável, nesse sentido, foram determinados seus conteúdos nutricionais e suas propriedades biológicas (clorofila a e carotenoides). Para isso, o trabalho foi dividido em três capítulos. No primeiro capítulo foram avaliadas a influência do processo de secagem (estufa e liofilização) nos parâmetros nutricionais e propriedades biológicas das amostras de bioflocos (fotoautotrófico e heterotrófico). Além disso, posteriormente as amostras foram dissolvidas em diferentes solventes (água, etanol e metanol) e avaliadas as suas propriedades bioativas. A liofilização favoreceu um maior conteúdo de proteína na amostra fotoautotrófica, enquanto em estufa, um maior nível proteico foi encontrado na amostra heterotrófica. Em relação ao perfil de aminoácidos (AA) totais (AAT) e hidrofóbicos (AAH), a liofilização contribuiu para valores superiores nos dois tipos de flocos avaliados, tendo a amostra heterotrófica maiores conteúdos de aminoácidos essenciais totais (AAET) em comparação com a fotoautotrófica. Os valores de clorofila a e de carotenoides foram superiores na amostra fotoautotrófica. A capacidade antioxidante (DPPH e ABTS), bem como o conteúdo de polifenóis foram superiores nas amostras processadas e em extrato aquoso, com valores superiores na amostra fotoautotrófica (seca e liofilizada), seguida da heterotrófica em estufa. O segundo capítulo comparou o uso de diferentes proteases no processo de hidrolise enzimática na farinha de tenébrio gigante. Maiores graus de hidrolise (GH) e menores valores de atividade de água (aa) foram obtidos nas amostras com as enzimas Alcalase e Protamex. Em relação as propriedades funcionais, o tempo de hidrolise influenciou negativamente na solubilidade em água nos hidrolisados, contudo, as amostras apresentaram boas capacidades de retenção de óleo, formação de espuma e emulsificação. O conteúdo nutricional foi aumentado nos hidrolisados (68,74 a 57,63%, proteína total) em comparação com a farinha de inseto (47,09%). Maiores níveis de AAET e AAT foram observados no hidrolisado obtido com a Alcalase, enquanto o de AAH foi encontrado com a enzima Protamex. Os hidrolisados obtidos com Alcalase e Protamex apresentaram efeito antimicrobianos frente a microrganismo Gram negativos (*Vibrio coralliyticus, Pseudomonas aeruginos, Acinetobacter baumannii* e *Escherichia coli*). Enquanto o hidrolisado com Flavourzyme não apresentou efeito a nenhum microrganismo avaliado. A atividade antioxidante mostrou que os hidrolisados apresentaram melhor capacidade de

eliminação do radical ABTS. Maior capacidade antioxidante foi observado no hidrolisado obtido com a Protamex, frente ao radical ABTS e o poder redutor férrico (FRAP). Finalmente, no Capítulo 3, foi avaliado o efeito da microencapsulação com diferentes materiais de parede (maltodextrina/MD e goma arábica/GA) nos hidrolisado obtidos com Alcalase e Protamex. Os hidrolisados microencapsualdos apresentaram menor solubilidade em água em comparação aos hidrolisados livres. Os resultados do potencial zeta mostraram que as amostras tendem a aglomeração em meio aquoso. A capacidade antioxidante mostrou o mesmo comportamento dos hidrolisados livres, tendo maior capacidade de eliminação para o radical ABTS. As análises térmica e de grupos funcionais (FTIR) mostraram que os hidrolisados foram encapsulados. E por fim, no ensaio *in vivo* com *C. elegans*, frente a condições de estresse térmico e oxidativo, os nematoides apresentaram crescimento, embora a sobrevivência tenha sido menor em comparação aos tratamentos dos hidrolisados livres. Em geral, a amostra de hidrolisado obtido com a enzima Protamex e microencapsulada com maltodextrina apresentou melhores resultados de crescimento e sobrevivência nos ensaios analisados em comparação aos demais tratamentos.

Palavras-chave: aditivos alternativos, processos biotecnológicos, composição nutricional, propriedades biotivas.

ABSTRACT

The present study aimed to evaluate the bioactive properties of microbial flakes from the BFT system and protein hydrolysates from the giant mealworm, *Zophobas morio*, to promote more sustainable aquaculture. Therefore, their nutritional contents and biological properties were determined. To this end, the work was divided into three chapters. In the first chapter, the influence of the drying process (oven and freeze-drying) on the nutritional parameters and biological properties of biofloc samples was evaluated. Furthermore, the samples were dissolved in different solvents (water, ethanol, and methanol) and their bioactive properties were evaluated. Freeze-drying favored a higher protein content in the photoautotrophic sample, whereas in the oven, a higher protein level was found in the heterotrophic sample. In relation to the profile of total (AAT) and hydrophobic (AAH) amino acids (AA), freeze-drying contributed to higher values in the two types of flakes evaluated, with the heterotrophic sample having higher contents of total essential amino acids (AAET) than the photoautotrophic sample. Chlorophyll a and carotenoid values were higher in the photoautotrophic samples. The antioxidant capacity (DPPH and ABTS) as well as the polyphenol content were higher in the processed samples and in the aqueous extract, with higher values in the photoautotrophic sample (dried and freeze-dried), followed by the heterotrophic sample in the oven. The second chapter compares the use of different proteases in the enzymatic hydrolysis of giant mealworm flour. Higher degrees of hydrolysis (DH) and lower water activity values (aw) were obtained in samples with Alcalase and Protamex enzymes. Regarding functional properties, hydrolysis time had a negative influence on the water solubility of the hydrolysates; however, the samples showed good oil retention, foaming, and emulsification capabilities. The nutritional content was increased in hydrolysates (68.74 to 57.63%, total protein) compared to insect meals (47.09%). Higher levels of TEAA and TAA were observed in the hydrolysate obtained with Alcalase, whereas HAA was detected with the Protamex enzyme. The hydrolysates obtained with Alcalase and Protamex showed antimicrobial effects against Gram-negative microorganisms (*Vibrio coralliyticus, Pseudomonas aeruginos, Acinetobacter baumannii* and *Escherichia coli*). While the hydrolyzate with Flavourzyme had no effect on any microor ganism evaluated. Antioxidant activity showed that the hydrolysates had a better ability to eliminate ABTS radicals. Greater antioxidant capacity was observed in the hydrolysate obtained with Protamex compared to the ABTS radical and ferric reducing power (FRAP). Finally, in Chapter 3, the effect of microencapsulation with different wall materials

(maltodextrin/MD and gum arabic/GA) on the hydrolysates obtained with Alcalase and Protamex was evaluated. The microencapsulated hydrolysates showed lower solubility in water than free hydrolysates. The zeta potential results showed that the samples tended to agglomerate in aqueous medium. The antioxidant capacity showed the same behavior as the free hydrolysates, with a greater ABTS radical elimination capacity. Thermal and functional group analyses (FTIR) showed that the hydrolysates were encapsulated. Finally, in the in vivo test with *C. elegans*, under conditions of thermal and oxidative stress, the nematodes showed good growth, although survival was lower than that of the free hydrolysate treatments. In general, the hydrolysate samples obtained with the Protamex enzyme and microencapsulated with maltodextrin showed better growth and survival results in the analyzed tests than the other treatments.

Keywords: Alternative additivies, biotechnological processes, nutritional composition, bioactive properties

1. INTRODUÇÃO GERAL

1.1. Uso de fontes sustentáveis na aquacultura

O futuro da aquacultura depende de estratégias que melhorem a produtividade e façam uso dos recursos naturais e de aditivos alternativos e mais sustentáveis, garantindo a qualidade nutricional das dietas fornecidas aos animais aquáticos. A substituição de farinha de peixe por fontes alternativas de proteína ambientalmente sustentáveis tem sido um dos alvos da aquicultura nos últimos anos, devido o preço crescente da farinha de peixe e seus efeitos nocivos ao meio ambiente (Han et al. 2018). Contudo, um dos maiores desafios é encontrar aditivos que supram as necessidades nutricionais das espécies garantindo resultados satisfatórios de crescimento e conversão alimentar (FAO, 2020).

Muitas alternativas podem levar a efeitos secundários adversos, como por exemplo de farinhas vegetais que causam distúrbios nutricionais e distúrbios metabólicos (Colombro, 2020) e subprodutos animais (farinha de sangue, carne e ossos e farinha de penas) que podem conter fatores antinutricionais, pepsina indigestível ou altos níveis de cinza bruta. Em outros casos podem gerar desequilíbrio de aminoácidos na dieta devido a altos níveos de prolina e glicina e baixos teores de triptofano e tirosina (Marques et al. 2023). Portanto, novas maneiras de desenvolver fontes sustentáveis de proteínas são necessárias. Nesse contexto, a utilização de ingredientes, como os resíduos sólidos, os chamados flocos microbianos, produzidos no sistema em BFT e os insetos produzidos a partir do uso de resíduos da agroindústria como substrato, vem ganhando destaque, como novos materiais com potencial aplicação na nutrição aquicola (Shao et al. 2017; Basto et al. 2020).

1.2. Aditivos alternativos

1.2.1. Flocos microbianos (bioflocos)

O cultivo de organismos aquáticos em sistema de tecnologia de bioflocos (BFT) é uma abordagem que promove a formação de agregados microbianos em sistemas de cultivo de alta densidade, utilizando aeradores para manter os sólidos em suspensão e fornecer oxigênio aos animais cultivados (Figura 1). Os bioflocos são constituídos de partículas orgânicas suspensas na água formados por vários organismos como invertebrados, fungos, detritos e diversos grupos de bactérias (Krummenauer et al., 2011), e por microalgas, protozoários rotíferos, copepodos, cladoceros e oligoquetas (Avnimelech, 2012) que, ao se agregar formam flocos (Henchion et al. 2017). Os

microrganismos presentes nesse sistema utilizam os resíduos orgânicos presentes no sistema, como amônia e matéria particulada, como fonte de energia e nutrientes. Além disso, os bioflocos contribuem para a biocontrole de patógenos, aumento da estabilidade ambiental do sistema e reciclagem de nutrientes, otimizando a conversão de resíduos em biomassa útil.

Figura 1: Sistema de cultivo em tecnologia de bioflocos (BFT)
Fonte: arquivo pessoal

Essa biomassa microbiana rica em macromoléculas, como proteínas e lipídios, pode ser diretamente consumida pelos organismos aquícolas, como camarões e peixes onívoros, melhorando a eficiência alimentar do sistema e reduzindo a necessidade de ração externa. Crab et al (2010), afirmam que a composição do bioflocos pode chegar até 58% de proteína bruta e 5,4% de lipídeos bruto (em base seca). No entanto, a composição nutricional pode variar dependendo da constituição da microbiota, a qual pela sua vez possui relação com a fonte de carbono usada para produção do biofloco. Dauda et al. (2018) avaliando diferentes relações C/N (10, 15 e 20) na composição proximal do bioflocos heterotrófico observaram variações nos teores de umidade (93.28, 96.10 e 96.70%), proteína bruta (44.27, 38.65 e 32.65%), lipídeos (5.84, 7.35 e 10.78%) e cinzas (4.55, 7.01 e 5.77%) em base úmida. Fleckenstein et al. (2019) relatam que bioflocos com dominância de microalgas (fotoautotróficos) convertem melhor os nutrientes dos resíduos

do sistema para proteína e ácidos graxos que são consumidos pelos animais cultivados, melhorando o seu desempenho de crescimento. Além disso, os bioflocos oferecem um perfil proteico equilibrado, contendo aminoácidos essenciais, ácidos graxos poliinsaturados, assim como outros compostos, como carotenoides, colorofila, minerais e vitamina C (Crab et al., 2012; Ju et al., 2008; Tacon et al., 2002), além de aumentar a resistência a doenças (Ge et al., 2017; Fleckenstein et al., 2019), e que podem melhorar a saúde e o crescimento dos animais

O controle da relação carbono:nirogênio (C:N) é fundamental para favorecer o crescimento de microrganismos heterotróficos que, ao assimilarem nitrogênio e carbono do meio, transformam-no em biomassa proteica (Oliveira et al. 2024). Estudos avaliaram o uso dos bioflocos como ingredientes para alimentação de organismos cultivados. Shao et al. (2017) substituíram farinha de pescado (FP) por farinha de bioflocos (FB) em rações para *Penaus vannamei* e constataram que a substituição de 15% de FB não difere do tratamento com FP, no que diz a respeito do crescimento do animal. Dantas et al. (2016) também avaliando a substituição de FP por FB para pós-larvas de *P. vannamei*, observaram que até 20% de substituição pode melhorar o crescimento do animal. Bauer et al. (2012) avaliaram dietas contendo uma mistura de farinha de bioflocos e concentrado proteico de soja em dietas para *P. vannamei*, onde não verificaram diferença significativa no desempenho zootécnico dos animais, em comparação com a dieta controle.

1.2.2. Proteína de inseto

1.2.2.1. Tenébrio gigante (*Zophobas morio*)

O tenébrio gigante (*Zophobas morio*) é uma das espécies que compõem a grande família dos besouros de Tenebrionidae, a qual também inclui o *Tenebrio molitor* e *Alphitobius diaperinus* (Rumbus e Athanassion, 2019). O tenébrio gigante trata-se de um animal onívoro, ou seja, se alimenta tanto de matéria vegetal quanto animal e costuma se desenvolver melhor em climas tropicais (Jabir et al. 2021). Possui quatro estágios básicos no decorrer da vida, passando pela fase de ovo, larva, pupa e por fim, após a metamorfose, o inseto adulto (Figura 2).

Os ovos do tenébrio gigante apresentam 1,7 mm de comprimento e 0,7 mm de largura, são levemente amarelados e eclodem 8 dias após a postura. A fêmea adulta pode pôr até 2200 ovos durante a vida. O estado larval é o que apresenta maior variabilidade de tempo, pois depende de diversos fatores que podem agir prolongando ou acelerando essa condição, como temperatura, alimentação, e as condições de isolamento ou

aglomeração das larvas. A fase de pupa se caracteriza pela transformação da larva do besouro adulto e tem duração de 13 a 15 dias. Nessa fase, os insetos ficam imóveis em formato de “c” respondendo a poucos estímulos. Após a fase de pupa, o inseto já se encontra na condição de adulto e pode viver por até 6 meses (Rumbus & Athanassion, 2019).

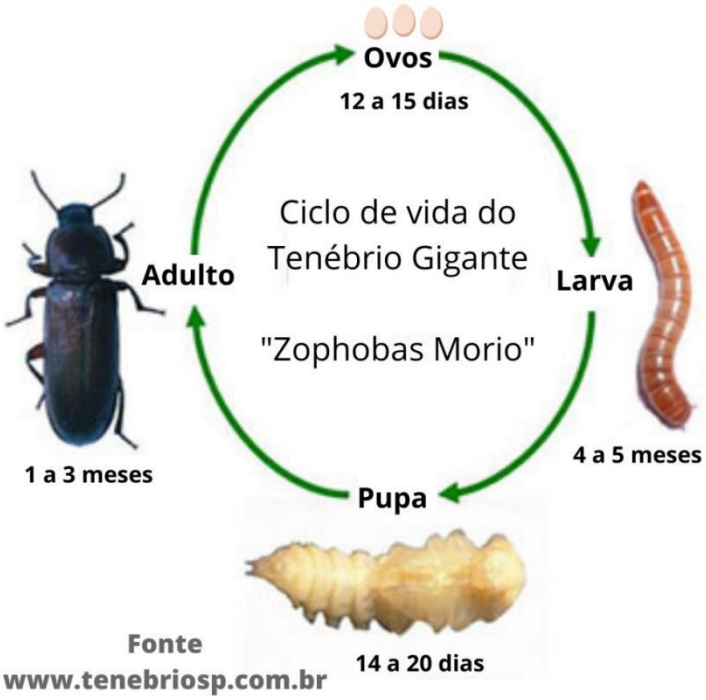

Figura 2: Metamorfose do Tenébrio gigante. Fonte: www.tenebriosp.com.br

O tenébrio gigante (*Zophobas morio*) é uma espécie promissora devido a facilidade na produção e no seu conteúdo nutricional satisfatório para inserção em alimentos da aquicultura. O ciclo de vida curto e a alta taxa de reprodução dos insetos, aliado à sua capacidade de serem criados em substratos de baixo custo, como resíduos orgânicos, fazem da farinha de insetos uma alternativa ambientalmente sustentável. Alguns estudos avaliaram a composição nutricional dessa espécie. Araújo et al. (2018) em estudo com larvas dessa espécie observaram valores em base seca de proteína, lipídeos, cinzas e carboidratos de 46,80%, 43,64%, 8,17% e 1,39%, respectivamente. No entanto esses valores se alteram durante os estágios de vida, como mostrado por Kulma et al. (2020), avaliando larvas de *Z. morio* com 60, 90 e 120 dias. Os autores constataram

que os valores de proteína (47,4, 47,0 e 48,1, respectivamente) e lipídeos (36,0, 31,3 e 34,0, respectivamente) variam com o tempo de desenvolvimento.

O consumo de insetos tem sido promovido como uma fonte alternativa de proteína sustentável para animais. Além disso, os insetos se destacam devido à sua alta eficiência na conversão de substratos orgânicos em proteína de alta qualidade para uso em dietas na aquacultura (Henry et al., 2015; Van Huis, 2013). Insetos possuem um perfil nutricional rico, com teor proteico variando de 35% a 65%, dependendo da espécie e do substrato utilizado para sua criação. Além disso, são fontes ricas em ácidos graxos, minerais e vitaminas, e têm um perfil de aminoácidos semelhante ao da farinha de peixe, o que os torna altamente adequados para rações aquícolas (Chia et al., 2020; Basto et al., 2020; Tubien et al., 2019).

Comparando com a atividade pecuária convencional, os insetos tem baixo impacto ambiental e ótima taxa de conversão alimentar (FCR) (Flores et al., 2020). O FCR é uma medida de eficiência de um animal para converter massa de ração em massa corporal aumentada. Estudos mostraram que a FCR para espécie de grilo é cerca de 1,7 sendo menor que o valor para outros fontes proteicas (Caparros et al., 2014; Dobermann et al. 2017). A união Europeia já aprovou a inclusão de proteínas de insetos em alimentos para formulações de ração para aquacultura (IPIFF, 2018). Assim, o uso de insetos surge como uma das alternativas alimentares em dietas na aquacultura, pois podem ser facilmente obtidos na natureza possibilitando a sua produção durante o ano, aportando valores nutricionais adequados que irão suprir as exigências dos organismos cultivados.

1.3. Processos biotecnológicos

1.3.1. Processos de secagem

A maioria dos produtos alimentícios são materiais altamente perecíveis devido ao seu alto teor de água. No caso dos bioflocos e insetos, a presença de água os torna suscetíveis a deterioração, como crescimento microbiano e reações química catalisadas por enzimas, causando mudanças rápidas em sua composição (Belwal et al. 2021). Nesse sentido, remover e/ou reduzir o teor de água desses materiais é uma medida para estabilizar tais biomateriais para o desenvolvimento posterior de produtos alimentícios. A secagem é um processo importante para redução o teor de umidade podendo ser aplicado em diversos tipos de materiais e é realizado através de uma matriz caracterizada por porosidade variável e envolve transição de fase (Jin et al. 2018; Orphanides & Gekas,

2016). O calor é aplicado diretamente ou indiretamente no material, provocando o movimento da água tanto da superfície quando das camadas internas (Belwal et al. 2021).

As técnicas de secagem podem afetar o aspecto final do produto, incluindo o conteúdo de compostos bioativos, propriedades físico-químicas e organolépticas, afetando suas atividades biologias e prazo de validade. Na área da aquacultura muitas pesquisas vêm sendo realizadas com aditivos alternativos e suplementos funcionais, para se obter um produto final de alta qualidade. Portanto, é fundamental adotar as melhores condições de secagem para manter a qualidade dos produtos secos e proteger os compostos bioativos das matrizes alimentares. Além disso, o processo de secagem tem papel importante economicamente em relação aos custos envolvidos na produção (Menon et al. 2020).

Algumas técnicas são usadas para a secagem desses materiais. O método convencional em estufa, é baseada na evaporação da água em temperaturas próximas ao ponto de ebulição pela aplicação de calor em um grau variável (Ele et al. 2016) e tem aplicações para inativação de enzimas, redução de microrganismos e redução da atividade de água na matriz alimentícia (Ozcan et al. 2021). No entanto, muitas vezes não é capaz de atender aos requisitos de uma alta qualidade para o produto final, pois mesmo reduzindo a umidade, condições em alta temperatura e tempo prolongado podem levar a degradação de compostos bioativos sensíveis e afetar a qualidade do produto, como volume, cor e forma (Lin et al. 1998). Já a liofilização é uma técnica de desidratação baseada na sublimação de um produto congelado (Ratti, 2001; Marques et al, 2005) e é considerado o melhor método para secagem de produtos de alto valor que sãos sensíveis ao tratamento térmico, pois é realizado em temperaturas muito baixas e produz materiais com alta porosidade, qualidade nutricional inalterada, sabor, aroma, e cor superiores (Krokid et al. 1998). Portanto, durante o processo de secagem, os efeitos das técnicas de secagem na qualidade física, química e nutricional do produto seco devem ser considerados para obter o produto da mais alta qualidade.

1.3.2. Produção de hidrolisados proteicos de insetos

Devido ao alto conteúdo proteico, os insetos podem ser melhor aproveitados através da produção de hidrolisados de proteína por hidrólise enzimática. Os hidrolisados proteicos são obtidos através da hidrólise de proteína pela ação de enzimas que hidrolisam a sua proteína nativa, isolando a fração proteica (Molla & Hovannisyan, 2011). Esses compostos são produzidos com o foco de solubilizar a fração proteica para melhorar o

seu valor biológico e nutricional visando a obtenção de produtos de alto valor agregado e interesse comercial (Nilsang et al. 2005). Segundo Nissan et al. (2014), os hidrolisados proteicos são considerados como importantes suplementos nutricionais e possuem uma ampla variedade de aplicações, considerando que podem ser facilmente absorvidos e que possuem a capacidade de desempenhar diferentes atividades metabólicas no organismo.

Esse processo altera as propriedades das proteínas diminuindo sua massa molecular, aumentando o número de grupos ionizáveis e permitindo a exposição de grupos hidrofóbicos (Villamil et al., 2017), gerando assim produtos que podem ser usados na elaboração de dietas funcionais e suplementos para a aquicultura.

