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PRÓ-REITORIA DE PÓS-GRADUAÇÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

EFEITO DA ADIÇÃO DE PREBIÓTICO E NUCLEOTÍDEO NA ALIMENTAÇÃO DE *Penaeus vannamei* (BOONE, 1931) EM SISTEMA SIMBIÓTICO

Danielle Alves da Silva

Tese apresentada ao Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura da Universidade Federal Rural de Pernambuco como exigênciapara obtenção do título de Doutora.

Prof. Dr. Luis Otavio Brito da Silva
Orientador

Prof. Dra. Suzianny Maria Bezerra Cabral da Silva
Co-orientadora

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EFEITO DA ADIÇÃO DE PREBIÓTICO E NUCLEOTÍDEO NA ALIMENTAÇÃO *Litopenaeus vannamei* (BOONE, 1931) EM SISTEMA INTENSIVO

Danielle Alves da Silva

Tese a ser julgada adequada para obtenção do título de Doutora em Recursos Pesqueiros e Aquicultura. Defendida em 30/07/2024 pela seguinte Banca Examinadora.

Prof. Dr. Luis Otavio Brito da Silva
Orientador
Departamento de Pesca e Aquicultura – UFRPE

Prof. Dr. Alfredo Olivera Gálvez
Universidade Federal Rural de Pernambuco – Membro interno

Profª. Dra. Roberta Borda Soares
Universidade Federal Rural de Pernambuco – Membro interno

Prof. Dr. Petrônio Alves Coelho Filho
Universidade Federal de Alagoas – Membro Externo

Profª. Dra. Gelcirene Albuquerque Costa
Universidade Federal do Rio Grande do Norte – Membro Externo

Dedicatória

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RESUMO

A carcinicultura, com ênfase na produção de *Penaeus vannamei*, tem se consolidado como um dos setores mais importantes da aquicultura global, especialmente devido à crescente demanda por proteínas de origem marinha. No entanto, a intensificação dessa produção trouxe à tona desafios críticos, como o aumento da incidência de doenças, que resultam em perdas significativas na produtividade. Para uma produção sustentável, é crucial desenvolver práticas e tecnologias que mitiguem esses desafios, como sistemas simbióticos intensivos e aditivos alimentares, que beneficiam os camarões cultivados. O presente estudo teve como objetivo avaliar os efeitos da suplementação dietética com nucleotídeos (NT) e mananoligossacarídeos (MOS) sobre o desempenho zootécnico, o estado de saúde e nos índices econômicos do camarão-branco-do-pacífico (*P. vannamei*) criado em um sistema simbiótico intensivo. No primeiro experimento, foram testados três níveis de suplementação com NT (75 mg kg^{-1}, 150 mg kg^{-1} e 300 mg kg^{-1}) em comparação a uma dieta controle sem nucleotídeos. Os camarões, com peso inicial de 1,78 ± 0,02 g, foram estocados a uma densidade de 100 camarões m^{-2} e alimentados quatro vezes ao dia durante 60 dias. Os resultados indicaram que a suplementação com NT promoveu um crescimento superior e melhorou a composição proteica dos camarões ($p < 0,05$), além de otimizar a morfologia intestinal, com aumento na altura das vilosidades e dos enterócitos. Essas melhorias refletem-se diretamente no desempenho zootécnico e na saúde dos camarões, embora não tenham sido observadas diferenças significativas na microbiologia intestinal (*Vibrio* spp. e *Bacillus* spp.) ou na resistência ao estresse por amônia e nitrito entre os tratamentos. O tratamento com 150 mg kg^{-1} de NT foi o que apresentou o maior retorno ao investimento (ROI), sugerindo que essa dosagem é a mais eficiente em termos de custo-benefício. No segundo experimento, avaliou-se a suplementação com MOS em três concentrações (0,6 g kg^{-1}, 1,2 g kg^{-1} e 2,4 g kg^{-1}). Os camarões, com peso inicial de 3,00 ± 0,04 g, foram também estocados a 100 camarões m^{-2} e alimentados quatro vezes ao dia por 60 dias. A suplementação com 0,6 g kg^{-1} de MOS proporcionou os melhores resultados em termos de crescimento, taxa de conversão alimentar e taxa de crescimento semanal, além de promover melhorias na morfologia tanto intestinal quanto branquial. Observou-se um aumento na altura das vilosidades e dos enterócitos, assim como uma menor incidência de lesões branquiais após os testes de estresse químico nos tratamentos com menores concentrações de MOS. Esses resultados sugerem que a suplementação moderada com MOS não só melhora o desempenho zootécnico, mas também contribui para a integridade morfológica dos tecidos críticos, aumentando a resiliência dos camarões em condições adversas, como o estresse por amônia e

nitrito. Apesar dessas melhorias significativas, não foram observadas diferenças na microbiologia intestinal entre os tratamentos. O lucro líquido foi mais elevado no tratamento com 0,6 g kg^{-1} de MOS (8,751 USD ha^{-1}), enquanto as dosagens mais altas não resultaram em benefícios adicionais e, em alguns casos, reduziram a rentabilidade. Os resultados obtidos nos dois experimentos demonstram que a administração de NT e MOS tem o potencial de melhorar significativamente o crescimento, a saúde intestinal e os retornos econômicos na criação de *P. vannamei* em sistemas simbióticos intensivos. Esses aditivos dietéticos, ao promoverem um melhor desempenho zootécnico e uma maior resiliência dos camarões, configuram-