O processo de hidrólise enzimática é uma forma de obter peptídeos bioativos de forma ambientalmente mais responsável em comparação com os métodos convencionais através da extração por solventes químicos. A hidrólise enzimática pode ser realizada através de enzimas endógenas (enzimas digestivas ou microbianas) (Moller et al. 2008) ou enzimas exógenas (como alcalase ou flavourzyme), sendo essas exógenas comumente escolhidas para a produção de hidrolisados devido a sua capacidade de obter um perfil e composição de peptídeos consistentes (Sousa et al. 2020). Além disso, a hidrólise enzimática se destaca devido a ausência de resíduos de solventes orgânicos e pro possibilitar um tempo de processo menor quando comparado a processos fermentativos (Salem et al., 2017).

Durante a hidrólise enzimática ocorre a quebra da ligação peptídica entre os aminoácidos pela adição de uma molécula de água (Rutherfurd, et al., 2009) e para cada ligação peptídica clivada ocorre liberação de um mol de grupo carboxila e um mol de grupo amina (Damodaran et al., 2010). A hidrólise enzimática é realizada nas condições ótimas de atuação das enzimas proteolíticas, onde variações de pH devem ser controladas com o auxílio de soluções básicas ou acidas (Clemente, 2001). A qualidade dos hidrolisados obtidos são influenciadas pela seleção da enzima usada, condições de hidrólise adequadas, relação enzima: substrato (E:S) temperatura, pH e tempo de duração da hidrólise (Sila e Bougatef, 2016).

Nos últimos anos várias pesquisas vêm sendo realizadas para avaliar as propriedades bioativas de hidrolisados proteicos de inseto, como potencial antioxidante (Tang et al., 2018), atividade anti-hipertensiva (Zhou et al., 2017), atividade antidiabética (Hall et al., 2018), e atividade antiinflamatoria (Zielinska et al., 2017). No entanto, existem algumas desvantagens na produção de hidrolisados proteico. Sarabandi et al. (2018) relata que, o processo de hidrólise envolve a formação de um sabor amargo, sendo

umas das limitações do uso desse produto na formulação de dietas, apesar das propriedades de melhoria da saúde. Esse sabor amargo está associado a liberação de pequenos peptídeos principalmente de aminoácidos hidrofóbicos. Outro ponto, é a liberação de peptídeos de baixo peso molecular, causando uma redução na temperatura de transição vítrea (Neto, 1998), podendo exibir uma alta higroscopicidade e serem mais susceptíveis a aglomeração e deterioração química (Kurozawa et al., 2011), sendo necessário a adoção de outros métodos para minimizar esses efeitos.

1.3.3. Encapsulação de compostos bioativos

O processo de encapsulação é uma forma de superar algumas características indesejáveis presentes nos hidrolisados proteicos. Além disso, peptídeos bioativos diferem de outros compostos bioativos, como polifenóis e vitaminas (Tkaczewska et al., 2019). Atualmente, há uma lacuna na literatura demonstrando os vários aspectos do encapsulamento de hidrolisados proteicos derivados de proteínas de insetos. Esse método é baseado no revestimento de pequenas partículas (no caso dos hidrolisados proteicos, solidas) com um material de parede polimérico para produzir microcápsulas (Correa-Filho et al., 2019). O uso do encapsulamento é um fator altamente significativo na proteção de moléculas bioativas (Tkaczewska et al., 2019), como é o caso dos hidrolisados proteicos.

O encapsulamento permite o desenvolvimento de um sistema de entrega por meio da melhoria na estabilidade, aumentando o tempo de residência em relação a circulação e uma liberação controlado do material no núcleo (Lemes et al., 2016). Dentre as vantagens do processo da encapsulação, ele reduz o sabor e gosto indesejado do material do núcleo, facilita a liberação controlada do material do núcleo e protege o material do núcleo frente a condições circundantes (Jeyakumari et al., 2016). Diferentes tipos de sistemas de entrega coloidais foram desenvolvidos, como por exemplo a produção de microcápsulas através da secagem por pulverização (usando um spray dryer). A microencapsulação é uma técnica que envolve o aprisionamento de um agente bioativo (material do núcleo) em um envelope protetor (material de parede) (Gharsallaoui et al., 2007).

Para esse processo, comumente são usados como material de parede, celulose, maltodextrina, goma arábica, alginatos, pectina, quitosana e inulina (Correa-Filho et al., 2019). Os polissacarídeos são geralmente ideais como agentes de entrega em encapsulados, devido serem estruturalmente estáveis, baratos e amplos por natureza.

Além disso, os polissacarídeos possuem grupos funcionais reativos (carboxil, amida, hidroxil e grupos sulfato) sendo bons candidatos como matrizes transportadoras (Mohan et al., 2015). A maltodextrina é frequentemente usada como material de parede devido a sua capacidade de formar uma película continua, que é fundamental para proteger o material do núcleo (Costa et al., 2015). Dentre as vantagens do seu uso, são mencionados seu custo relativamente baixo, aroma e sabor neutros, alta solubilidade e baixa viscosidade em concentrações de sólidos alta (Justus et al., 2022).

No entanto, o uso de diferentes materiais de parede pode afetar algumas características físico-químicas das microcápsulas, como o tamanho, estrutura, densidade, porosidade, teor de umidade e retenção de compostos bioativos (Muzaffar et al., 2016; Seerangurayar et al., 2018; Silva et al., 2012; Tonon et al., 2012). Materiais de parede com alto peso molecular usados no processo de microencapsular hidrolisados proteicos pode contribuir no processo de secagem diminuindo a adesão do produto na câmara do secador e aumentando a sua estabilidade em condições ambientais adversas (Muzaffar et al., 2016; Santana et al., 2017). Logo, o estudo sobre as propriedades é necessária para otimizar o processo, melhorar a funcionalidade e reduzir custos de operação.

1.4. Efeito de compostos bioativos na aquacultura

Apesar dos avanços no setor aquícola, o bem-estar animal e a redução ao estresse induzido pelos cultivos, continuam sendo um desfaio e superá-los é fundamental para contribuir no desemprenho zootécnico dos animais e a resistência as ameaças ambientais (como surtos de doenças e mudanças climáticas) (Marmelo et al., 2024). Encontrar maneiras mais sustentáveis e econômicas para auxiliar o sistema imunológico e as respostas antioxidantes dos animais é importante, pois auxiliam na resistência frente a condições que podem causar prejuízo a suade dos animais (FAO, 2022). Para contornar esses desafios, as rações biofortificadas com aditivos naturais bioativos, surgem como uma estratégia promissora para aumentar a imunidade dos organismos aquáticos.

O efeito protetor de compostos bioativos em organismos aquáticos ocorre por meio de diversas ações biológicas que visam aumentar a resistência ao estresse oxidativo, patógenos, inflamações e outras condições adversas. Esses compostos bioativos incluem antioxidantes, peptídeos antimicrobianos, polissacarídeos imunomoduladores, entre outros, que atuam em várias frentes para proteger e melhorar a resposta fisiológica dos organismos, tornando-os mais resilientes a desafios ambientais e patogênicos (Gomes et al., 2024; Colombo et al., 2023). Compostos como flavonoides, carotenoides, e vitaminas

presentes nos bioflocos (Crab et al., 2012; Ju et al., 2008; Tacon et al., 2002) desempenham papel essencial na neutralização de radicais livres, reduzindo o estresse oxidativo e os danos às células e tecidos. Em sistemas de aquicultura intensiva, como no caso do sistema BFT, onde os camarões são expostos a altas densidades de estocagem e flutuações de qualidade de água, o estresse oxidativo aumenta, levando a imunossupressão e morte celular.

A produção de ROS (espécies reativas de oxigênio) acontece normalmente no metabolismo aeróbico dos organismos, no entanto, em altas concentrações e a longo prazo pode danificar as células animais e levar ao envelhecimento celular e redução da imunidade (Tsumbu et al. 2012; Lobo et al. 2010). O estresse oxidativo em peixes como resultado da resposta imunológica pode levar ao comprometimento da saúde (Blier, 2014). Ingredientes bioativos podem eliminar auxiliar na redução de produção do ROS, diminuindo os danos no corpo dos animais. Proteínas após processo de hidrólise apresentam propriedades bioativas, e demonstraram contribuir na saúde dos animais (Zamora-Sillero et al., 2019). Alguns peptídeos de cadeia curta e aminoácidos livres são conhecidos por possuir atividade antioxidante. Essas moléculas podem facilmente diminuir a produção de ROS e radicais livres (Elias et al., 2008). No entanto a maioria dos estudos avalia apenas essa atividade *in vitro*.

A modulação da microbiota intestinal contribui para uma barreira intestinal mais forte, reduz a colonização de patógenos e promove uma digestão mais eficiente, resultando em melhor aproveitamento dos nutrientes e uma menor produção de resíduos (Schelder et al. 2017). Isso é especialmente importante em sistemas de aquicultura, onde a saúde intestinal está diretamente relacionada à capacidade de crescimento e à resistência a doenças.

2. OBJETIVO GERAL

Obtenção, caracterização e aplicação das propriedades bioativas obtidas a partir de hidrolisados proteicos de tenébrio gigante (*Zophobas morio*) e flocos microbianos dos sistemas fotoautotróficos e heterotróficos produzidos no meio em bioflocos -BFT.

2.1. Objetivos específicos

- Obter flocos microbianos (heterotrófico e fotoautotrófico) utilizando diferentes métodos de secagem (estufa e liofilização);
- Determinar a composição proximal dos bioflocos concentrado (*in natura*), secos e liofilizados;
- Caracterizar os bioflocos processados em relação ao seu conteúdo de aminoácidos;
- Analisar as propriedades biológicas (clorofila a e carotenoides) dos bioflocos processados;
- Avaliar as propriedades bioativas dos extratos (aquoso, etílico e metílico) nas amostras de bioflocos;
- Obter hidrolisados proteicos de tenébrio gigante (*Zophobas mori)* desidratado utilizando diferentes proteases (Alcalase, Protamex e Flavourzyme);
- Caracterizar os hidrolisados proteicos com relação a sua composição aminoacídica;
- Avaliar as propriedades funcionais dos hidrolisados proteicos obtidos;
- Avaliar os hidrolisados proteicos como agentes antimicrobianos;
- Analisar a capacidade antioxidante dos hidrolisados proteicos antes e após a simulação gastrointestinal in vitro;
- Microencapsular os hidrolisados proteicos mediante secagem por "spray-dryer" usando diferentes materiais de parede (maltodextrina e goma arábica);
- Caracterizar os hidrolisados microencapsulados quantos as suas características físico-químicas;
- Avaliar o efeito da microencapsulação sobre as atividades antioxidantes dos hidrolisados proteicos;
- Analisar os hidrolisados microencapsulados como agente protetor frente a condições de estresse (oxidativo e térmico) no nematóide *Caenorhabditis elegans*.

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CAPÍTULO 1

Microorganisms, nutritional composition, and biological properties of the processed microbial floc produced in a BFT system

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Microorganisms, nutritional composition, and biological properties of the processed microbial floc produced in a BFT system

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Abstract

The aim of this study was to evaluate the influence of the drying process on the proximal composition, amino acid profile, and biological properties of photoautotrophic and heterotrophic bioflocs samples to analyze the effects of different bio-based solvents (water, ethanol, and methanol) on the antioxidant capacities and polyphenol content. The samples were first dried in hot-air oven (60 ºC) or lyophilized (0 ºC). After this process, aqueous, ethanolic or methanolic extracts were obtained. The freeze-drying process resulted in higher amino acid values among the samples, as well as higher concentrations of bioactive molecules, such as chlorophyll a and carotenoids. After preparation of the extracts, the highest antioxidant activity against DPPH radicals was observed in the freeze-dried photoautotrophic bioflocs (LPB) extract. The aqueous extract sample of the oven-dried photoautotrophic bioflocs for ABTS radicals showed the highest antioxidant activity. Total polyphenol content (TPC) was higher in the aqueous extracts of the photoautotrophic samples (DPB and LPB). The processed bioflocs samples demonstrated promising potential as supplementary additives for the nutrition of cultivated organisms. In addition, the obtained extracts exhibited antioxidant properties and contained natural compounds suitable for biotechnological applications.

Keywords: microbial aggregates, drying conditions, nutritional value, bioactive compounds.

1. Introduction

Microbial aggregates or bacterial biomass produced in the super-intensive biofloc system (BFT) have been reported as a mechanism for controlling water quality and providing nutritional sources for farmed animals (Martínez-Cordova et al., 2015). Bioflocs are aggregates of microorganisms, including bacteria, algae, protozoa, and particulate organic matter, which are formed in intensive aquaculture systems where water is recirculated and enriched with nutrients. Several studies have observed that the nutritional composition of bioflocs depends on factors such as the carbon source, carbon:nitrogen (C:N) ratio, type of substrate, environmental conditions of the culture, culture time, and species of organism produced (Wasielesky et al., 2006; Ferreira, 2008; Avnimelech, 2009; Vinatea et al. 2010; Emerenciano et al. 2011; Krummenauer et al., 2020; Oliveira, Wasielesky & Tesser, 2024).

Understanding the use of nutrients within the BFT system contributes to sustainable activity. In this context, systems can be classified into different categories based on the predominant type of microorganism (Ebeling, et. al, 2006; Emerenciano et al., 2017; Jimenez-Ojeda et al., 2018). For example, in the system with a predominance of photoautotrophic microorganisms, there is a predominance of microalgae that perform photosynthetic processes and convert nutrients present in the water into biomass (Fimbres-Acedo et al. 2020). These systems are highly dependent on light and play an important role in water quality, conversion of nitrogen compounds and a nutrient-rich food source (Jia & Yuan, 2016; Jimenez-Ojeda et. al., 2018). On the other hand, in the heterotrophic system, heterotrophic bacteria play a fundamental role, converting organic matter, such as food scraps and excretions from animals produced, into bacterial biomass (Ebeling et al. 2006). The addition of carbon sources, such as molasses, is essential to promote the growth of these microorganisms, which act directly in recycling waste,

improving water quality and providing supplementary food for the cultivated organisms (Gomez-Ramirez et al., 2019; Luo et al., 2020). Furthermore, these bioflocs can contribute to unfavorable conditions in the cultivation environment such as temperature (Prates et al. 2023) and nitrogen compounds (Prates et al., 2024) that interfere with animal growth.

The increase in the generation of biofloc waste derived from animal production in the BFT system is linked to population growth and the demand for quality proteins for the population. These microbial aggregates are rich in macromolecules (proteins, lipids, ash, and fibers) and serve as a potential low-cost source for animal nutrition. They have a protein content that can vary between 25% and 50% (Krummenauer et al., 2020; Braga et al. 2023; Ramiro et al., 2024) depending on the system conditions, and are rich in essential fatty acids, especially polyunsaturated fatty acids (PUFAs) (Castro, Pinto & Nunes, 2021), which play a crucial role in the growth and health of aquatic organisms. Proteins derived from bioflocs residues have a composition rich in essential amino acids and serve as an alternative feed during production the shrimps and fishes. Studies have used bioflocs meal in the formulation of animal diets exhibit positive responses such as improved growth, physiological responses, feed efficiency, nutrient metabolism, immune response and intestinal microbiota (Nethaji et al. 2022; Durigon et al. 2024; Rostro et al. 2024; Wei et al. 2024). However, drying to obtain a meal for prolonged periods at high temperatures can promote proximal chemical composition (Emerenciano et al. 2013) and loss of biological constituents (Santos & Martins, 2023). Furthermore, some points need to be evaluated regarding the effect of different bioflocs systems and drying methods on the nutritional composition of dried in hot-air oven and freeze-dried bioflocs.

The biological properties of bioflocs extend beyond their nutritional composition. In recent years, studies have shown that they contain phytochemicals (polyphenols,

flavonoids, and carotenoids), providing bioactive ingredients for aquaculture nutrition (Gomes et al. 2024; Colombo et al. 2023; Léon et al. 2018). The presence of these biocompounds can benefit the immune system of animals, reducing the need for antibiotics and protecting cells against damage caused by free radicals and oxidizing agents present in the culture environment (Reis et al. 2024.). However, most of these studies have evaluated the incorporation of other enrichers in bioflocs, and it is necessary to determine the bioactive characteristics of different types of bioflocs systems. Regarding, it is necessary to understand some points regarding the use of waste from the BFT system for biotechnological applications.

Therefore, the aim of this study was to evaluate the effect of the drying method on the nutritional composition and amino acid profile of microbial bioflocs from photoautotrophic and heterotrophic systems in BFT and subsequently analyze the influence of the extraction solvent on the content of phenolic compounds and antioxidant activity. The results of this study may contribute to better use of the solids produced in the BFT system for the formulation of diets or supplement products and generate information on obtaining extracts rich in bioactive compounds.

2. Material and methods

2.1. Material

Photoautotrophic and heterotrophic bioflocs samples were obtained from greenhouse cultures at the Marine Aquaculture Station of the Federal University of Rio Grande (Rio Grande, Brazil). The following chemical reagents were purchased from Sigma-Aldrich: ABTS (2, 2′ -Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)); DPPH (2,2-Diphenyl-

1-picrylhydrazyl); Trolox; Gallic acid and Quercetin. All chemicals reagent used were of analytical grade purity.

2.2. Experimental cultivation conditions and bioflocs formation

Penaeus vannamei post-larvae were stocked in two different biofloc systems: (1) photoautotrophic biofloc system and (2) heterotrophic biofloc system, with an initial average weight of 0.05 g (± 0.005 g) at an initial stocking density of 1500 shrimp/m^3 and 0.01 g (± 0.009 g). The experimental units were two raceways filled with 237 m^3 of a mixture of filtered seawater with sand (185 m^3, 23 ppt salinity) and groundwater (52 m^3, 5 ppt salinity), The tanks were completed with seawater prior treated with a chlorine solution (20 ppm) and neutralized using ascorbic acid powder (1 ppm).

To form a heterotrophic system, the C/N ratio was initially fixed at 15:1, according to the methodology described by Avnimelech (1999) and Ebeling et al. (2006). Organic fertilization was performed using sugarcane molasses with 37.27% carbon, during the first three days in clear water to stimulate bacteria, to maintain good water quality in relation to total ammonia, nitrite, nitrate, total suspended solids, and phosphate. Shading (70% shade) was used to prevent light entry. For the photoautotrophic system, 15% of the heterotrophic system inoculum was used to stimulate the proliferation of microorganisms, a longer period of light incidence (around 16 h) to favor the appearance of microalgae to control water quality in relation to the same parameters as the heterotrophic system Furthermore, commercial probiotics (INVE® Sanolife PROeW) at a proportion of 0.5 ppm were added to the culture water.

At the time of bioflocs sample collection, shrimp from the photoautotrophic and heterotrophic systems had an average weight of 0.4 g (± 0.25 g) and 1.91 g (± 0.35 g), respectively. Values of water quality parameters for water from the system considered photoautotrophic after 4 weeks were: TAN 0.00 mg/L, NO_2 0.65 mg/L, NO_3 0.00 mg/L,

SST 75 mg/L, and PO_4 0.00 mg/L, respectively. For water from a system considered heterotrophic after 11 weeks were: TAN 0.30 mg/L, NO_2 0.12 mg/L, NO_3 5.0 mg/L, TSS 200 mg/L, and PO_4 1.9 mg/L, respectively.

2.3. Collection and processing of bioflocs

Water samples from photoautotrophic and heterotrophic cultures (PB and HB, respectively) were collected manually and left to rest in 1000 mL water tanks to separate the solid and liquid fractions. The solid fraction was then centrifuged (Novatecnica, NT 825) at 800 x g for 15 min at 4 °C. The concentrated biomass was placed in 50 m Falcon tubes. For the drying process, the bioflocs were divided into batches: the first, concentrated biomass (*in natura*) was used as a control to evaluate the drying process. In the second batch, samples were dried in an oven (Nova Ética) for 24 h at 55 °C. In the third batch, the samples were freeze-dried in a horizontal benchtop freeze dryer (SL-405/E) for 168 h at 0 °C and -760 mmHg. The samples processed in these different forms were called: concentrated photoautotrophic bioflocs (CPB), concentrated heterotrophic bioflocs (CHB), oven-dried photoautotrophic bioflocs (DPB), oven-dried heterotrophic bioflocs (DHB), lyophilized photoautotrophic bioflocs (LPB), and lyophilized heterotrophic bioflocs (LHB). After these steps, the samples were kept in an ultrafreezer (TECTALMAQ) at -95 ºC until further analysis.

2.4. Identification of microorganisms

To quantify and characterize the microbial community present in the heterotrophic and photoautotrophic bioflocs samples, water samples from the systems were collected, fixed in 4% formalin in a ratio of 18:2 mL (water: formalin; v/v), and kept in amber glass (Reis

et al., 2019). To determine the microorganisms present in the samples, the groups were counted using an inverted microscope (Zeiss Axiovert) at 400 x magnification as described by Utermohl (1958). The diversity of microorganisms was estimated using the Shannon-Weaver index (*H'*) method (equation 1).

$$H' = \sum_{i=1}^{S} (p_i)(\ln p_i) \qquad \text{Equation 1.}$$

Where *H´* is the Shannon diversity index, ∑ pi is the number obtained by dividing Ni by N, Ni is the total number of individuals of a species, N is the total number of individuals from the entire sampled area, and ln is the natural logarithm applied to pi.

2.5. Proximal composition

The proximal compositions of the photoautotrophic and heterotrophic bioflocs samples (concentrated biomass, oven-dried, and lyophilized) were determined according to the methodology described by AOAC (2006). Moisture content was determined in a drying oven at 105 ºC. The crude protein content was determined using the micro-Kjeldahl method with a conversion factor of 6.25. The lipid levels were determined using a Soxhlet extractor. The ash content was determined in a muffle furnace at 550 ºC, and the crude fiber content was estimated according to the method proposed by Silva and Queiroz (2009) by washing with acid and alkaline solutions, followed by incineration in a muffle furnace at 550 ºC. All analyses were performed in triplicates.

2.6. Amino acid composition

The total, essential and hydrophobic amino acid (TAA, TEAA, HAA, respectively) profile compositions of the photoautotrophic and heterotrophic bioflocs samples were determined using the method described by White et al. (1986). The analysis was performed using high-precision liquid chromatography.

2.7. Chlorophyll content

Chlorophyll a values were estimated according to the method proposed by Jeffrey and Humphrey (1975) with modifications. For this, 100 mg of photoautotrophic and heterotrophic bioflocs samples (concentrated biomass, oven-dried, and lyophilized) were added to a cryopreservation tube together with 10 mL of acetone (90%). The samples were sonicated for 3 min under refrigeration (QSonica Sonicators) and then placed on an orbital shaker (KLA-210) for 24 h in the dark at room temperature (aprox. 25ºC). Finally, 2 mL aliquots of the samples were collected and their optical density was read in a spectrophotometer (BEL PHOTONICS) at absorbance wavelengths of 630 nm, 647 nm, and 664 nm. To estimate the values of chlorophyll a (Cla), equation 2, proposed by Jeffrey and Humphrey (1975), was used.

$$Cla\ \left(\mu\frac{g}{L}\right) = \frac{(11.85\ E_{664} - 1.54\ E_{647} - 0.08\ E_{630})\ x\ v}{V\ x\ L} \qquad Equation\ 2$$

Where E λ = reading at the respective λ, corrected by the value of the cuvette blank and subtracted from the value at 750 nm (turbidity), v= volume of the aliquot in the acetone extract (mL), V = volume of the filtered sample (L) and L = optical length of the cuvette (cm)

2.8. Carotenoid analysis

Carotenoid content was extracted from the photoautotrophic and heterotrophic bioflocs samples (concentrated biomass, oven-dried, and lyophilized) according to the protocol proposed by Torrisen and Naevdal (1984), with modifications. For this, 1.00 g of bioflocs sample was placed in a Falcon tube. Then, 10 mL of acetone and 100 mg of sodium sulfate were added, followed by vortexing and left to stand for three days at 25 ºC in the dark. After this period, the samples were centrifuged at 5,000 x g for 4 min at 4 ºC, the supernatant was collected, and the optical density was read using a spectrophotometer (BEL PHOTONICS) at an absorbance wavelength of 480 nm. Total carotenoid content (µg/g) was estimated using equation 5:

$$TCC\ \left(\frac{\mu g}{g}\right) = A\lambda = 480\ \text{nm} * \text{K} * \frac{\text{V}}{\text{E}(1\%;1\text{cm}) * \text{G}} \qquad \text{Equation 3.}$$

Where Aλ=480 nm is the absorbance value, K is a constant (104), V is the volume of the extract solution (mL), E(1%;1 cm) =1.900 is the extinction coefficient used as a basis for the calculation to compensate for the presence of cis isomers (Foss et al., 1984), and G is the weight of the sample (g).

2.9. Biochemical analyses

2.9.1. Extract preparation

The photoautotrophic and heterotrophic bioflocs samples (concentrated biomass, oven-dried, and lyophilized) were homogenized (1:5, w/v) in three different solvents to obtain aqueous (Milli Q water), ethanolic (100%), and methanolic (100%) extracts of each sample. The samples were subjected to sonication (QSONICA SONICATORS) at a frequency of 50 kHz for 1 min in the dark, homogenized for 3 h on a benchtop shaker

under refrigeration, centrifuged at 1000 x g for 5 min at 4 ºC, and the supernatant was collected and stored in an ultra-freezer for analysis.

2.9.2. Antioxidant activity

2.9.2.1. Antioxidant capacity against DPPH [2,2-diphenyl-1-picrylhydrazyl] radical

The scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined according to the methodology described by Sicari et al. (2004) with some modifications. A 50 µL aliquot of extract and 150 µL of DPPH solution (60 µM) diluted in absolute methanol (P.A) were added to the ependorff, incubated for 30 min in the dark, centrifuged at 10,000 x g for 5 min at 4 ºC, the supernatant was collected, and 150 µL was placed in the wells of a transparent microplate with 96 wells and the absorbance was read at 515 nm in a microplate reader (Biotek Synergy HT). Antioxidant activity was expressed as the equivalent antioxidant capacity of Trolox against DPPH radical per gram of sample (µM Trolox/g).

2.9.2.2. Antioxidant activity by scavenging the free radical ABTS 2,2'-azinobis (3-ethylbenzothiazoline sulfonic acid)

The ABTS radical-scavenging capacity of the extracts was determined according to the methodology proposed by Nenadis et al. (2004). The cation radical ($ABTS^{+}$) was obtained by dissolving the ABTS stock solution (7 mM) in potassium persulfate (140 mM) and kept in the dark at room temperature for 16 h. Then, 30 µL of the bioflocs extract and 3.0 mL of $ABTS^{+}$ radical were added to a cryopreservation tube, homogenized in a benchtop vortex, and the solution was kept at room temperature for 6 min. The $ABTS^{+}$ radical was read using a microplate reader (BioTek Synergy HT) and measured at 734 nm. Ethanol (100%) was used as the blank and a Trolox solution was used as the standard curve. The

values are expressed in relative micrograms of the standard per gram of sample (μM Trolox/g).

2.9.3. Polyphenol analysis

2.9.3.1. Total polyphenols content (TPC)

The polyphenol content (PC) of the extracts was determined as described by Dias et al. (2013). A 25 μL aliquot of the extract and 625 μL of 0.1 M Folin-Ciocaletu were added to clear microplate wells. After 5 min, 500 μL of 7.5% (w/v) Na_2CO_3 was added. A standard curve was prepared using gallic acid previously diluted in 100% methanol. The samples were kept in the dark for 60 min at room temperature, and absorbance was measured at 765 nm using a microplate reader (Biotek Synergy HT). The total polyphenol content was expressed as μg of gallic acid per gram of the sample.

2.9.3.2. Flavonoid analysis

To determine flavonoid content (FC), a protocol according to Gajula et al. (2009) and modified by Léon et al. (2018), was adopted. Aliquots of the extracts (50 μl) were added to an eppendorf tube, followed by 50 μl of distilled water and 37.5 μl of sodium nitrite (5%), incubated in the dark for 6 min, then 75 μl of aluminum chloride (10%) was added and incubated again for 5 min. Finally, 250 μl of sodium hydroxide (1 M) was added, homogenized, and 250 μl of the samples were removed and added to the transparent microplates. The absorbance was read at 510 nm using a spectrofluorimeter (BioTek Synergy HT). Quercetin solution was prepared for the standard curve. The data are expressed as μg of quercetin per gram of the sample.

2.10. Statistical analyses

All results were analyzed using a two-way analysis of variance (ANOVA) to evaluate the effect of the dried (fixed factor with three levels: control, over-dried and lyophilized) and the types of extracts (fixed factor with three levels: aqueous, ethanolic and methanolic). The treatment means were compared using the Tukey's test. Previously, the assumptions of normality and homogeneity of variances were evaluated using the Shapiro-Wilk and Levene tests, respectively. The significance level of all the tests was set at 5%.

3. Results

3.1. Composition of groups of microorganisms

The behavior of the community of microorganisms found in the water samples from the photoautotrophic and heterotrophic bioflocs systems is shown in **Figure 1**. Significant differences were observed in all groups of microorganisms evaluated between the two systems ($p<0.05$). The Shannon index indicated a greater abundance in the water samples from the photoautotrophic system ($p<0.05$) (**Fig. 1a**). In addition, higher values ($p<0.05$) of oocyster concentration ($0.38x10^5$) and total flagellates ($1.75x10^5$) were observed in this system (**Fig. 1b** and **1c**). The heterotrophic sample had a higher concentration of ciliates ($0.002x10^5$), diatoms ($0.051x10^5$) and filamentous microalgae ($0.0009x10^5$) (**Fig. 1d**, **1e**, and **1f**).

3.2. Proximal analysis and amino acid profile

The results of the proximal analysis of photoautotrophic and heterotrophic bioflocs samples according to the drying process are presented in **Table 1**. The moisture content

of the bioflocs samples decreased with the application of the oven-drying and freeze-drying processes, with no differences observed between them ($p>0.05$). Higher levels of moisture, lipids, and fibers were observed in the concentrated samples ($p<0.05$). For protein content, differences were observed between the bioflocs samples ($p<0.05$), with the most expressive value being found in DHB (40.01%), while lower values were observed in the DPB (34.08%) and LHB (24.70%) samples. The amount of ash increased significantly in the ovend-dried and lyophilized bioflocs ($p<0.05$) compared to the concentrated ones, with the DPB sample having the highest value (51.70%); however, in these treatments, there was a reduction in the fiber content compared to the control samples ($p<0.05$).

Table 2 presents the amino acid profiles of the oven-dried and lyophilized of photoautotrophic and heterotrophic bioflocs samples. In general, glutamic acid was the most abundant amino acid in the samples (ranging from 2.13 to 2.49%), whereas aspartic acid contributed with 2.62% in the LPB sample. Our results showed that freeze-drying increased the amino acid values in the photoautotrophic sample; however, the same behavior was not observed in the heterotrophic bioflocs. Other major amino acids found in the samples were leucine, glycine, alanine, proline, and valine. In addition, more expressive values for hydrophobic, total, and essential amino acids were obtained for both freeze-dried samples (LPB and LHB). The LPB sample presented the highest values of HAA and TAA (9.10 and 21.35, respectively), whereas the most expressive value of TEAA was observed in the LHB sample (6.92%).

3.3. Chlorophyll a and carotenoid levels

Chlorophyll a content, was significantly different between the bioflocs samples ($p<0.05$) (**Table 3**). The lyophilized photoautotrophic bioflocs (LPB) treatment resulted in higher

concentrations of chlorophyll than the other treatments ($p<0.05$). In addition, CHB treatment resulted in lower levels for all concentrations of chlorophyll a. Regarding carotenoid levels, significant differences were found between samples ($p<0.05$) (**Table 3**). The photoautotrophic and heterotrophic bioflocs samples that underwent freeze-drying presented higher carotenoid contents (2.33 and 1.93 µg/kg) and differed significantly ($p<0.05$) from their concentrated samples. A lower content was observed in the CHB sample (0.96 µg/kg).

3.4. Bioactive properties

3.4.1. Antioxidant activity of bioflocs extracts

The antioxidant capacities of the bioflocs extract samples are shown in **Figure 2**. Regarding the DPPH radical scavenging capacity, the bioflocs extracts showed greater activity according to the drying process between treatments, showing bioflocs-biological solvent behavior ($p<0.05$) (**Fig. 2a**). The LPB samples in aqueous and ethanolic extracts (2242.28 and 2286.17 µg Trolox/g bioflocs, respectively) showed greater antioxidant capacity than the other samples ($p<0.05$). On the other hand, lower activities were found in both concentrated bioflocs samples (CPB and CHB), which did not differ from each other ($p>0.05$).

However, the same behavior was not observed for the ABTS radical scavenging capacity (**Fig. 2b**). More significant values were found in the samples of aqueous extracts in photoautotrophic and heterotrophic bioflocs dried in an oven (16526.67 and 16421.11 µg Trolox/g bioflocs, respectively), but did not differ from each other ($p>0.05$). Furthermore, lower antioxidant activities against the $ABTS^{+}$ radical were observed in both concentrated bioflocs samples (CPB and CHB), which did not differ significantly from each other ($p>0.05$).

3.4.2. Polyphenol content in bioflocs extracts

The average values of total polyphenols and flavonoids in the extracts of the bioflocs samples with different solvents are presented in **Figure 2**. A significant increase in the polyphenol content (PC) was observed in the samples composed of the aqueous solvent that underwent the drying process, with higher values in the photoautotrophic samples DPB and LPB (475.26 and 476.59 mg gallic acid/g bioflocs, respectively) than in the other samples ($p<0.05$) (**Fig. 2c**). Lower contents were observed in the CHB sample (235.32 mg gallic acid/g bioflocs) obtained using the methanolic solvent. A similar trend was observed for the mean values of flavonoids in the bioflocs extracts (**Fig. 2d**). The highest content was observed in the LPB sample (1696.00 mg quercetin/g bioflocs) compared to the other samples ($p<0.05$), while a lower content was observed in the CHB sample (63.22 mg quercetin/g bioflocs).

4. Discussion

The diversity and abundance of microorganisms are directly correlated with the environment; that is, they will vary according to the environment in which they are inserted. In this study, it was possible to determine the occurrence of microorganisms in the photoautotrophic and heterotrophic systems in the evaluated bioflocs. In a photoautotrophic system, there is a longer period of light, favoring the proliferation of microalgae at first, serving as a natural food source for the development of the zooplankton community (Fimbres-Acedo et al. 2020). Studies have shown that treatments exposed to light present a greater abundance of photoautotrophic organisms, whereas heterotrophic systems have a greater predominance of heterotrophic organisms (Reis et

al. 2019; Jiang et al. 2020). In general, the presence of light contributed to a greater abundance of microorganisms, than in the heterotrophic system. In the present study, it was possible to find a greater number of oocysters and flagellates in the photoautotrophic system than in the heterotrophic system, which had higher concentrations of ciliates and diatoms. This result may be related to the predation of autotrophic flagellates by ciliates, resulting in ecological succession among microorganisms in culture tanks (Reis et al. 2019). It is worth mentioning that, even at low concentrations, filamentous microalgae were only found in the heterotrophic system. They serve as a habitat and food source for other zooplankton and invertebrate organisms that are part of bioflocs, such as rotifers and copepods (Ferreira, 2008).

The drying process aims to reduce the moisture content of the food matrices and retain the content of other macromolecules (e.g., proteins, amino acids, and lipids). Microbial aggregates are known as a supplementary food source for animals in bioflocs production systems and are sources of these macromolecules, as well as minerals and vitamins. In this study, samples that underwent hot air drying (oven) or freeze-drying (DPB, DHB, LPB and LHB, respectively) showed a reduction in moisture content compared with CPB and CHB (Table 1). Drying food matrices, such as bioflocs, causes partial or complete dehydration of the free water content, which is easily removed from samples rich in this component (Hannef et al. 2024).

The crude protein content did not show significant differences between the samples ($p>0.05$); however, in the culture water, the bioflocs are diluted, which may suggest that in this state, the content is lower, indicating that dehydration may hinder protein retention in the sample. The protein content values in this study may be related to the abundance and protein contribution of the microorganisms in each system. Studies have shown that the main microorganisms that contribute to crude protein content in bioflocs samples are

coccoid bacteria, *Bacillus* bacteria, and nematodes (Silva et al. 2008; Rocha et al. 2012), and nematodes were not observed in the water samples in this study.

The oven-dried and freeze-dried photoautotrophic and heterotrophic bioflocs samples presented lower lipid values than the concentrated samples because the high moisture content may have facilitated the removal of other components from the samples. When subjected to drying, it causes the transport of this compound to the surface of the sample, facilitating the dissolution and removal of lipids (Puente et al. 2021). The lipid content observed in this study may be related to the presence of flagellates, ciliates and microalgae (oocysters and filamentous). In addition, filamentous bacteria, coccoid bacteria, cyanobacteria, and unicellular heterotrophic bacteria can contribute to the lipid content of bioflocs samples (Silva et al. 2008; Rocha et al. 2012).

In this study, the ash and crude fiber contents were inversely proportional to drying. The ash content increased in the samples that underwent drying, demonstrating that the bioflocs had high levels of solids in their composition. The main component in bioflocs is ash, which comprises soluble and total solids generated during the management cycle (Schveitzer et al. 2023). The crude fiber content showed significant differences between the samples ($p<0.05$), indicating that the drying process caused changes in the content of the insoluble fiber fraction, contributing to higher levels in the concentrated samples.

Amino acid composition is related to the characteristics of the food matrix, such as the composition of microorganisms and nutritional value. Among the amino acids present in bioflocs, glutamic acid stands out for presenting values between 2.13 and 2.49 % of the total AA content. This result is important because it is a flavor enhancer (Witono et al. 2016), indicating that bioflocs are attractive to animals and serve as a complementary nutritional source in the BFT system As previously reported, the most abundant amino acids in this study were glutamic acid, aspartic acid, glycine, alanine, and leucine (Table

2). Similar to this study, the main amino acids found in lyophilized heterotrophic bioflocs were glutamic acid, glycine, proline, and phenylalanine + tyrosine (Oliveira et al., 2021). In a study to rapidly produce bioflocs biomass from different carbon and nitrogen sources, the bioflocs were found to be composed mostly of glutamic acid, aspartic acid, glycine, alanine, and leucine, with lower levels of cysteine, methionine, and proline (Luo et al., 2022), which were present at lower levels in this study. In general, samples subjected to the drying process showed higher levels of AA than the concentrated samples (control). Amino acids play important roles in the nutrition of aquatic organisms, such as in muscle metabolism.

A higher concentration of chlorophyll a in the BFT system is associated with the growth of photoautotrophic algae in the cultivation environment (Wei et al. 2016). In this study, higher concentrations of chlorophyll a were observed in the photoautotrophic bioflocs samples (LPB and DPB) and are associated with cultivation conditions, where the water was influenced by a higher light frequency. In addition, higher levels of chlorophyll a in this system may be associated with lower values of soluble and total solids that allow the penetration of sunlight into the water column and favor the growth of algae (Green, 2015). Some studies have shown that BFT production systems with photoperiod treatment with 24 h of light presented higher concentrations of chlorophyll a compared to the control treatment (12 h light: 12 h dark) and 24 h of dark (Reis et al. 2019; Khoa et al. 2020). Lower concentrations were observed in the heterotrophic samples in the present study. In this type of system, lower chlorophyll a values are related to the use of molasses to adjust the ammonia levels. Additionally, carbon sources promote the succession and predominance of heterotrophic bacteria in algae (Baloi et al. 2013; Espirito Santo et al. 2017). It is important to emphasize that heterotrophic systems using shadows reduce the photosynthetic rate and have a constant production of solids, contributing to lower

chlorophyll a values (Costa et al. 2023), which justifies the concentrations observed in this study.

Microbial bioflocs produced in the BFT system have been shown to be a source of carotenoids that contribute to the pigmentation of cultivated species of organisms (Cunha et al. 2020; Costa et al. 2023). In this study, the application of a drying process influenced the concentration of carotenoids among the samples as well as the type of biofloc evaluated, which were significantly different. Treatments with photoautotrophic biofloc samples exhibited higher carotenoid values than heterotrophic samples, possibly due to the presence of photosynthetic organisms (microalgae, such as oocysters). The photoautotrophic sample dried by freeze-drying (LPB) presented significantly higher amounts of carotenoids (2.33 μg/kg) than the other treatments. The predominance of a phytoplankton community acts in the biosynthesis and dispersion of this compound in the cultivation water, owing to the light intensity assisting in the photosynthesis process and the development of this pigment as a form of protection (Huang et al. 2017). In addition, the carbon source and feed remains can serve as substrates for microorganisms and contribute to carotenoid production (Cunha et al. 2020). It is important to report that, even at lower concentrations, the heterotrophic samples presented carotenoid values in this study. Our results showed that heterotrophic samples that underwent the drying process presented an increase in carotenoid concentration compared to the concentrated samples. In this case, a lower light intensity due to the use of shade cloths to obtain a predominantly heterotrophic system favors the development of heterotrophic bacteria that produce metabolites as defense mechanisms against the microbial community (Manon Mani et al. 2015). Megana et al. (2023) demonstrated that the presence of bacteria in bioflocs, such as cocci and rod strains, are responsible for the production of bacterial pigments (chlorophyll a and carotenoids) and can minimize photodynamic lipid peroxidation in

liposome and protect against oxidative damage. Despite this, there is still a lack of research to identify which microorganisms are responsible for the production of carotenoids in the BFT system, especially in heterotrophic systems, where bioflocs are available to animals 24 h a day.

Solvents ares used to extract biocompounds, and the efficiency of the process is influenced by the affinity of the solvent for the sample matrix (Bordin Viera et al., 2021; Martínez-Ramos et al., 2020). As expected, the drying process applied to both types of bioflocs in this study provided greater antioxidant activities in the DPPH and ABTS tests, as shown in Fig. 2a and 2b, respectively. The DPPH and ABTS radicals tests were used to evaluate antioxidant capacity, where DPPH has greater reactivity to compounds soluble in organic solvents (hydrophilic), while ABTS evaluates the activity of fat-soluble compounds (Santos et al., 2021). In addition, antioxidant activity is associated with polyphenols of the samples (Zhu et al. 2020). Regarding the solvents, water showed the highest scavenging activity for DPPH and ABTS radicals in the DPB, DHB, and LPB samples. This result can be explained by the interaction of bioactive components with the hydrophilic characteristics of the extract (e.g. anthocyanins) with the polar solvent used as a food simulant (Santos, Silva & Martins, 2022). Antioxidant activity was higher using the ABTS method, indicating that lipophilic compounds were present at lower concentrations in the bioflocs samples. A recent study reported the same behavior when evaluating the antioxidant activity in a sample of heterotrophic bioflocs lyophilized using the DPPH and ABTS methods (Gomes et al., 2024). Regarding the DPPH assay, the LPB sample showed higher antioxidant activity in water and ethanol. This behavior may be related to the concentration and quality of the compounds present, such as polyphenols and , flavonoids, which have greater DPPH scavenging activity (Orsavová et al. 2023). In contrast, the samples of extracts in ethanol with the bioflocs showed a low level of

ABTS radical scavenging of the ABTS radical, unlike the aqueous and methanolic extracts. This can be explained by the presence of more polar antioxidant compounds, such as phenolic acids and flavonoids, with highly polar hydroxyl groups (Shi et al. 2022), making it difficult to eliminate this radical.

The extraction efficiency of phenolic compounds depends on several factors, such as the solid-liquid ratio, temperature, time, particle size, pH, and type of solvent (Santos & Martins, 2022). Regarding the extracts evaluated, the aqueous extracts of DPB, DHB, and LPB presented the highest levels of total polyphenols (TPC), with no significant differences between them ($p > 0.05$) (Fig. 2C). A similar result was reported by Gomes et al. (2024), that carried out a polyphenol optimization process in a sample of heterotrophic bioflocs residues. The authors observed that extracts prepared using water as the solvent achieved higher polyphenol concentration than the ethanolic extracts. However, a higher recovery of polyphenols has been reported using ethyl extracts because it is composed of hydroxyl groups and hydrogen bonds, promoting a greater recovery of these compounds (Martinez-Ramos et al. 2020). Regarding the flavonoid content (FC), the extraction may vary depending on the food matrix, type of solvent and technique applied (Ali et al. 2016). In this study, it was not possible to determine the flavonoid content of aqueous extracts. A greater release of FC was observed in the photoautotrophic biofloc samples freeze-dried with methanol (Fig. 2d), because of its ability to extract hydrophilic and lipophilic flavonoids, which may be present in the LPB samples.

5. Conclusion

This study demonstrated an alternative drying process for the better utilization of bioflocs produced in the BFT system. Furthermore, when evaluating the effect of drying on bioflocs, it was observed that they could provide higher levels of essential and total amino acids, as well as chlorophyll a and carotenoid levels in photoautotrophic and heterotrophic

bioflocs samples. Finally, the DPB, DHB, and LPB samples obtained using water as the solvent showed better bioactive capacities against free radicals and the recovery of phenolic compounds in the bioflocs. Therefore, this study shows that hot-air-dried and/or freeze-dried bioflocs present an alternative protein source with biological properties for biotechnological applications.

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TABLES

Table 1. Proximal chemical composition of photoautotrophic and heterotrophic bioflocs as a function of drying process (dry basis). **CPB**: concentrated photoautotrophic bioflocs. **CHB**: concentrated heterotrophic bioflocs. **DPB**: oven-dried photoautotrophic bioflocs. **DHB**: oven-dried heterotrophic bioflocs. **LPB**: lyophilized photoautotrophic bioflocs. **LHB**: lyophilized heterotrophic bioflocs.

Samples (%)*	Moisture	Protein	Lipids	Ashes	Fiber
CPB	79.76±6.41^{a}	39.47±6.57^{a}	2.69±1.05^{ab}	26.14±2.55^{d}	21.26±6.64^{a}
CHB	82.41±0.68^{a}	37.95±3.02^{a}	4.30±1.17^{a}	33.10±3.07^{c}	23.10±4.57^{a}
DPB	3.24±0.22^{b}	34.08±1.50^{b}	0.47±0.07^{c}	51.70±0.19^{a}	1.45±0.73^{c}
DHB	3,98±0,23^{b}	40.01±0.33^{a}	0.71±0.27^{c}	46.65±0.67^{b}	1.02±0.23^{c}
LPB	6.85±0.19^{b}	38.72±0.59^{a}	0.64±0.06^{c}	43.64±0.17^{b}	3.85±0.15^{b}
LHB	7.59±0.03^{b}	34.70±1.34^{b}	1.00±0.37^{bc}	47.90±0.23^{ab}	4.73±0.48^{b}

* Mean values ± standard deviation of triplicates (n=3). Different lowercase letters superscript in the same column demonstrates significant differences ($p<0.05$).

Table 2. Percentage composition of amino acids in photoautotrophic and heterotrophic bioflocs as a function of drying process. **CPB**: concentrated photoautotrophic bioflocs. **CHB**: concentrated heterotrophic bioflocs. **DPB**: oven-dried photoautotrophic bioflocs. **DHB**: oven-dried heterotrophic bioflocs. **LPB**: lyophilized photoautotrophic bioflocs. **LHB**: lyophilized heterotrophic bioflocs.

Amino acids (%)	**Bioflocs**			
	DPB	**LPB**	**DHB**	**LHB**
Aspartic acid	2.11	2.62	2.33	2.29
Glutamic acid	2.13	2.49	2.48	2.36
Serine	0.95	1.14	1.01	1.09
Glycine	1.33	1.60	1.38	1.46
Histidine	0.44	0.54	0.44	0.46
Taurine	<0.01(LQ)	<0.01(LQ)	<0.01(LQ)	<0.01(LQ)
Arginine	0.88	1.14	0.95	1.05
Threonine	0.85	1.10	0.96	0.94
Alanine	1.32	1.74	1.56	1.46
Proline	0.84	1.26	0.91	1.14
Tyrosine	0.53	0.69	0.65	0.57
Valine	0.96	1.27	0.97	1.09
Methionine	0.31	0.38	0.36	0.32
Cystine	0.44	0.53	0.35	0.49
Isoleucine	0.64	0.88	0.68	0.74
Leucine	1.37	1.85	1.55	1.57
Phenylalanine	0.85	1.09	0.94	0.93
Lysine	0.81	1.06	0.97	0.87
Hydroxyproline	0.18	0.17	0.16	0.18
HAA[a]	6.82	9.10	7.65	7.82
TAA[b]	16.94	21.55	18.65	19.01
TEAA[c]	6.23	6.32	6.87	6.92

[a]Hydrophobic amino acids (HAA): Alanine, Proline, Tyrosine, Valine, Methionine, Isoleucine, Leucine and Phenylalanine.

[b]Total amino acids (TAA)

[c]Total essential amino acids: (TEAA): Histidine, Threonine, Valine, Methionine, Isoleucine, Leucine, Phenylalanine, Lysine.

LQ: limit of quantification

Table 3. Chlorophyll a and carotenoids contents measured in samples of photoautotrophic and heterotrophic bioflocs as a function of the drying process. **CPB**: concentrated photoautotrophic bioflocs. **CHB**: concentrated heterotrophic bioflocs. **DPB**: oven-dried photoautotrophic bioflocs. **DHB**: oven-dried heterotrophic bioflocs. **LPB**: lyophilized photoautotrophic bioflocs. **LHB**: lyophilized heterotrophic bioflocs.

Compound*	**CPB**	**CHB**	**DPB**	**DHB**	**LPB**	**LHB**
Chlorophyll a (μg/g)	635.53± 18.8^{c}	94.69± 7.96^{d}	1881.09± 12.5^{ab}	1692.42± 13.5^{b}	1965.43± 1.68^{a}	830.69± 5.65^{c}
Carotenoid (μg/g)	1.57± 0.04^{bc}	0.96± 1.58^{c}	1.58± 0.19^{bc}	1.70± 0.50^{ab}	2.33± 0.35^{a}	1.93± 0.03^{ab}

*Mean values ± standard deviation of triplicates (n=3). Different lowercase letters superscript in the s demonstrates significant differences ($p<0.05$).

FIGURES

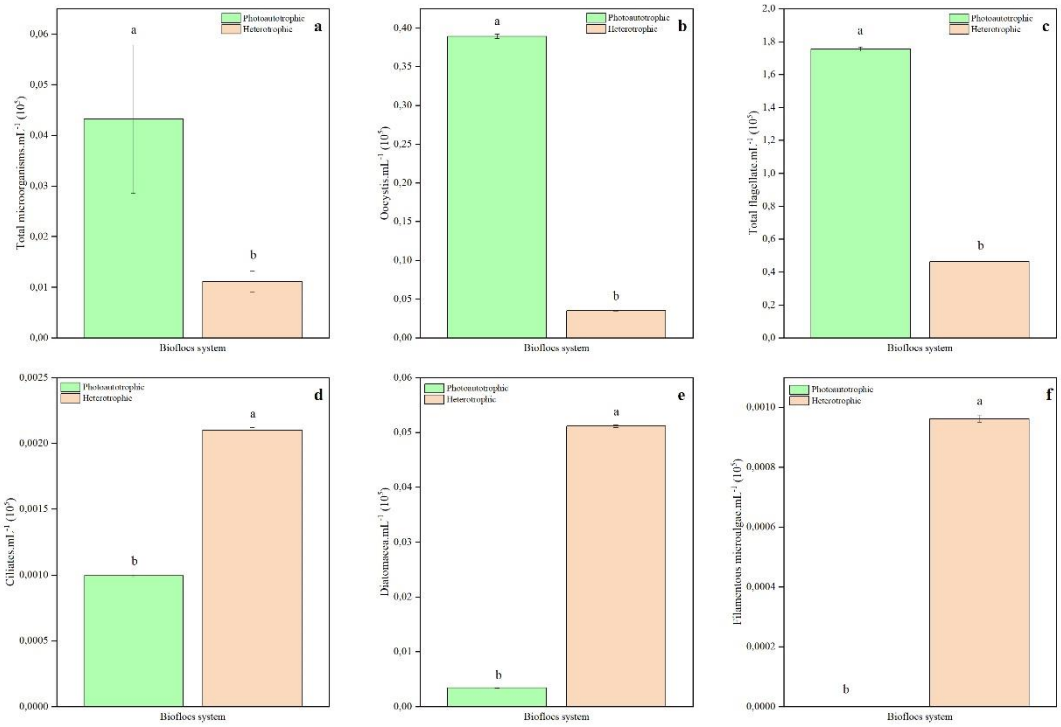

Figure 1. Mean values (±SD) (**a**) total microorganisms, (**b**) oocystis, (**c**) total flagellate, (**d**) ciliates, (**e**) diatomacea and (**f**) filamentous microalgae in water samples from photoautotrophic and heterotrophic biofloc systems. **CPB**: concentrated photoautotrophic bioflocs. **CHB**: concentrated heterotrophic bioflocs. **DPB**: oven-dried photoautotrophic bioflocs. **DHB**: oven-dried heterotrophic bioflocs. **LPB**: lyophilized photoautotrophic bioflocs. **LHB**: lyophilized heterotrophic bioflocs. Data area expressed as mean values ± standard deviation of triplicates (n=3). Different lowercase letters superscript in the s demonstrates significant differences ($p<0.05$).

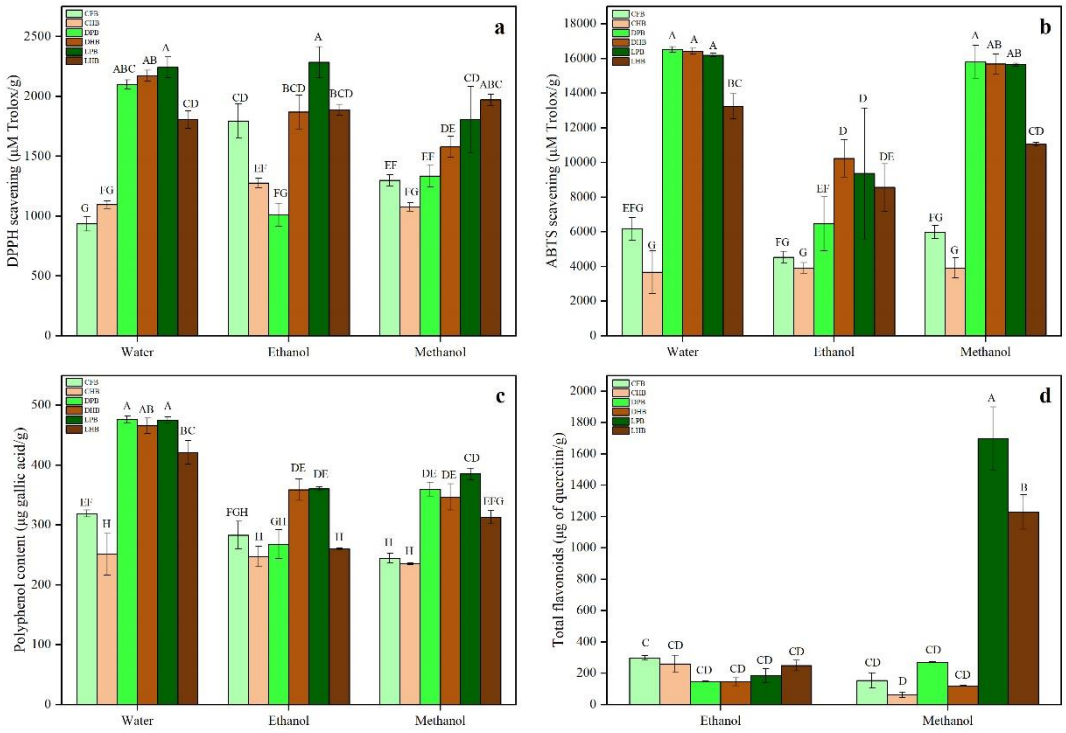

Figure 2: Antioxidative activity by radical scavenging DPPH (**a**), radical scavenging ABTS (**b**), Polyphenol content (**c**) and total flavonoid content (**d**) of aqueous, ethanolic and methanolic extracts of photoautotrophic and heterotrophic bioflocs as a function of the drying process. **CPB**: concentrated photoautotrophic bioflocs. **CHB**: concentrated heterotrophic bioflocs. **DPB**: oven-dried photoautotrophic bioflocs. **DHB**: oven-dried heterotrophic bioflocs. **LPB**: lyophilized photoautotrophic bioflocs. **LHB**: lyophilized heterotrophic bioflocs. Data are expressed as mean values ± standard deviation of triplicates (n=3). Different lowercase letters superscript in the s demonstrates significant differences ($p<0.05$).

CAPÍTULO 2

Protein hydrolysates derived from superworm (*Zophobas morio*): Composition, bioactivity and techno-functional properties

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Protein hydrolysates derived from superworm (*Zophobas morio*): Composition, bioactivity and techno-functional properties

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Abstract

This study aimed to produce protein hydrolysates from superworm (*Zophobas morio*) flour using the enzymes alcalase (HA), protamex (HP), or flavourzyme (HF), and to characterize their nutritional composition, techno-functional properties, bioactive capacity, and bioaccessibility index. The enzymatic process increased the total amino acid and crude protein contents of the hydrolysates by approximately 36% and 46%, respectively, generating better foaming capacity, oil retention, and emulsification capacity, when compared to raw flour. Although 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical capture was similar between the hydrolysates, HA (1479,66 µM $FeSO_4$/g) and HP (1514,66 µM $FeSO_4$/g) showed greater antimicrobial and iron reducing power (FRAP) activity, while HF has a higher scavenging efficiency for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (27.53%). The best antimicrobial activity was observed for HA against *Vibrio corallilyticus* (400 mg/mL), and HP showed a better antioxidant response scavenging for DPPH radical. The antioxidant capacity against ABTS radical after *in vitro* simulation of gastrointestinal digestion (GID) was as follows: HA (79.07 ± 1.53%), HP (74.65 ± 5.85%), and HF (57.95 ± 8.31%). Therefore, insect flour is a promising ingredient for the production of protein hydrolysates and their application in animal and human feeds.

Keywords: Insect proteins, protein hydrolysate, bioactive capacity, gastrointestinal *in vitro* digestion

1. Introduction

The increase in production of farmed organisms as a source of protein is driven by population growth and improved living standards. With the world population expected to reach approximately 9.7 billion people by 2050 estimated by the United Nations (UN), the aquaculture sector will play a crucial role in providing food with the necessary nutritional values for human growth and health [1]. The search for sustainable and accessible protein sources from various ingredients to maintain the essential connection between aquaculture and human nutrition has intensified [2,3]. The production of farmed organisms stands is a fundamental sector for generating high-quality food, including fish, crustaceans, and mollusks, which are essential components of the human diet.

One of the challenges faced by the aquaculture feed industry is the use of fishmeal as the primary protein source due to increasing demand, environmental concerns related to overfishing, variations in nutritional content, and high market value [4]. These factors have driven the search for alternative sources of protein. Among the emerging options, plant and insect proteins have proven to be viable alternatives capable of providing the essential amino acids needed in aquaculture [5,6]. In particular, insect proteins have gained prominence owing to their balanced nutritional profile and low environmental impact, offering an efficient and sustainable source of essential nutrients [7]. Insects are rich in macromolecules, such as proteins, lipids, and chitin [8]. Additionally, the use of insect proteins for human and animal consumption has emerged as a promising solution to address challenges related to food security and sustainability.

Due to their high protein content, insects are considered a promising source to produce protein hydrolysates. These hydrolysates, obtained through enzymatic hydrolysis, offer advantages, such as improved digestibility, bioactivity, and functionality, making them suitable for incorporation into a variety of food products [9]. The enzymes used in this

process play crucial roles in influencing the composition and properties of hydrolysates in unique ways [10], making them suitable for different industrial applications. Insect-based protein hydrolysates generated by different types of enzymes have been gaining prominence for demonstrating good antioxidant [11][10], antimicrobial [12] and high nutritional value [13] effects and, when used as ingredients in diets for cultured organisms, they performed satisfactory zootechnical effects, modulating biochemical parameters of the animal's blood and intestinal microbiota [14].

The bioactive activity of hydrolysates and bioactive peptides can contribute to improving the functional and sensory properties of a food, minimizing lipid oxidation processes that can be harmful to human and animal health. Studies have shown that hydrolysates obtained from aquatic organisms have positive immunomodulatory effects [15], Ca chelating effects [16] and angiotensin-I converting enzyme (ECA) inhibitors [17]. There is a growing interest in the use of antioxidant supplements for incorporation into foods; however, most studies with hydrolysates and bioactive peptides obtained from insects have not evaluated their availability after in vitro gastrointestinal digestion (GID)[18]. Peptides that can resist this process can exert physiological effects, and GID is a method that can evaluate the availability of bioactive peptides.

Among the various types of insects studied, *Zophobas morio*, also known as superworm, can reach up to 5 cm in length and has a higher nutritional density than *Tenebrio molitor* [19]. Despite their composition being rich in proteins, fats, and essential micronutrients, there is little research on the production and characterization of superworm protein hydrolysates. Therefore, this study aimed to obtain protein hydrolysates from superworms using different proteolytic enzymes (Alcalase, Protamex, and Flavourzyme) and to evaluate their physicochemical, technological, functional, and bioaccessibility properties. The results presented here will allow further exploration of the use of insect proteins in

more sustainable food practices, both in animal nutrition and in the production of food intended for human consumption.

2. Material and methods

2.1. Raw material and reagents

Dehydrated samples of superworms (*Zophobas morio*) (4.34%, 47.09%, 36.95%, 2.75%, and 8.87% of moisture, protein, lipids, ash, and carbohydrates, respectively) obtained from Insetos Kaisara (Além Paraíso, MG, Brazil) were crushed in a homogenizing processor (Walita Mega Máster Plus) for 5 min to obtain insect flour and sieved through a 42 μm sieve. The proteolytic enzymes, Alcalase 2,4 L (Batch: PLN0552; protease from *Bacillus licheniformis*; endopeptidase) and Flavourzyme 1000L (Batch: HPN 00498; exopeptidase), were supplied by Novozymes Latin America (Araucaria, PR, Brazil), whereas Protamex (1,5 AU/g; Batch: SLBQ6409V; protease from *Bacillus* sp.; mix exo- and endopeptidase) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals used were of analytical grade.

2.2. Obtaining insect protein hydrolysates

The hydrolysates were produced as described by [20]. The insect flour was homogenized in distilled water at a proportion of 10% (w/v; protein:water) at 90 °C for 15 min to inactivate endogenous enzymes. The optimal pH and temperature conditions of Alcalase (pH 8.0/50 °C/HA), Protamex (pH 7.0/50 °C/HP), and Flavourzyme (pH 7.0/50 °C/HF) were adjusted, and hydrolysis was initiated by addition of the enzyme (3 g_{enzyme} /100 $g_{protein}$) to the mixture. The pH was kept constant for 5 h of the hydrolysis process by the addition of NaOH solution (1 M), and the volume used was checked every 5 min to determine the degree of hydrolysis (DH) using the pH-stat method [15,16]. The mixture was subjected to enzymatic thermal inactivation (90 °C for 10 min), followed by

centrifugation (11,806 × g for 20 min at 35 °C), and the protein-hydrolyzed fraction of the supernatant was lyophilized (Liotop, L108) and stored at -18 °C until further analysis.

2.3. Amino acid profile and crude protein content

The amino acid profile was determined using the method proposed by [23], using a high-precision liquid chromatograph with a fluorescence detector (HPLC-FL, 20ADShimadzu) coupled with post-column derivatization and Shim-Pack Amino-Na (100 mm × 6 mm) column. Chromatograms were obtained at 350 nm (excitation) and 450 nm (emission) for a reading time of 45 min. Amino acid quantification and identification were determined using retention time and curve analysis of commercial amino acid standards. Crude protein content was determined using the Kjeldahl method according to the official protocol [24] and a conversion factor of 5.6 [25].

2.4. Morphological, functional groups and water actitivy characterization

The morphology of the hydrolysates was visualized by Scanning Electron Microscopy (SEM) using a microscopy (JSM 6610LV, JEOL) at 15 kV and ×100. The hydrolysates were placed on aluminum stubs with carbon tape and covered with a layer of gold using a Denton Vacuum (Desk V, USA). The functional groups were determined by Fourier transform infrared spectroscopy (FTIR) using a spectrometer (IRPrestige-21, SHIMADZU), and the spectral patterns were measured in the range of 650–4000 cm^{-1} in 16 scans with a resolution of 4 cm^{-1}. Water activity (Aw) was measured using an analysis equipment (Novasina, model LabTouch) at room temperature (aprox. 25 °C) [26].

2.5. Functional properties

The functional properties of the insect flour and hydrolysate samples were evaluated. The solubility index (SI) was evaluated at pH values of 2.0, 4.0, 6.0, 8.0, and 10.0. The sample

(0.5 g) was homogenized in 50 mL of distilled water, and the pH was adjusted with HCL (1 M) and NaOH (1 M), followed by shaking on a magnetic stirrer for 30 min at room temperature and centrifuged at 8667 × g for 20 min at 4 °C. The sample was filtered, and the supernatant obtained was measured according to the protein content using the [27] The SI was calculated as the ratio of soluble protein content to total protein content [28].

Foaming capacity (FC) and foam stability (FS) were determined using the method described by [29]. The sample (0.5 g) was dispersed in 50 mL of distilled water in a graduate beaker, and the pH of the solution was adjusted to 2.0, 4.0, 6.0, 8.0, and 10.0, using HCl (1M) or NaOH (1M). The solutions were stirred in Ultra-Turrax at 10000 rpm for 1 min at 25 °C, and the volume of foam was measured to determine the FC. After 20 min of standing, the foam volume of the solution was measured to determine FS. The water-holding capacity (WHC) and oil-holding capacity (OHC) were evaluated according to [30], with modifications. The samples were homogenized with distilled water or soybean oil at a ratio of 1:10 (w/v) for 1 min in previously tared tubes, left to rest for 30 min, and centrifuged at 2000 × g for 15 min at room temperature. Finally, the supernatant was discarded and the tubes containing the absorbed samples were weighed. WHC and OHC were determined by the difference between the initial weight (Wi) and final weight (Wf) of the samples.

The emulsification capacity index (EAI) and emulsification stability index (ESI) were determined according to the methods of Pearce and Kinsella [31]. Briefly, a solution with 0.06 g of the sample and 30 mL of distilled water was adjusted to pH 4.0, 7.0, and 10.0, with HCL (1 M) and NaOH (1 M). This mixture was homogenized with 10 mL of soybean oil in Ultra-Turrax at 10,000 rpm for 1 min at 25 ºC and 50 µL was diluted (0.1%, w/v)

in 5 mL of sodium dodecyl sulfate (SDS). The absorbance was measured at 500 nm at reaction times of 0 and 10 min to determine EAI and ESI, respectively [28].

2.6. Antimicrobial properties

The minimum inhibitory concentrations (MIC) of hydrolysates against *Vibrio corallilyticus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Escherichia coli* were determined by the broth microdilution method using 96-well polyethylene microplates according to the Clinical and Laboratory Standards Institute protocol [32]. 50 µL of Mueller Hinton (MH) broth, five serial concentrations of each protein hydrolysate sample (800.0, 400.0, 200.0, 100.0; and 50.0 mg/mL), and 50 µL of inoculum (5.10^5 cells/mL) were incubated at 37 °C for 24 h. Ciprofloxacin (32 µg/mL) was used as a positive control. After incubation, 15 µL of resazurin (0.02%) was added to each well, and the plate was incubated again for 1 h at 37 °C [33]. The MIC was estimated by colorimetric interpretation; pink indicates metabolic activity, and blue indicates no metabolic activity. All analyses were performed in duplicates.

2.7. Bioaccessibility

2.7.1. In vitro simulation of gastrointestinal digestion (GID)

The bioaccessibility of the flour and insect hydrolysate samples was evaluated using the *in vitro* test method applied by de Quadros et al. [34]. The simulation consisted of two phases: gastric digestion and intestinal digestion. The gastric phase was carried out by mixing pepsin (4 g/100 g of sample on a dry basis) and phosphate buffer (35 g/L) at pH 2.0, followed by incubating at 37 °C for 2 h. Subsequently, the intestinal digestion phase was performed by adjusting the solution to pH 5.3 with $NaHCO_3$ (0.9 M), followed by a new adjustment to pH 7.5 with NaOH (1 M), with the addition of pancreatin (4 g/100 g

sample on a dry basis), and homogenization at 37 °C for 4 h. The gastrointestinal simulation was completed by inactivating the solution at 90 °C for 10 min, centrifuging at 9000 x g for 30 min 25°C , and lyophilizing the supernatant. The generated product was used to evaluate the protein bioaccessibility and antioxidant capacity.

2.7.2. Antioxidant properties

Antioxidant activity assays were performed before and after the gastrointestinal simulation at a concentration of 2.5 mg/mL.

2.7.2.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was estimated as described by [22]. Briefly, 200 µL of the sample was mixed with 3 mL of DPPH methanolic solution in glass tubes and kept in the dark for 30 min at room temperature, and the absorbance was measured at 517 nm. DPPH assays were used to estimate radical scavenging activity (%).

2.7.2.2. 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was estimated using the method described by [35]. Initially, a stock solution of 7 mmol/L ABTS radicals was prepared with 140 mmol/L potassium persulfate (PBS, pH 7.4) and kept in the dark for 16 h at room temperature. To determine the values, the stock solution was first diluted with methyl alcohol to an absorbance between 0.8 and 1.0 at 734 nm. Then, 200 µL of the sample was mixed with 150 mL of diluted ABTS, and after 6 min of reaction in the dark at room temperature the absorbance was measured at 734 nm. ABTS assays were used to estimate radical scavenging activity (%).

2.7.2.3. Iron reducing power (FRAP)

The iron reducing power (FRAP) was estimated according to the methodology described by Santos and Martins [36]. Distilled water (270 µL) and the sample (90 µL) were mixed

with 2700 µL of FRAP solution (50 mL of buffer solution, 5 mL of TPTZ (2 4 6-tris(2-piridil)-s-triazina), and 5 mL of $FeCl_3$) and incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm. FRAP using a standard curve of ferrous sulfate in relation to the equivalent antioxidant capacity of ferrous sulfate per gram of sample (µM $FeSO_4$/g).

2.8. Statistical analysis

The assays were performed in triplicate. Data were evaluated using a one-way analysis of variance (ANOVA). Previously, ANOVA assumptions were evaluated using the Shapiro Wilks test (normality) and Levene's test (homogeneity of variances). Statistical differences between the treatments were analyzed using Tukey's test. In all the cases, the significance level was set at 5%.

3. Results and Discussion

3.1. Degree of hydrolysis (DH)

The protease type exerts an important effect on the protein substrate because of the affinity of the enzyme to the substrate, cleaving the protein chain, and generating smaller particles [21]. Furthermore, the antioxidant, antimicrobial, and functional capacities of hydrolysates depend on the DH, and generally, lower DH levels can significantly enhance the biological activity of the hydrolysate. During a period of 300 min of hydrolysis (**Fig. 1a**), insect protein hydrolysates with different proteases showed DH values of 23.20±5.7%, 17.81±1.3%, and 5.20±1.0% for HA, HP, and HF, respectively. The use of Alcalase provided a higher DH than other enzymes, which can be explained by its mechanism of action by breaking peptide bonds within the protein chain and due to the affinity of the for insect flour [37]. This behavior was observed for cricket protein and mealworms with the use of Protease A, whereas lower DH levels were observed in samples after hydrolysis with Flavourzyme [9].

3.2. Amino acid and total protein content

The amino acid composition is related to the characteristics of the raw material and its nutritional content. Enzymatic hydrolysis can improve the biological activities of raw materials. Compared to superworm flour, the hydrolyzed samples showed an increase in the content of total amino acids (TAA), hydrophobic amino acids (HAA), and essential amino acids (EAA) by up to 36%, 41%, and 48%, respectively (**Table 1**). Because of the higher DH, which is indicative of a greater rupture of the protein chain in the enzymatic hydrolysis process, the HP and HA samples presented similar EAA, HAA, and TAA contents, which were higher than those of HF. Each protease acts in a different manner to break down the protein chain, releasing different levels or types of amino acids from the same sample. Previous studies have shown that hydrolysates rich in EAA, HAA, and TAA can contribute to the formation of functional protein aggregates, increasing their efficiency in gastrointestinal system adsorption and offering better bioactivity properties [38].

3.3. Functional groups, Water activity (a_W) and morphological characteristics

The FTIR spectra of the hydrolysate samples are shown in **Fig. 1b**. The spectra of the hydrolysates showed bands in the regions of 3560-3384 cm^{-1} (O-H stretching), 3225-2995 cm^{-1} (C-H stretching), 1616-1608 cm^{-1} (C=C stretching), 1480-1465 cm^{-1} (angular deformation of CH and CH_2 bonds), 1195-1180 cm^{-1} (C-O stretching) and 780-630 cm^{-1} (vibrations of aromatic rings and halide compounds). The hydrolysate samples showed amide I and amide II bands (1616-1608 cm^{-1}), as reported in other studies [16,39] however, enzymatic breakdown of the structure resulted in lower intensity in HA, possibly due to lower disruption of amide I and II bonds [40]. Enzymatic hydrolysis with different proteases also showed lower intensities in peaks 6 and 7 for hydrolysate

produced with Flavourzyme and peak 8 in hydrolysates with Protamex, respectively, due to lower exposure of the carboxyl groups and aromatic rings.

The SF (Superworm flour), HA, HP, and HF samples presented a_w of 0.414, 0.058, 0.087, and 0.244, respectively (Fig. 1c). a_w values lower than 0.06 indicate greater stability in the growth of bacteria, yeasts, and molds that spoil the products [41]. Furthermore, the lower a_W values in hydrolyzed samples may be related to the osmoregulatory effects of free amino acids and low-molecular-weight peptides, given its incorporation as an ingredient, reducing moisture due to the increase in solids in the formulation, favoring the effectiveness of the hydrolyzate in reducing water activity [42].

The hydrolysate samples presented a fragmented form with irregular particles and rough surfaces (**Fig. 2**). The shape and size of the particles are related to the specificity of each enzyme (endo- and exopeptidases). The presence of fragmented particles is characteristic of hydrolyzed products, which may occur because of the breakdown of the protein structure during enzymatic hydrolysis. Smaller particles were found in hydrolysates with Alcalase and larger particles with Flavourzyme. Variations in particle sizes between samples may have been influenced by enzyme activity during the hydrolysis process, such as DH, time, temperature, and the mechanism of action of the enzyme [43].

3.4. Functional properties

Functional properties are important characteristics for evaluating an ingredient to be applied in food or feed products. The hydrolysis process allows hydrophilic groups to become more available, which may contribute to the greater solubility compared to the raw sample (**Fig. 3a**), which was observed in this study. A lower solubility was observed in native insect flour at all pH values ($p < 0.05$). The higher protein solubility index in hydrolysates than in flour can be explained by the breakdown of protein units during the

hydrolysis process, favoring greater interactions between peptides (proteins with lower molar mass) and hydrogen bonding interactions with water molecules [44]. Furthermore, hydrolysates with a higher DH generally have greater solubility than those with a low DH [21], which was confirmed in this study. Hydrolysates with higher DH have more polar residues, such as ionizable amino and carboxylic groups [45][46], which promote electrostatic repulsion and increase the formation of hydrogen bonds with water [47], contributing to greater solubility. Solubility aids in the mixing and homogenization of ingredients in a product formulations.

All hydrolysate samples showed low water holding capacity (WHC) compared to native insect flour (**Fig. 3b**). This behavior is related to the high solubility of hydrolysate samples, as previously reported. WHC can be influenced by several factors such as amino acid profile composition, protein concentration, conformity, pH, hydrophobicity, and temperature [48]. Lower WHC values in hydrolysate samples can also be correlated with the composition of soluble peptides that cannot retain water, experimental conditions, and parameters used in the hydrolysate process [49]. Using hydrolysis conditions of pH 8.5, 3% E/S (enzyme/substrate) concentration, and Alcalase as protease, Dion-Poulin et al. [50] produced two types of protein hydrolysates: cricket hydrolysate (*G. sigillatus*) and bran hydrolysate (*Tenebrio molitor*). The authors reported a higher WHC in all pH ranges evaluated for the cricket hydrolysate. The higher WHC in native flour is related to a composition rich in hydrophilic compounds (chitin, lipids, etc.).

The oil retention capacity (OWC) was higher for the hydrolysates, and the hydrolysis process and protein size favored a higher ORC. In addition, hydrolyzed proteins are structurally modified, exposing hydrophobic amino acids from native insect flour and promoting oil trapping [51–53]. As shown in **Fig. 3b**, the HP and HF samples did not exhibit significant differences ($p<0.05$). A lower value was obtained for the HA sample.

These results may be related to the proteolytic mechanism of action of the enzymes and the lower DH obtained in the hydrolysates with Protamex and Flavourzyme.

Figure 3c shows the influence of pH and the enzyme used on foaming capacity (FC) and foam stability (FS). The hydrolysate samples exhibited a low foaming capacity (5-15%). Low values or no foam formation and stability have been reported in the literature owing to the lipid content of insects [44]. Compared with flour, the hydrolysates presented FC values greater than or equal to pH 6.0. The hydrolysate obtained by Flavourzyme demonstrated greater foaming capacity at pH 4.0 and Protamex at pH 2.0, and the values of the three hydrolysates were statistically (p>0.05) similar at pH 6.0 and 10.0, indicating that the specificity of breaking the bonds of each enzyme can release amino acid residues with improved foaming properties depending on pH [28]. The hydrolyzed samples showed higher FC at all evaluated pH values compared to native insect flour, due to enzymatic hydrolysis generating low molar mass peptides and producing residues that stabilize on the air-water surface, contributing to rapid migration, flexibility, and rearrangement of the interface, thereby improving foaming properties [47][54]. In contrast, foam stability (**Fig. 3d**) was lower in the hydrolysates than in native insect flour. This behavior may be related to the lower molar mass of the hydrolysate, which affects the resistance of the film interface and the formation of foam [20]. Larger HF particles, as shown in the SEM images, may have generated less stability in the foam compared to HP and HA.

The emulsion index (EAI) and emulsion stability (ESI) varied within this pH range. The HP and HF samples presented higher emulsification values at pH 10.0 (**Fig. 3e**), whereas SF and HA had higher values at pH 7.0. The emulsion capacity of hydrolysates is associated with the amphipathic nature of the peptides produced during hydrolysis [55], and the emulsifying properties can be improved at alkaline pH because of the unfolding

of negatively charged polypeptide chains in this pH range [28]. The HP and HF treatments were statistically similar (p<0.05), which may be related to the fact that the OHC values in both samples were similar, contributing to this effect on emulsification. Furthermore, the lower capacity of HA at pH 10.0 may be related to the lower OHC in HA, reducing its ability to interact with oil-water molecules and the emulsification capacity. In contrast, lower emulsifying capacity values were found at pH 4.0, which was expected because the pH 4.0 is close to the isoelectric point of insect proteins, where the protein molecules precipitate, reducing their emulsifying properties [28]. Regarding emulsion stability (**Fig. 3f**), the activity of the hydrolysates was reduced due to the generation of peptides with a small molar mass during hydrolysis, which have rapid migration and adsorption at the interface [56] and are unable to unfold and reorganize itself [47]. In addition, the composition and distribution of amino acids produced during hydrolysis can influence the strength of the interfacial layer, thereby minimizing the emulsion stability [20][57]. The hydrolysates presented varied techno-functional performances depending on the pH, enabling their application in the development of various products.

3.5. Antimicrobial properties

Table 2 shows the antimicrobial activities (minimum inhibitory concentration - MIC) of the protein hydrolysates. The HA and HP exhibited an inhibitory effect against the Gram-negative microorganisms evaluated (*V. coralliyticus*, *P. aeruginosa*, *A. baumannii* and *E. coli*), while HF did not show inhibition at all concentrations tested. None of the samples exhibited an inhibitory effect on *S. aureus*. Our findings were better compared than those of Sousa et al.[58], where tenebrio hydrolysate samples (*Alphitobius diaperinus*) did not show antimicrobial effects. A concentration of 400 mg/mL of HA was the most efficient against *V. coralliyticus*, while the MIC for HP was 800 mg/mL. This effect can be explained by the higher degree of hydrolysis of HA compared to HP and HF, making

compounds with antimicrobial action more available to act against microorganisms (MOO). Our results can be explained by the products obtained at the end of the hydrolysis process, where specific amino acids with antimicrobial effects were released, for example, arginine and lysine, at higher concentrations in HP (1.32 and 16.05%) and HA (1.04 and 19.84%), respectively. The with low DH in HF resulted in lower antimicrobial activity.

3.6. In vitro bioaccessibility

3.6.1. Antioxidant capacity

Antioxidant properties evaluated by DPPH, ABTS, and FRAP assays were performed before and after the gastrointestinal simulation (**Table 3**). Our results suggest that for the ABTS, DPPH, and FRAP radicals, the hydrolysates showed greater activity than the insect flour sample because of the release of essential amino acids that confer greater bioactivity and bioavailability.

Since DPPH has a greater capacity for activity with lipophilic bioactive compounds, due to the lower DH of HP and HF, there is an indication that these samples have a higher content of lipophilic compounds (amino acids), precisely because they had the highest OHC. Therefore, they can react and reduce DPPH radicals more efficiently than HA can. The ability to scavenge DPPH radicals was greater in superworm hydrolysates with a lower DH obtained at the end of 300 min of hydrolysis. This lower DPPH radical scavenging capacity was also reported by [59] using the enzyme Alcalase in mealworm hydrolysis. However, it is known that hydrolysates with lower DH exhibit weak radical scavenging activity, which improves with an increase in DH [58][60]. The hydrolysis process with different enzymes can provide peptides with different hydrophobic amino acid sequences [58][61], which influence the scavenging of the DPPH radical.

The ABTS radical scavenging results shown in Table 3 exhibit higher ABTS values for the hydrolysates than for the native insect flour. The ABTS radical is more susceptible to hydrophilic compounds and as hydrolysates are highly soluble, they are able to spread their bioactive action and eliminate ABTS radicals more efficiently than flour [62]. Regarding FRAP, due to the high degree of hydrolysis of HA and HP, they presented greater antioxidant capacity than HF, indicating that such samples are capable of releasing amino acids (cysteine and tryptophan) and oligopeptides with chelating effects [63][64] during hydrolysis.

After gastrointestinal simulation (GID), the DPPH radical scavenging assay showed a decrease in activity (**Table 3**) in all hydrolysates, with lower values for HA and HP (4.25 and 4.74 %, respectively). Insect meal contains more compounds that can affect antioxidant activity after digestion, such as fats (fatty acids), which also have antioxidant effects, mainly against DPPH. HF may have maintained its action against DPPH due to its low DH, indicating that the lipophilic structure of the amino acids was still intact and was not affected by the simulated gastrointestinal digestion (GID). Unlike HP and HF, which could already have a more "damaged" structure. Peptide fractions are more soluble in water after digestion, which reduces their ability to eliminate this radical [65]. There was a decrease in ABTS radical scavenging capacity ($p<0.05$) in the hydrolysates exposed to GID, and HA showed greater activity than the other hydrolysates. A similar behavior was observed in the ferric reduction assay (FRAP), in which the HA and HP samples showed a decrease in activity, but were higher than the other samples. HF has less action against ABTS because of its higher content of lipophilic bioactive compounds, whereas HP and HA have a higher hydrophilic content.

3.6.2. Protein content and bioaccessibility

The protein content and bioaccessibility results after GID are presented in **Table 3**. As previously reported, enzymatic hydrolysis favored an increase in protein content in all hydrolysates compared with native insect flour (47.09%). Protein content is related to DH, mainly due to greater protein availability, enzymatic effects, and separation of other high-proportion components found in flour, such as lipids. Our results showed that after the GID process, protein values decreased in the hydrolysates, whereas the protein content of insect flour was high, demonstrating the bioaccessibility of the protein after GID. The digestibility of insect proteins *in vitro* can be affected by components such as chitin, which is not degraded or absorbed by the intestine [66,67].

4. Conclusion

The present study demonstrated that the hydrolysis process contributed to an increase in the protein content and content of total amino acids in superworm flour. Hydrolysates obtained using Alcalase and Protamex showed greater antimicrobial activity against gram-negative bacteria, good antioxidant activities, and techno-functional properties (i.e., oil retention, foam formation, and emulsifying capacity). Such hydrolysate samples were bioaccessible after gastrointestinal digestion, showing that the enzymatic hydrolysis process is a promising alternative for obtaining functional and emerging ingredients based on superworm flour, which can be capable of being used in the development of products for human or animal food.

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FIGURES

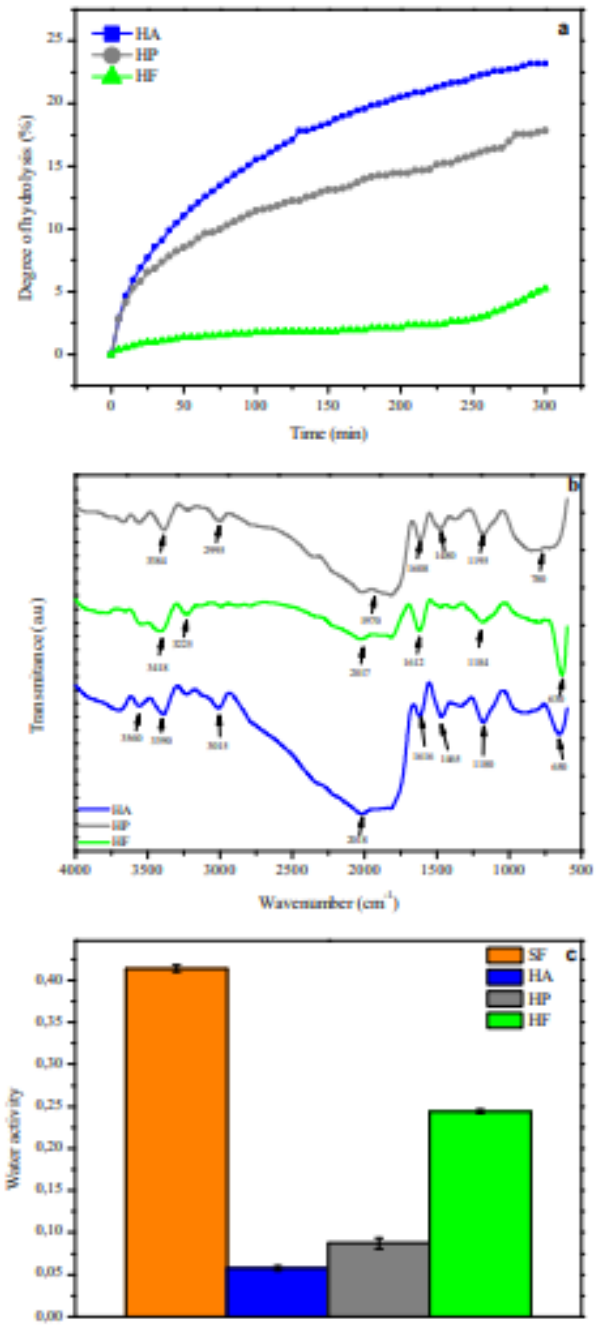

Figure 1. Parameters of insect protein hydrolysates: **(a)** degree of hydrolysis, **(b)** Fourier transform infrared spectroscopy (FTIR), and **(c)** water activity.

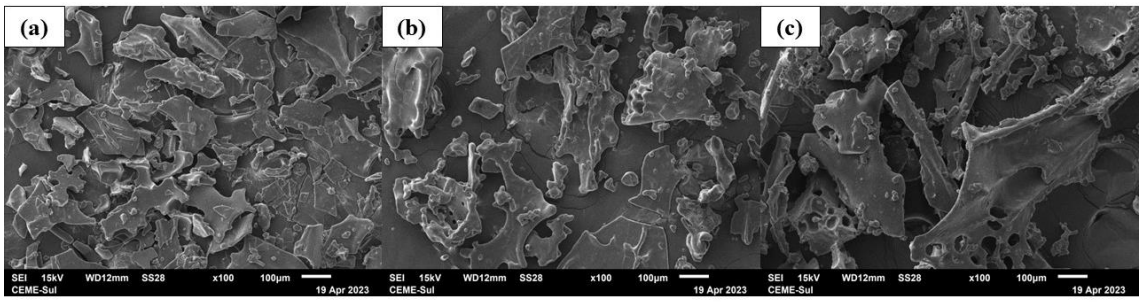

Figure 2. Morphological characteristics of **(a)** Alcalase hydrolysate, **(b)** Protamex hydrolysate, and **(c)** Flavourzyme hydrolysate

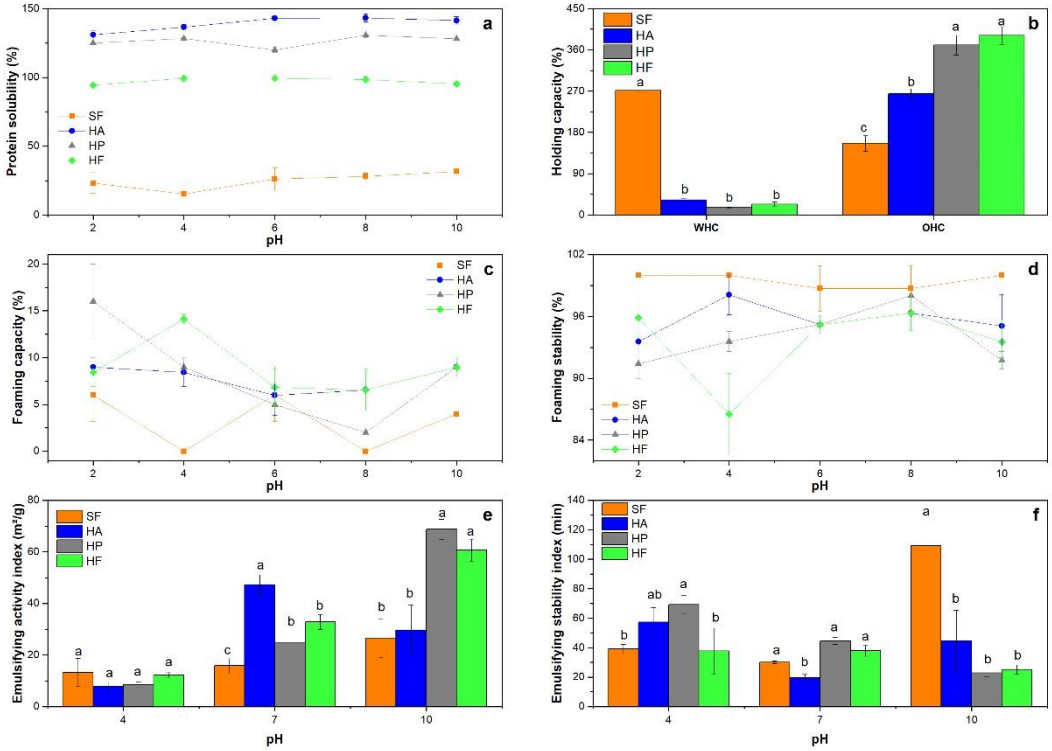

Figure 3. Functional properties from protein hydrolysate insect: **(a)** protein solubility, **(b)** water and oil holding capacity, **(c** and **d)** foaming properties, and **(e** and **f)** emulsifying properties. (n=3) Different lowercase letters superscript in the s demonstrates significant differences ($p<0.05$).

TABLES

Table 1. Amino acid composition and total protein content of native insect flour (SF) and protein hydrolysates using enzymes Alcalase (HA), Protamex (HP) and Flavourzyme (HF).

Amino acid (%AA/g protein)	SF	AH	PH	FH
Aspartic Acid	1.70 ± 0.26^{a}	2.34 ± 0.24^{a}	1.81 ± 0.46^{a}	2.32 ± 0.25^{a}
Threonine	0.51 ± 0.02^{c}	0.80 ± 0.02^{b}	0.98 ± 0.04^{a}	0.77 ± 0.01^{b}
Serine	0.68 ± 0.13^{a}	0.87 ± 0.09^{a}	0.98 ± 0.21^{a}	0.79 ± 0.15^{a}
Glutamic acid	1.41 ± 0.56^{a}	1.52 ± 0.39^{a}	2.22 ± 0.71^{a}	2.55 ± 0.70^{a}
Proline	7.62 ± 0.40^{b}	8.47 ± 0.44^{ab}	8.90 ± 0.45^{a}	8.82 ± 0.43^{a}
Glycine	1.16 ± 0.04^{a}	0.84 ± 0.78^{a}	1.64 ± 0.10^{a}	1.14 ± 0.07^{a}
Alanine	2.15 ± 0.11^{b}	3.21 ± 0.01^{a}	3.37 ± 0.02^{a}	2.23 ± 0.08^{b}
Valine	0.86 ± 0.01^{c}	1.29 ± 0.02^{ab}	1.24 ± 0.01^{b}	1.34 ± 0.05^{a}
Methionine	0.17 ± 0.01^{b}	0.28 ± 0.01^{b}	1.06 ± 0.81^{a}	0.18 ± 0.00^{b}
Isoleucine	0.51 ± 0.01^{c}	0.72 ± 0.01^{b}	0.68 ± 0.01^{b}	0.92 ± 0.05^{a}
Leucine	0.83 ± 0.64^{b}	2.24 ± 0.04^{a}	2.18 ± 0.10^{a}	1.94 ± 0.01^{a}
Tyrosine	3.25 ± 0.02^{c}	4.59 ± 0.05^{a}	4.33 ± 0.10^{b}	2.41 ± 0.08^{d}
Phenylalanine	0.64 ± 0.03^{c}	1.00 ± 0.03^{a}	0.86 ± 0.04^{b}	0.65 ± 0.00^{c}
Histidine	0.45 ± 0.01^{c}	0.63 ± 0.05^{b}	0.80 ± 0.10^{a}	0.71 ± 0.05^{ab}
Lysine	14.14 ± 0.42^{c}	19.84 ± 0,.5^{a}	16.05 ± 0.32^{b}	14.31 ± 0.20^{c}
Arginine	0.43 ± 0.04^{b}	1.04 ± 0.18^{a}	1.32 ± 0.17^{a}	1.05 ± 0.14^{a}
EAA (% AA/g protein)	18.14	26.83	23.88	20.86
TAA (% AA/g protein)	36.60	49.75	48.48	42.19
HAA (% AA/g protein)	15,52	21,08	21,94	17,67
Total Protein (%)	47.09 ± 1.10^{c}	68.74±1.47^{a}	63.48±6.31^{ab}	57.63±5.32^{b}

EAA = essencial amino acid; TAA = total amino acid; HAA = Hydrophobic amino acids. Different letters in the same line indicate statistical difference ($p < 0.05$).

Table 2. Minimum inhibitory concentration (MIC) of protein hydrolysates of superworm (*Zophobas morio*) using the enzymes Alcalase (AH), Protamex (PH), and Flavourzyme (FH) against different bacterial strains.

Samples	MIC Concentration (mg/mL)				
	Vibrio coralliyticus	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Acinetobacter baumannii*	*Escherichia coli*
AH	400	800	NI	800	800
HP	800	800	NI	800	800
FH	NI	NI	NI	NI	NI

NI: no inhibition

Table 3. Bioaccessibility of antioxidant activity and total protein content of superworm *Zophobas morio* flours (SF) and their hydrolysates using enzymes Alcalase (HA), Protamex (HP), and Flavourzyme (HF).

Samples	Gastrointestinal digestion		Gastrointestinal digestion	
	DPPH radical-scavening (%)		ABTS radical-scavening (%)	
SF	8.47±1.03^{c}	26.70±2.97^{a}	45.71±1.30^{b}	49.76±2.83^{bc}
HA	15.87±0.81^{b}	4.25±1.15^{b}	95.74±0.56^{a}	79.07±1.53^{a}
HP	21.10±0.42^{a}	4.74±0.14^{b}	94.96±0.33^{a}	74.65±5.83^{a}
HF	27.53±0.99^{a}	22.30±2.00^{a}	93.19±3.14^{a}	57.95±8.31^{b}
	FRAP (μM FeSO4/g)		Total Protein (%)	
SF	503.0±39.0^{c}	22.33±26.0^{b}	47.09±1.10^{c}	47.36±1.1^{a}
HA	1479.66±50.3^{a}	167.0±5.0^{a}	68.74±1.47^{a}	49.40±1.07^{a}
HP	1514.66±16.0^{a}	143.0±36.0^{a}	63.48±6.31^{ab}	45.84±2.15^{a}
HF	1316.33±15.2^{b}	133.6±20.2^{a}	57.63±5.32^{b}	37.88±0.88^{b}

Different letters in the same column indicate statistical difference ($p < 0.05$).

CAPÍTULO 3

Structural, functional and biological activity (*in vitro* and *in vivo*) characteristics of microencapsulated superworm protein hydrolysate

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Structural, functional and biological activity (*in vitro* and *in vivo*) characteristics of microencapsulated superworm protein hydrolysate

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Abstract

This study aimed to microencapsulated protein hydrolysates from superworm (*Zophobas morio*) procuced by Alcalase (HA) and Protamex (HP) by spray drying, using maltodextrin and gum arabic as wall material. The physicochemical aspects and *in vitro* antioxidant capacity were evaluated to understand the effect of the enzyme type and wall material on the microencapsulated samples. Both microencapsulated hydrolysate samples showed low water activity (<0.258) and reduced hygroscopicity (<26.86%). The microcapsules presented a spherical shape (particle size ranging from 230.74 to 419.69 nm) and infrared spectroscopy showed that the hydrolysates were encapsulated, evidencing the wall material-hydrolysate interaction. The *in vivo* study showed that treatments with hydrolysate produced with Protamex contributed significantly to the biological aspects (growth and survival) of the nematode *Caenorhabditis elegans* even under conditions of oxidative and thermal stress, including survival and growth. Therefore, the microencapsulation process may be an alternative to maintain the stability and bioactive capacity of the hydrolysates, acting as a protective agent against stress conditions in *C. elegans*, indicating its potential use as a nutraceutical supplement.

Keywords: Insect protein hydrolysate, wall material, spray-drying, bioactivity.

1. Introduction

There is great interest in insect proteins as supplements and food additives based on their nutritional composition and biological properties that have contributed to animal nutrition. Insect proteins hydrolyzed via enzymatic procedures have been shown to be effective because of their spectrum of bioactivities such as antioxidant, antimicrobial, and functional properties (Dion-Poulin et al. 2020, Fashakin et al. 2023, Flores et al. 2020). In addition, their incorporation into diets for feeding aquatic organisms contributes to the antioxidant and immune systems, and intestinal microbiota (Mikolajzak et al. 2020; Roques et al., 2020). However, there are some limitations to be overcome before more effective use of insect bioactive proteins, such as their solubility in water and hygroscopicity. In a previous study, the degree of hydrolysis (DH) was found to negatively influence the water solubility of insect hydrolysates (unpublished data). Therefore, microencapsulation is a technique that can minimize the effect of protein hydrolysates.

The microencapsulation process of insect protein hydrolysate can minimize the negative effects mentioned above, and the spray-drying technique is used by the food industry because it produces powders with high stability, process simplicity, and low thermal stress (Simoes et al. 2017). Furthermore, microencapsulation allows bioactive molecules to be maintained in a polymeric matrix and their release to be controlled, minimizing degradation and sufficient absorption of the encapsulated compound (Feng et al. 2022). Numerous studies have described the efficiency of protein microencapsulation through a polysaccharide matrix (Parandi et al. 2024; Chen et al. 2021; Akbarbaglu et al., 2019). Among the wall materials used in this process, carbohydrates such as maltodextrin (MD) and gum arabic (GA), are commonly used because of their characteristics such as ease of handling, ability to form protective capsules, and good stability under storage conditions

(Annamalai et al. 2020, Pieczykolan et al. 2019). No studies were found on the microencapsulation of superworm protein hydrolysates by spray drying.

In vivo evaluation using animal models provides useful information on possible effects that may occur during the use of protein hydrolysates as a food source, with physiological analyses being a support for predicting the effects of supplying encapsulated bioactive compounds (Lima et al. 2021, Chen et al. 2020; Zhou et al., 2018). Among animal models, the nematode *Caenorhabditis elegans* is an attractive model for evaluating physiological responses after *in vivo* exposure to bioactive compounds, such as antioxidants, due to its ease of manipulation and high reproduction rate (Benedetto et al., 2019). A previous study by our group (Lima et al. 2021) showed that microencapsulated protein hydrolysates from the fish *Cynoscion guatucupa* improved the growth and reproductive status of *C. elegans.* These results prompted us to evaluate the effects of supplying microencapsulated superworm protein hydrolysates on physiological parameters under thermal and oxidative stress conditions in the same biological model.

Thus, the present study aimed to evaluate the effect of microencapsulation of insect protein hydrolysate (*Zophobas morio*) using different wall materials (maltodextrin and gum arabic) on physicochemical, structural characteristics and bioactivities *in vitro*. In addition, we verified the *in vivo* effects on resistance to thermal and oxidative stress as well as physiological parameters (survival and growth) in the nematode *C. elegans*.

2. Material and methods

2.1. Materials

Dehydrated samples of superworms (*Z. morio*) obtained from Insetos Kaisara (Além Paraíso, MG, Brazil) were crushed in a homogenizing processor (Walita Mega Máster Plus) for 5 min to obtain insect flour and sieved through a sieve of 42 μm., The proteolytic enzyme Alcalase (Novozymes Latin America) and Protamex (Sigma-Aldrich) were used

for hydrolysis process. Maltodextrin (Dextrose equivalency/DE - 20) and gum arabic was purchased from Pryme Foods (Sorocaba, Brazil). The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl radical was purchased Sigma. All the other chemicals used were of analytical grade.

2.2. Production of protein hydrolysates

The protein hydrolysates were prepared according to the method described by Hall et al. (2017). The insect flour was homogenized in distilled water at a proportion of 10% (w/v; protein/distilled water) at 90 °C for 15 min to inactivate endogenous enzymes. Subsequently, the mixture was kept under mechanical stirring at 50 ºC and the optimum pH of Alcalase (pH 8.0) and Protamex (pH 7.0) was achieved by adding NaOH solution (1 M). The hydrolysis process was initiated by addition of the enzyme in a proportion of 3% (w/w; enzyme/protein) and, after 5 h of process, the solution was submitted to enzymatic thermal inactivation (90 °C for 10 min), followed by centrifugation (11,806 × g for 20 min at 35 °C) and the protein hydrolyzed fraction of the supernatant was lyophilized (Liotop, L108) and stored at -18 °C until further analysis. The degree of hydrolysis (DH) was determined by the pH-stat method (Adler-Nissen, 1986).

2.3. Encapsulation process

The hydrolysate samples obtained with the enzymes Alcalase and Protamex are called as HA and HP, respectively. For encapsulation by spray drying, two formulation of wall material was used. For the first treatment (En1), the MD was used according to the process adopted by Lima et al. (2021) and for the second treatment (En2), a mixture of MD and GA in the proportion 1:3 (MD:GA) described by Fernandes et al. (2021) was used. For encapsulation, the wall materials and hydrolysates were homogenized in distilled water at room temperature (around 25 ºC), constituting 10% of the total solids. Then, the

solutions were atomized using a Mini Spray-Dryer (Labmaq, Model PS1, Brazil) at 0.3 L/h with an inlet temperature of 120 ºC, air flow of 1.65 m^3/h, air atomization pressure of 5 bars, and drying air with 40% relative humidity (RH). The encapsulated hydrolysates were named HAEn1 (obtained with Alcalase enzyme and MD), HAEn2 (obtained with Alcalase enzyme and MD+GA), HPEn1 (obtained with Protamex enzyme and MD), and HPEn2 (obtained with Protamex enzyme and MD+GA). The yield of the encapsulation process was calculated as the ratio of the mass of the powder produced in relation to the total solids content in the spray drying solution and the initial amount in the dispersion volume and was expressed as a percentage (Akbarbaglu et al., 2019).

2.4. Morphological characterization

The morphology of the free and microencapsulated hydrolysates was visualized by Scanning Electron Microscopy (SEM; JSM 6610LV, JEOL) at 15 kV with magnifications of 100× and 1000×. The hydrolysates were placed on aluminum stubs with carbon tape and covered with a layer of gold using a Denton Vacuum (Desk V, USA).

2.5. Physicochemical characterization

2.5.1. Moisture, water activity (Aw), water solubility (WS) and hygroscopicity

Moisture values were determined according to the official protocol proposed by the AOAC (2006). Water activity (Aw) was measured using an analysis equipment (Novasina, model LabTouch) at room temperature (around 25 °C). Water solubility (WS) was estimated according to the method described by Choi et al. (2019), with modifications. Approximately 0.5 g of the sample was dispersed in 50 mL of distilled water, followed by stirring on a magnetic stirrer for 30 min. Subsequently, the dispersions

were centrifuged at 8667 x g for 20 min at 4 ºC. The % WS was estimated by multiplying the dry weight of the supernatant and the sample weight multiplied by 100.

The hygroscopicity of the microencapsulated hydrolysate samples was determined according to the protocol described by Cai and Corke (2000), with modifications. Approximately 100 mg of the sample was weighed and placed in a desiccator containing a saturated NaCl solution (RH ≈75%) at room temperature for 7 days. After this period, the samples were weighed, and the hygroscopicity was expressed in g of adsorbed moisture per 100 g of dry sample.

2.5.2. Protein contend and color parameters

Total protein content was determined for the free and microencapsulated hydrolysate samples (10.0 mg/mL of protein diluted in distilled water) using the Biuret method (Amado et al., 2009). Briefly, 20 µL of the sample and 1 mL of Biuret reagent were added to a 2 mL eppedendorf tube, vortexed for 2 min, and left to rest for 10 min. After this time, the samples were placed in transparent 96-well plates and the reading was performed on a microtiter plate reader (Biotek Synergy HT) at a wavelength of 550 nm

The color parameters (*L*, *a**, and *b**) of the spray dried powders were using a colorimeter equipment (Minolta, model CR400, Japan) with the CIELab scale. Measured color attributes were lightness (L^*, ranges between 0-black and 100-white), red/green chromaticity [a^*, ranges from negative (green) to positive values (red)], and yellow/blue chromaticity [b^*, ranges from negative (blue) to positive values (yellow)].

2.6. Zeta potential and particle size

The zeta potential and particle size values in the microencapsulated insect hydrolysate samples (0.025 mg/mL, diluted in distilled water) were determined using a

electrophoretic light scattering technique (Litesizer^{TM} 500, Anton Paar, Austria). Three parallel tests were performed.

2.7. Encapsulation efficiency

The encapsulation efficiency of the samples was estimated from the total protein content of the free hydrolysates and the microencapsulates obtained after spray drying and expressed as a percentage (%).

2.8. Funcional groups (FTIR) and thermal analysis (DSC)

The functional groups were analyzed by Fourier transform infrared spectroscopy (FTIR) using a spectrometer (IRPrestige-21, SHIMADZU), and the spectra patterns were measured in the range of 650–4000 cm^{-1} in 16 scans with a resolution of 4 cm^{-1}. KBr was used to press the samples in this slice before spectral capture. The thermal properties were evaluated by differential scanning calorimetry (DSC) using a calorimeter (DSC60-Shimadzu) with a sealed aluminum pan and 1 mg of the sample. The temperature ranged between -25 to 300 ºC with a heat rate of 10 ºC/min and the onset temperature (T_0), temperature of denaturation (T_d), and enthalpy of denaturation (ΔH) were obtained from thermograms using the TA-60WS Software.

2.9. Amino acid composition

The amino acid profile of the free hydrolysate samples was determined using the method described by Santos and Martins (2024). The analysis was performed using high-precision liquid chromatography coupled with a fluorescence detector and commercial standards were used to identify and quantify the amino acids compounds of the samples.

2.10. In vitro activity

2.10.1. Antioxidant activity

The antioxidant activity of the microencapsulated hydrolysates was performed by ABTS and DPPH method using an aqueous solution (10.0 mg/mL). The (ABTS) radical scavenging activity was estimated using the method described by Chi et al. (2015). The scavenging of the DPPH radical was estimated as described by Zamora-Sillero et al. (2018).

2.11. In vivo assay

2.11.1. Experimental design

The wild-type strain *C. elegans* N2 (Bristol) was used for the experimental assays. The strain was maintained in accordance with the conditions described by Lima et al. (2021). The bacterium *Escherichia coli* OP50 (OD = 1 at 600 nm) was used as food in the culture on medium plates for nematode growth. To obtain a generation of L1 larvae, the animals were collected by filtration.

2.11.2. Experimental procedure

The assays were performed in S-basal liquid medium (5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 mL of cholesterol, and 5 mg/mL per 1 L of ultrapure water) containing the food source (*E. coli* OP50), free and microencapsulated hydrolysates, or wall materials. To determine growth, 20–30 L1 larvae were transferred to each well of a 24-well plate and exposed for 96 h. The treatments adopted in the assay were as follows: (1) control group containing only *E. coli*; (2) group containing *E. coli* and En1 (MD; final concentration 1 mg/mL); (3) group containing *E. coli* and En2 (MD+GA:1:3; final concentration 1 mg/mL); (4) group exposed to free Alcalase hydrolysate and *E. coli* (final concentration 1 mg/mL); (5) group exposed to HAEn1 treatment and *E. coli* (final

concentration 1 mg/mL); (6) group exposed to HAEn2 treatment and *E. coli* (final concentration 1 mg/mL); (7) group exposed to free Protamex hydrolysate and *E. coli* (final concentration 1 mg/mL); (8) group exposed to HPEn1 treatment and *E. coli* (final concentration 1 mg/mL); and (9) group exposed to HPEn2 treatment and *E. coli* (final concentration 1 mg/mL). The experiments were performed in triplicate and the animals were kept in the dark at 20 ºC.

2.11.3. Physiological parameters

The growth rate was evaluated as a physiological parameter based on the ISO 10872:2010 protocol. Briefly, after 96 h of exposure, 50 µL of Bengal Rose was added to each well, and the plates were heated at 60 ºC for 30 min for dye incorporation into the nematodes. Images of each well were obtained using a stereomicroscope with an attached camera (Leica S8 APO, São Paulo, SP, Brazil). All images were analyzed using the software ImageJ. Length was calculated by measuring the total body length (mm) of individuals worm from the top of the head to the tail. The growth was estimated as the difference between the length after 96 h and at time 0.

2.11.4. Oxidative and thermal stress resistance test

In assay 1, the resistance to oxidative stress was determined according to the method described by Zhou et al. (2018). L1 larvae (20–30 animals) were transferred to each well of a 24-well plate and exposed for 48 h to the corresponding treatment group (1, 2, 3, 4, 5, 6, 7, 8 and 9, described above). After 48 h, the worms were exposed to 10 mM H_2O_2 for 2 h. Finally, the animals were washed with S-basal and allowed to recover in the same medium for 12–16 h, after which live animals were counted. In assay 2, resistance to heat stress was determined according to the protocol described by Michalski et al. (2001), with

modifications. L1 larvae (20–30 animals) were placed on a plate and exposed for 48 h to the treatments . After 48 h, the worms were exposed to a temperature of 35 ºC for 6 h. After, the animals were kept at 20 ºC for 18 h for recovery, and the survival of the animals was evaluated.

2.12. Statistical analysis

The assays were performed in triplicate. Analysis of variance (ANOVA) was applied to evaluate statistical differences between treatments. Previously, Variance homogeneity and data normality were assessed using the Levene test and the Shapiro-Wilk test. Mean differences between treatments were assesed using the Tukey test. In all cases, significance level (α) was fixed in 0.05.

3. Results and Discussion

3.1. Yields of process

Insect microencapsulates with different wall materials showed spray drying yields of 12.80±0.07%, 17.52±3.77%, 13.52±2.07%, and 20.93±5.84% for HAEn1, HAEn2, HPEn1, and HPEn2, respectively. The conditions adopted (inlet temperature 120 ºC and pump flow 0.3 L/h) in the atomization process negatively influenced the yield. Fernandes et al. (2021) observed that low temperatures associated with high feed rates decreased the microencapsulation yield of chia oil obtained by the spray drying process. These authors reported that the process yield decreased from 50 to 40.3%T when conditions of 100 ºC and 0.3 L/h were used. The authors associated the reduction in yield with the shorter contact time of the drying air with the emulsion, which remained adhered to the wall of the drying cylinder. In this study, the high flow rate used was not sufficient to promote a high yield.

3.2 Morphological characterization

The scanning electron microscopy (SEM) images of the free hydrolysate and microencapsulated samples showing their distinct morphological characteristics are presented in Figure 1. The free hydrolysate samples (HA and HP) presented characteristics similar to those reported by Lima et al. (2021): large, irregular particles and rough surfaces. The microencapsulated hydrolysates had a smooth surface with concavities and were spherical in shape and of different sizes. Garzo et al. (2023) reported that higher concentrations of wall material in the microcapsules formulation can generate aggregation and serrated surfaces in the capsules, which was not observed in this study, showing that the formulation of the solutions are determinants of the morphology of the microcapsules. The presence of concavities occurs due to the loss of water in the spray drying process, causing the capsules to shrink and wrinkle (Favaro-Trindade et al. 2010).

3.2. Physicochemical characterization

Table 1 shows the physicochemical properties of insect hydrolysate samples microencapsulated with different wall materials. Moisture values range from 3.25 to 5.05%, these values are low and contribute to the storage, stability, transport and controlled release of active substances. In general, the samples that were microencapsulated with the mixture of MD and GA presented significantly lower values ($p<0.05$) than the other samples. The hydrolysate obtained with the enzyme Protamex (HPEn2) presented the lowest value (3.25±0.17%; Table 1). Pieczykolan et al. (2019) suggested that variations in moisture content are related to the ability of the wall materials to bind with water, being influenced by the hydrophilic groups present in their structural molecules.

All samples presented low Aw values (maximum value of 0.258 – HPEn1, Table 1) in the microcapsules produced with different wall materials, indicating a low capacity for

microbial growth. Lower water activity may be related to the low DE in capsules with the presence of MD (Lima et al. 2021). However, samples HAEn1 and HPEn1 showed higher values than samples containing MD and GA (Table 1). This may indicate that formulations with a higher moisture content have a higher amount of free water (a_w), being more susceptible to chemical, enzymatic and microbiological reactions.

The water solubility and surface charge (zeta potential) of the microencapsulated hydrolysate samples were evaluated to verify their behavior as a potential additive or enrichment for animal feed. All microencapsulated hydrolysate samples presented a water solubility of approximately 90%, and the type of enzyme used for hydrolysis or the encapsulating material did not contribute to significant differences ($p>0.05$). Higher solubility has been reported for formulations containing MD as a wall material compared to other materials, such as GA, MD, and Whey protein concentrate (WPC) (Jafari et al. 2019). Which may justify the results observed in these studies, where MD was used in the capsule formulations, in addition, protein hydrolysates are highly soluble. This result may be related to the particle size of the samples, as smaller particles have a larger surface area exposed to the solvent (Fazaeli et al. 2012). Furthermore, solubility can be correlated with moisture and Aw the lower these parameters, the greater is the solubility of the sample (Kalajahi et al. 2023).

Hygroscopicity refers to the ability of hydrolysates to absorb moisture from the environment, and this characteristic can affect the physicochemical properties and stability of the microcapsules (Akbarmehr et al. 2023, Sarabandi et al. 2018). The hygroscopicity values ranged from 20.12 to 26.86% (Table 1), showing no significant differences between the samples ($p> 0.05$). These results are consistent with the solubility data; for example, the wall materials provided the same percentage of solubility and hygroscopicity. Breternitz et al. (2017) reported that low-DE MD has a high molecular

mass, which is higher than protein hydrolysates, and is therefore considered hygroscopic, which may justify the results obtained in this study. Furthermore, protein hydrolysates favor the formation of films in the microcapsules with wall materials, leading to the migration of low-Mw proteins to the surface of the microcapsule (Jayasundera et al., 2011). Likewise, hydrophobic functional amino acids present in the hydrolysates can repel water molecules, reducing the affinity of the molecules to enter the microcapsules (Nottagh et al. 2020). Hydroxyl groups from the wall material and hydrolysates bonded together to form a cohesive network, and the available lipophilic or nonpolar compounds prevented high water uptake.

The production of microcapsules by spray drying can affect color parameters. The use of polysaccharides as wall material increases browning reactions (such as caramelization and Maillard reactions) when subjected to a hot-air drying process (Santos & Martins, 2024; Bohm et al. 2023). Table 2 shows the color parameters for the microencapsulated hydrolysates. The L^* values were > 50 and did not present significant differences ($p>0.05$) among the treatments, indicating that the samples showed a clear color. The same behavior was observed for the a* values, presenting low values, indicating that they tended towards a more greenish coloration, but not enough to have a noticeable color. In addition, the microcapsule samples had more positive b^* values, making them closer to yellow. The color of the wall materials (MD and GA) may be one of the reasons that influenced this result (Arepally & Goswami, 2019). Likewise, the results of chroma a^* and b^* indicated a tendency towards yellow-greenish, which may be associated with the color of the free hydrolysates.

3.3. Zeta potencial and particle size

The higher the zeta potential value, regardless of whether it is negative or positive, the greater is the electrostatic repulsion of the compounds present in the sample (Yan et al., 2022). The microencapsulated hydrolysates in this study presented electronegative zeta potential values that were distant from ±30 mV (Table 1), showing no significant differences between treatments ($p > 0.05$). This negative value in the samples may be related to the functional groups of protein hydrolysates that are ionized at pH (6-7 in distilled water), as well as, at pH above 4, the hydroxyl groups deprotonate and acquire a negative charge. According to Santos et al. (2016), suspensions with good stability should present zeta potential above ≥ +30 or ≤ -30 mV, to prevent aggregation in aqueous media because of the electrostatic repulsion of the particles. This result can be explained by the fact that functional groups are bonding together, and thus, contribute to the agglomeration of particles. Chen et al. (2021) states that good particle stability is related to the low molecular weight of the hydrolysates and numerous charges on their surface.

The dispersion capacity and solubility of microcapsules depend on the particle size, and sizes above 50 μm can significantly affect the solubility and dispersion (Mohan et al., 2015). The particle size was more heterogeneous, depending on the type of hydrolysate produced and the wall material used. The microencapsulated hydrolysate samples presented an average particle diameter ranging between 230.74 and 419.69 nm. Hydrolysate samples prepared with Alcalase resulted in smaller particle sizes (HAEn1: 239.02 nm; HAEn2: 230.74 nm) and the contrary was observed with Protamex that promoted larger particle sizes (HPEn1: 340.61 nm; HPEn2: 414.69 nm). Microencapsulated particles larger than 50 μm can generate undesirable sensations, such as roughness, grittiness, or hardness (Sarabandi et al. 2019).

3.4. Encapsulation efficiency (EE)

Encapsulation efficiency is an important parameter for understanding the interaction between the encapsulated active material (molecular and physicochemical properties of the bioactive peptides) and the different wall materials used in the process (Mohan et al. 2015). The prepared microencapsules showed EE ranging from 50.89 to 54.63%, with no significant differences between the samples, that is, the type of hydrolysate in combination with the wall material did not interfere with the encapsulation efficiency. Similar to those found by Alcantra et al. (2019) when microencapsulating chia oil using MD and GA as wall material (42.0–63.8%). The results of this study demonstrate that EE is correlated with the characteristics of the active material and wall material used. Rhamani-Manglano et al. (2020) reported that there is a correlation between EE and DE of the carbohydrate used as wall material, where the presence of smaller oligosaccharides allows for a larger, uniform and dense packing of the material in the core. McClements (2018) reported that bioactive peptides interact with the wall material by hydrophobic or hydrophilic domains and by electrostatic interactions between oppositely charged groups. Therefore, the composition ratio of the wall materials used was adequate to produce a structural matrix capable of retaining protein hydrolysates.

3.5. Funcional groups (FTIR) and termal analysis

FTIR spectroscopy was used to verify the physicochemical bonds and interactions between the protein hydrolysates (HA and HP) and wall materials (MD and GA). Figure 2 shows the spectra of the samples. In this study, the spectrogram comparisons show that the peaks in the 11820 cm^{-1} band increased slightly owing to the formation of a complex between the wall materials and HP. This interaction may have occurred via hydrogen bonds, elongation of the N-H group, and bending of the C-H group (Nenadis, Lazridou & Tsimidou, 2007). However, the bands in the 1497-1620 cm^{-1} range were almost similar,

with a difference between them only in the free hydrolysate samples. After the microencapsulation process, the bonds in the 1200-1497 cm^{-1} bands were displaced between the free HP and microencapsulated samples. In this study, the band at 1200-800 cm^{-1} indicated the presence of a carbohydrate region with C-O and C-O-H vibrations being more stereotic owing to the uniform distribution of peptides in the structure of the wall materials (MD and GA) (Kumari et al. 2023). According to Annamalai et al. (2020), the FTIR spectra band of maltodextrin (3415 cm^{-1}, 1643 cm^{-1}) and gum arabic (3415 cm^{-1}, 1611 cm^{-1}) indicate the presence of O-H bond and amide complex. Curley et al. (1998) reported that the peaks of amide I (C=O stretching) and amide II (N-H bending and C-N stretching) can be observed at 1646 cm^{-1} (HA), 1641 cm^{-1} (HP), and 1533 cm^{-1} (HA and HP). After microencapsulation, the amide I peak exhibited an upward shift in the microencapsulated samples. This increase in the frequency of the amide I peak may be related to the strong crosslinking between the MD and hydrolysate due to peptide dehydration (Lima et al. 2021).

The denaturation enthalpy (ΔH) and peak denaturation temperature (Td) are shown in Figure 3. Due to microencapsulation, the Td value decreased in the samples with HA, while in the HP sample the increase was more pronounced in the HPEn2 treatment (165.22ºC). This enthalpy variation represents the amount of thermal energy involved in sample transition processes, such as melting, crystallization, glass transitions, chemical reactions, among others, therefore, a lower energy means a lower stability. The lower ΔH in microcapsules compared to free hydrolysates may result from a decrease in the amount of secondary structure (α-helix and β-sheet) or from the breakdown of intramolecular forces caused by covalent conjugation with wall materials (Pirestani et al. 2018).

3.6. In vitro activity

3.6.1. Amino acid composition of hydrolysates

The amino acid profiles plays an important role in the biological properties of hydrolysates. The HA and HP samples presented a similar amino acid profile (Supplementary Material, Table SM1), with glutamic acid, proline, glycine, alanine, valine, leucine, tyrosine, and lysine being the most abundant. The contribution of hydrophobic amino acids, such as alanine, valine, isoleucine, leucine, phenylalanine, proline, and methionine, in relation to the amino acid contribution was approximately 21%. Lysine was the major essential amino acid in the samples, with HP presenting 1357.30 μmol/g protein.

3.6.2. Antioxidant activity

The biological activity (e.g., antioxidant activity) of encapsulated actives can demonstrate the efficiency of preservation/preservation of the capsule core biocompound (Akbarbaglu et al. 2019). There were significant differences ($p < 0.05$) in the DPPH radical assay between the treatments (Table 2). The HAEn1 treatment showed the lowest activity (29.21%), the others did not differ from each other. This result can be explained by the degradation of the liposoluble biocompounds during the drying process, or even, they were bound enough to cause the loss of activity. For ABTS, the HPEn1 sample showed the lowest capacity compared with the other samples. All samples presented values above 90% for the elimination of ABTS radical, while for DPPH they varied between 29.31 and 41.35% (Table 2). A similar result was reported by Salleh et al. (2022) that encapsulated of edible bird´s nest (EBN) hydrolysate only with MD (27.77%). Rodriguez-Diaz et al. (2014) reported that microcapsules obtained with MD with DE above 15% improve the antioxidant characteristics of protein hydrolysates. Although there were no differences in radical scavenging capacity, samples prepared with a mixture of MD and GA tended to

have greater activity in both hydrolysates. Maqsoudlou et al. (2020) observed that the combination of MD and Whey Protein Concentrate (WPC) can increase the protection efficiency of the encapsulated material. Domian et al. (2017) reported that the a carbohydrate/protein combination offers better oxidative stability and protection during the microcapsule drying process.

3.7. In vivo evaluation of free and microencapsulated hydrolysates in *C. elegans*

According to the results, supplementation with the samples contributed to distinct responses when different stress modes (oxidative and thermal) were applied (Fig. 4). To determine the antioxidant effects of free and microencapsulated hydrolysates, the survival of the animals during the stress assays was observed. In assay 1, with oxidative stress, animal survival was lower in the treatments that used microencapsulated hydrolysates (Figure 4a). In assay 2, with thermal stress, the same behavior was observed, except for HPEn1 treatment (81.24%), which it was significantly like the free hydrolysates and controls (Figure 4c). Our results differ from those reported by Lima et al (2021), that evaluated hydrolysates of *Cynoscion guatucupa* residues microencapsulated with MD. The authors found higher growth and survival rates in treatments with free and microencapsulated hydrolysates than in the control treatment. Our results indicate that free hydrolysates (HA and HP) favored antioxidant protection under stress conditions. Regarding microcapsules, the composition of the wall material may have made the microcapsules more rigid and thick because of the protein composition, forming a more complex layer and hindering the availability of hydrolysates to the worms. In addition, since the protein hydrolysates were encapsulated, the nematodes may have lacked food, reducing their survival.

In the oxidative stress test, significant higher growth ($p>0.05$) was observed for treatments HPEn1 (0.2386 mm) and HPEn2 (0.2313 mm) when compared with the other

treatments after 96 h of exposure to an oxidizing agent (H_2O_2) (Figure 4b). This behaviour was different in the heat stress test. The highest growth ($p<0.05$) was observed with HAEn2 treatment (0.1853 mm), followed by the free and microencapsulated hydrolysates (Figure 4d). Lower growth rates in both assays were observed in treatments with empty capsules (CEn1 and CEn2) (Figure 4b and 4d). This growth result may be associated with the amino acid content (Supplementary Material, Table 1), as certain amino acids influence the growth of the organism (Wu, 2010).

4. Conclusion

Samples of protein hydrolysate from a superworm (*Zophobas morio*) produced with the enzymes Alcalase and Protamex were microencapsulated with maltodextrin and gum arabic as wall materials, with low recovery yields (12.8 to 20.3%, HAEn1 and HPEn2, respectively). However, these hydrolysates exhibited low water activity and hygroscopicity. The morphological and structural characteristics demonstrate the effectiveness of the microencapsulation process. The surface charge (zeta potential) of the samples was negative and distant from -30 mV, which indicated that the microcapsules tended to agglomerate. In the *in vitro* antioxidant study, the samples presented greater capacity to scavenge the ABTS radical (>90.0%) with more expressive values with the enzyme Protamex. In *in vivo* assays with the nematode *C. elegans*, microencapsulated hydrolysates (HPEn1 and HPEn2) increased the growth rate of the worms. Overall, the microcapsule (HPEn1) contributed to the survival of the nematodes when exposed to heat stress conditions, demonstrating that the production of microencapsulates did not affect the bioactivity of the hydrolysates.

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FIGURES

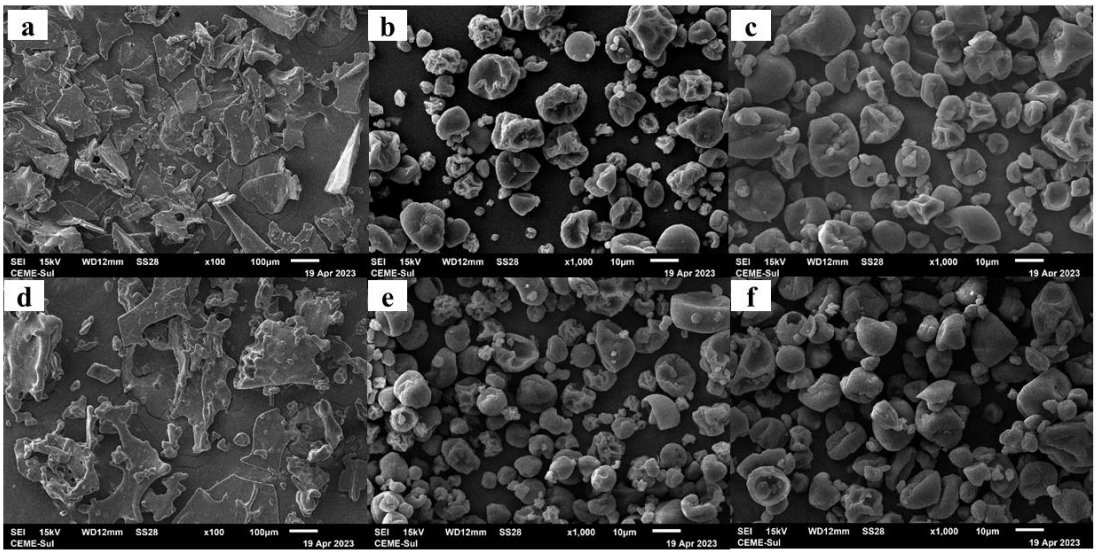

Figure 1. Morphological characteristics using a Scanning Electron Microscopy. (A) free hydrolysate using Alcalase. (B) microencapsulated hydrolysate using Alcalase with maltodextrin (MD). (C) microencapsulated hydrolysate using Alcalase with MD and gum arabic (GA). (D) free hydrolysate using Protamex. (E) microencapsulated hydrolysate using Protamex with MD. (F) microencapsulated hydrolysate using Protamex with MD and GA.

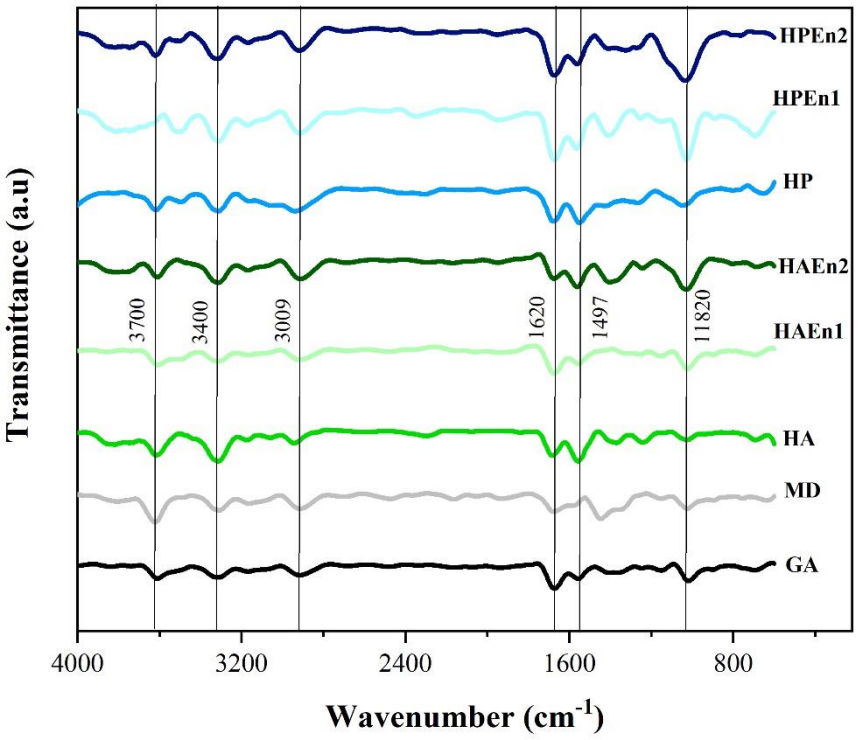

Figure 2. Infrared Spectroscopy for free and microencapsulated hydrolysate obtained with Alcalase and Protamex using different wall materials (MD-maltodextrin; GA-gum arabic). HA: hydrolysates produced using alcalase. HP: hydrolysates produced using protamex. En1: microencapsulation using MD as wall material. En2: microencapsulation using MD and GA as wall materials.

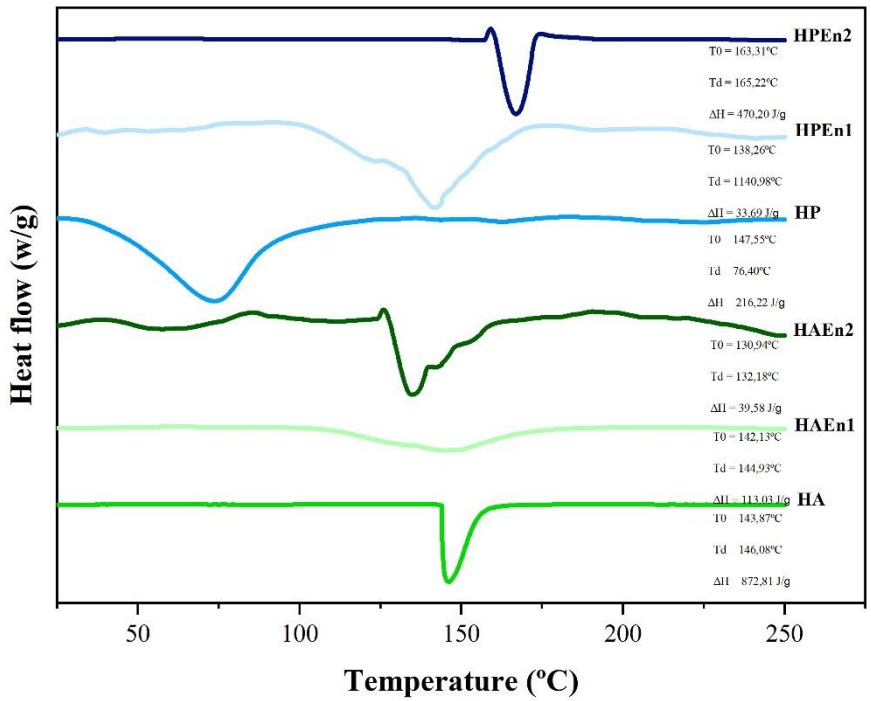

Figure 3. Thermal characteristics for free and microencapsulated hydrolysate obtained with Alcalase and Protamex using different wall materials (MD-maltodextrin; GA-gum arabic). HA: hydrolysates produced using alcalase. HP: hydrolysates produced using protamex. En1: microencapsulation using MD as wall material. En2: microencapsulation using MD and GA as wall materials.

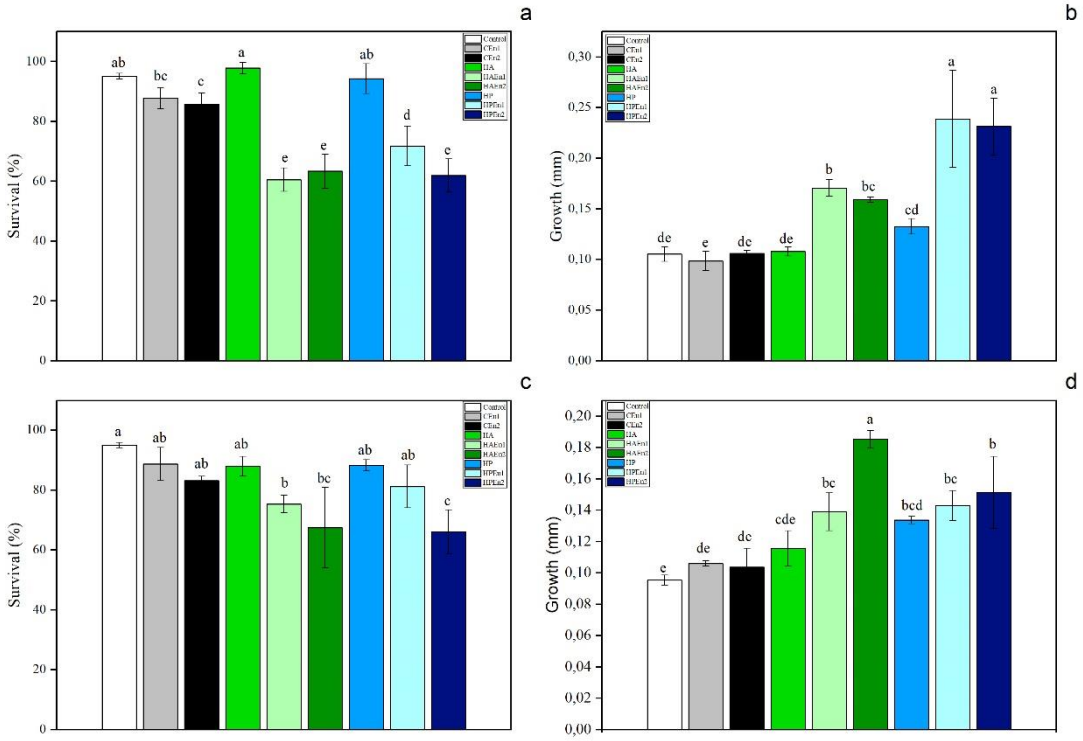

Figure 4. Effects of different treatments on survival and growth after oxidative stress (a and b) and survival and growth after termal (c and d). (1) control group containing only *E. coli*; (2) group containing *E. coli* and En1 (MD); (3) group containing *E. coli* and En2 (MD+GA); (4) group exposed to free Alcalase hydrolysate and *E. coli*; (5) group exposed to HAEn1 treatment and *E. coli*; (6) group exposed to HAEn2 treatment and *E. coli*; (7) group exposed to free Protamex hydrolysate and *E. coli*; (8) group exposed to HPEn1 treatment and *E. coli*; and (9) group exposed to HPEn2 treatment and *E. coli*. Different lowercase letters indicate a significant difference between samples ($p < 0.05$).

TABLES

Table 1. Physicochemical characteristics of microencapsulated protein hydrolysate of insect with different wall materials. HA: hydrolysates produced using alcalase. HP: hydrolysates produced using protamex. En1: microencapsulation using maltodextrin as wall material. En2: microencapsulation using maltodextrin and gum arabic as wall materials.

Parameter	HA En1	HA En2	HP En1	HP En2
Moisture (%)	4.91 ± 0.10^{c}	3.99 ± 0.16^{b}	5.05 ± 0.16^{c}	3.25 ± 0.17^{a}
Water activity (Aw)	0.232 ± 0.03^{bc}	0.189 ± 0.01^{ab}	0.258 ± 0.01^{c}	0.179 ± 0.02^{a}
Water solubility (WS)	91.32 ± 0.43^{a}	91.54 ± 0.27^{a}	90.82 ± 0.43^{a}	90.99 ± 0.78^{a}
Hygroscopicity (%)	24.82 ± 7.87^{a}	21.80 ± 2.73^{a}	20.12 ± 3.26^{a}	26.86 ± 7.10^{a}
Zeta potencial (mV)	-11.94 ± 0.74^{a}	-13.34 ± 0.70^{a}	-12.21 ± 0.30^{a}	-13.24 ± 0.39^{a}
Particule sizes (nm)	$239,02\pm46,93^{a}$	$230,74\pm64,92^{a}$	$340,61\pm75,15^{ab}$	$414,69\pm112,52_{b}$

Different lowercase letters in the same line indicate that there is a statistical difference between the samples ($p < 0.05$).

Table 2: Encapsulation efficiency, protein content, color parameters and antioxidant activity of microencapsulated protein hydrolysate of insect with different wall materials. HA: hydrolysates produced using alcalase. HP: hydrolysates produced using protamex. En1: microencapsulation using maltodextrin as wall material. En2: microencapsulation using maltodextrin and gum arabic as wall materials. EE: encapsulation efficiency. L*: lightness. *a**: red/green chromaticity. *b**: yellow/blue chromaticity.

Parameters	HA En1	HA En2	HP En1	HP En2
EE (%)	50.89±2.73^{a}	52.68±4.17^{a}	54.63±4.32^{a}	52.78±2.83^{a}
Protein (mg/mL)	5.65±0.18^{c}	6.90±0.28^{a}	5.05±0.28^{cd}	6.65±0.18^{ab}
Color				
*L**	60.33±7.72^{a}	60.80±8.51^{a}	57.62±0.01^{a}	60.81±7.66^{a}
*a**	1.58±0.14^{a}	1.37±0.07^{a}	1.42±0.01^{a}	1.43±0.03^{a}
*b**	11.37±1.44^{a}	12.76±1.65^{a}	10.21±0.01^{a}	12.23±1.29^{a}
DPPH (%)	29.31±4.93^{b}	36.73±8.78^{ab}	37.16±4.10^{a}	41.35±7.74^{a}
ABTS (%)	96.89±0.31^{a}	97.94±1.52^{a}	93.82±1.66^{b}	98.69±0.46^{a}

Different lowercase letters in the same line indicate that there is a statistical difference between the samples ($p < 0.05$).

Supplementary Material

Table SM1. Amino acid composition of protein hydrolysates using enzymes Alcalase (HA) and Protamex (HP). EAA = essential amino acid; TAA = total amino acid; HAA = hydrophobic amino acid.

Amino acid (µmol/g protein)	HA	HP
Aspartic Acid	98.65	176.40
Threonine	82.84	67.89
Serine	93.51	83.46
Glutamic acid	151.55	103.40
Proline	773.16	735.82
Glycine	219.38	112.58
Alanine	378.37	361.40
Valine	105.95	110.68
Methionine	71.47	18.77
Isoleucine	51.85	55.14
Leucine	166.28	170.81
Tyrosine	239.30	253.45
Phenylalanine	52.43	60.59
Histidine	51.79	40.90
Lysine	1098.28	1357.30
Arginine	75.81	59.92
EAA (µmol/g protein)	1680.89	1882.09
TAA (µmol/g protein)	3710.63	3768.54
HAA (% AA/g protein)	21.08	21.94

4. DISCUSSÃO GERAL

O crescimento da atividade aquícola está associado a fatores como intensificação da atividade, uso de tecnológicas ambientalmente sustentáveis (como a produção em sistemas de bioflocos), aliados a necessidade de fornecer proteína de boa qualidade à população. Atualmente o setor da aquacultura já ultrapassou o setor da pesca em questão de produção de alimento (FAO, 2024). Isso foi possível graça aos avanços citados acima e ao uso de fontes proteicas alternativas em substituição da farinha de peixe. No entanto, o uso de ingredientes inadequados pode causar sérios danos fisiológicos e nutricionais aos animais, prejudicando o seu crescimento e sobrevivência e diminuindo o lucro ao final da produção. Nos últimos anos, as pesquisas estão sendo voltados para ingredientes proteicos mais sustentáveis, entre eles, os resíduos sólidos produzidos no sistema em bioflocos e a farinha de insetos (como os tenébrios) (Jackqulinwino et al. 2024; Rostro et al. 2024; Auzins et al. 2024; Ido et al. 2024). Neste estudo, avaliamos as características dessas matérias-primas através da aplicação de processos tecnológicos como forma de melhor aproveitamento deles, não somente como fonte proteica alternativa, mas também, como fonte de compostos bioativos que podem favorecer uma produção mais segura, frente a diversos fatores ambientais.

Estudos já comprovaram que os bioflocos são uma fonte proteica alternativa dentro do sistema de cultivo aos animais confinados. Contudo, em sua maioria eles relatam apenas os resultados da contribuição dos bioflocos na manutenção dos parâmetros de qualidade da água do cultivo e composição proximal dos bioflocos (Kring et al. 2023; Blancaflor & Baccay, 2022). Fica evidente a necessidade de se ter um maior conhecimento sobre as propriedades biológicas (clorofila a, carotenoides) e compostos bioativos (antioxidantes e polifenóis) dos bioflocos produzidos devido ao metabolismo dos microrganismos presentes na água do cultivo e interações com outros elementos (restos de rações, fezes dos animais, entre outros). Neste estudo foi comprovado, através do perfil de aminoácidos, que os bioflocos estudados podem ser usados como fonte nutricional complementar devido aos valores dos aminoácidos encontrados. O ácido glutâmico foi um dos majoritários entre os 18 aminoácidos descritos. Esse resultado é interessante pois o ácido glutâmico é um realçador de sabor do produto (Witono et al. 2016), o que pode justificar o consumo pelos animais de cultivo.

Os ensaios com os extratos usando diferentes solventes associados ao processo de secagem mostraram um efeito do tipo de floco, tipo de secagem e tipo de solvente, onde os melhores resultados encontrados nesse estudo foram nas amostras de bioflocos

liofilizadas em extrato aquoso. Esse resultado é interessante, pois naturalmente, os bioflocos ficam dispersões dentro da água de cultivo, e demonstra que os animais ao consumir esse bioflocos podem aproveitar não somente o conteúdo proteico, como também outros compostos bioativos presentes neles.

Os insetos são fonte proteica usadas na nutrição de organismos aquáticos, sem comprometer seu desempenho zootécnico e melhorando a comunidade da microbiota intestinal desses animais (Tegtmeier et al. 2021). Como forma de melhor aproveitamento, nesse estudo, avaliamos o processo de hidrólise enzimática a partir de diferentes enzimas proteolíticas. Nossos resultados mostram uma contribuição do processo de hidrólise no perfil de aminoácidos totais e essenciais e no conteúdo de proteínas bruta. Tendo em vista que um novo ingrediente para ser usada na nutrição de animais deve apresentar características que tragam benefícios a eles (Gasco et al. 2020), os hidrolisados produzidos nesse estudo apresentaram boas propriedades funcionais que podem contribuir para uma maior acessibilidade dos alimentos durante a digestão nos animais.

Devido a maiores densidades de estocagem dentro do ambiente de cultivo, os animais ficam estressados e suscetíveis a ação de microrganismos que podem prejudicar a sua saúde. Comprovamos nesse estudo, que os hidrolisados proteicos obtidos com as enzimas Alacalse e Protamex apresentaram efeito antimicrobiano frente a microrganismo gram-negativos (*Vibrio coralliyticus, Pseudomonas aeruginos, Acinetobacter baumani* e *Escherichia coli*). Esse achado é muito interessante, pois adicionar um ingrediente que contribua não somente no balanço nutricional das dietas como também um agente frente a possíveis patógenos, demonstra o potencial uso dos insetos na produção de hidrolisados proteicos com aplicação na aquacultura. Devido aos resultados de solubilidade em água e acessibilidade após digestão gastrointestinal, investigamos o uso do processo de microencapsulação, para minimizar esses efeitos. O processo de microencapsulação diminuiu a solubilidade dos hidrolisados livres (para aproximadamente 90%), demonstrando a eficiência de usar materiais de parede para proteger o material núcleo (hidrolisados proteico de inseto).

De fato, microencapsular os hidrolisados não somente diminuiu essa propriedade funcional (solubilidade), como apresentou um efeito no crescimento do nematoide *C. elegans*, mesmo em condições de exposição a fatores estressantes (oxidantes e térmicos). Esse animal é um modelo para ensaios de toxicidade, devido suas características biológicas (hermafrodita, ciclo de desenvolvimento rápido) e fácil manipulação em condições de laboratório. Poucos estudos na literatura mostram os benefícios de

hidrolisados proteicos nos parâmetros fisiológicos e bioquímicos em *C elegans* (Lima et al. 2021). Nossos resultados mostraram que, os hidrolisados livres foram mais eficientes na sobrevivência dos animais em comparação aos microencapsulados. Esse resultado foi esclarecedor, pois pode comprovar que o material do núcleo estava bem protegido dentro das microcápsulas, e sua liberação foi mais controlada e lenta. Outro ponto, os nematoides parecem ter mais bem aproveitado as microcápsulas para crescimento em vez de usar para sobrevivência. Vale salientar que, os efeitos do uso de suplementos (como os hidrolisados microencapsulados) pode variar dependendo da espécie estudada.

Por fim, os resultados obtidos nos três experimentos desse estudo mostraram que a adoção de processos tecnológicos (secagem, hidrólise enzimática e encapsulação) podem produzir novos ingredientes a partir das amostras de bioflocos (fotoautotróficos e heterotróficos) e farinha de tenébrio gigante com características distintas da matriz original (inata), com boas características nutricionais e propriedades bioativas, a serem adotadas na nutrição de organismos aquáticos provenientes da aquacultura.

5. CONCLUSÕES GERAIS

O conhecimento das interações da matéria-prima (bioflocos e insetos) e processos tecnológicos aplicados em relação aos parâmetros de composição nutricional e propriedades biológicas reforçam o potencial uso desses produtos. A série de estudos executadas nesta tese, geraram informações para melhor aproveitamento dessas amostras como ingrediente em dietas ou até mesmo como suplemento alimentar na alimentação de peixes e camarões produzidos na aquacultura.

Os pontos a seguir merecem destaque como as principais conclusões desta Tese:

A. O processo de secagem em amostras de flocos microbianos (fotoautotrófico e heterotrófico) para obtenção de novos produtos desidratados, teve efeitos positivos sobre o perfil de aminoácidos e compostos biológicos (carotenoides e clorofila a). Além disso, a secagem por liofilização dos flocos fotoautotrófico e heterotrófico foi superior no conteúdo de aminoácidos totais (21,55 e 19,01%, respectivamente) e essenciais (9,10 e 7,82%, respectivamente). No entanto, a composição proximal nas amostras avaliadas não apresentou grandes diferenças no conteúdo de proteínas e lipídeos, correlacionando com a composição dos microrganismos encontrados dentro na água dos sistemas. Considerando o ensaio de bioatividade nos extratos com as amostras, recomenda-se o uso de água aliado a secagem por liofilização na amostra de floco fotoautotrófico e secagem em estufa na amostra heterotrófica, uma vez que assim, a atividade antioxidante e o conteúdo de polifenóis foram superiores.

B. A produção de hidrolisados proteicos com as enzimas Alcalase e Protamex aumentou o conteúdo de proteínas totais e aminoácidos, em comparação com a farinha do tenébrio não processada. Este resultado reflete uma forte relação entre o tipo de substrato, tipo de enzima proteolítica usada, tempo de hidrólise, contribuindo para explicar as melhores atividades antioxidantes (frente aos radicais DDPH e ABTS) e o poder de redução férrica, antes da simulação gastrointestinal e antimicrobianas (inibindo, em sua maioria, no crescimento de microrganismos gram-negativos na concentração de 800 mg/mL) promovido pelos hidrolisados produzidos com essas enzimas.

C. A aplicação do processo de encapsulação nos hidrolisados promoveu uma redução na solubilidade (para aproximadamente 90%) e na higroscopicidade (20% aproximadamente) sobre as amostras. As análises de eficiência de encapsulação (EE) e FTIR mostraram que os hidrolisados foram encapsulados, enquanto a análise de DSC

garantiu que a microencapsulação protegeu os hidrolisados livres sob a influência da degradação térmica, com melhores resultados para os tratamentos com a enzima Protamex (HPEn1 e HPEn2). O potencial zeta mostrou que os hidrolisados microencapsulados tendem a aglomeração, o que pode ser interessante, quanto a sua aplicação como suplemento alimentar a animais, porque podem ser inseridos na formulação de dietas funcionais. Além disso, a atividade antioxidante não foi reduzida pelo processo de microencapsulação dos hidrolisados, pelo contrário, a capacidade de eliminação do radical DPPH aumentou nas amostras em comparação com os hidrolisados livres, vistos no capitulo 2. A partir da inclusão de 1 mg/mL das microcápsulas no ensaio com o nematoide *C elegans*, obteve-se um aumento no crescimento dos animais no tratamento usando a enzima Protamex com o material de parede com a MD, mesmo frente a condições de estresse oxidativo e térmico. Desta forma, sugere-se a inclusão de HPEn1 para o uso como ingrediente em dietas ou como suplemento alimentar para aquacultura.

6. TRABALHOS FUTUROS

Os resultados obtidos no presente estudo apontaram para o efeito direto do processo de secagem na composição nutricional e propriedades biológicas nas amostras de bioflocos dos sistemas fotoautotrófico e heterotrófico, propõem-se estudos avaliando a composição de bactérias presentes nesses sistemas, para verificar a contribuição desses organismos na composição nutricional dos bioflocos. Aliado a isso, estudos avaliando o comportamento nutricional em diferentes estágios do cultivo e sua relação direta no perfil de aminoácidos, podem favorecer um melhor entendimento sobre a contribuição dessas amostras de bioflocos na nutrição de organismos aquáticos (camarões e peixes). Sabe-se também que, sistemas quimioautotróficos vem sendo realizados para a produção em sistemas em bioflocos, e dados na literatura sobre a contribuição desse sistema ainda são poucos conhecidos, carecendo realizar estudos semelhantes ao realizado no primeiro capítulo da tese.

Como informações quanto ao uso do tenébrio gigante (*Zophobas morio*) na literatura para produção de hidrolisados proteicos é mínima, os resultados do processo de hidrólise mostraram que, um maior tempo de hidrólise usado nesse estudo (300 min) causou efeito negativos na solubilidade das amostras, consequentemente, diminuindo sua atividade antioxidante durante a simulação gastro-intestinal (SGI) (capítulo 2), propõem-se avaliar hidrolisados com menores graus de hidrólise (GH) fixados (5, 10 e 15%), como já realizado para espécies de organismos aquáticos, e determinar seus efeitos nas propriedades funcionais e propriedades antimicrobianas e antioxidante *in vitro,* assim como o perfil de aminoácidos totais, essenciais e hidrofóbicos desses hidrolisados proteicos.

Baseado nos resultados obtidos no capítulo 3, propõem-se estudos com condições de operação com menor taxa de vazão de bomba (<0,3 L/h) e menor temperatura de entrada (<120,0ºC) no spray-dryer, e avaliar seu efeito para um maior rendimento do processo de microencapsulação, consequentemente, influenciando uma maior eficiência de encapsulação. Aliado a isso, estudos com outros materiais de parede distintos citados na literatura, como por exemplo concentrado proteico de soro de leite, óleo de pescado e/ou vegetal, carragenanas e mucilagem de chia, ou uma combinação de dois ou mais materiais de parede, podem favorecer as propriedades físico-químicos das microcápsulas. Além disso, avaliar as características das microcápsulas durante período de armazenamento, podem verificar possíveis alterações nas características físico-químicas

e bioativas, que comprometam o uso delas como ingrediente ou suplemento em dietas para a nutrição de organismos aquáticos.

Além disso, assim como no ensaio *in vivo* com o nematoide *C. elegans*, o uso dos hidrolisados de tenébrio gigante microencapsulados pode ser avaliado como enriquecedor na água durante o cultivo de camarões em sistema de bioflocos. Fatores como o uso da maltodextrina e goma arábica como materiais de parede, podem influenciar no tipo e predominância de bactérias dentro do sistema; o efeito antioxidante dos hidrolisados microencapsulados pode contribuir para uma maior resistência dos animais de cultivo durante condições ambientais desfavoráveis, como concentrações baixas de oxigênio dissolvido, temperaturas baixas ou maiores do que a faixa de tolerância para a espécie e o efeito crônico e agudo dos compostos nitrogenados (amônia e nitrito) durante um período de exposição. Por fim, a bioconversão dos hidrolisados microencapsualdos pode contribuir na qualidade nutricional dos camarões produzidos e favorecer um maior tempo de prateleira desse produto durante o armazenamento sob condições de refrigeração e congelamento.

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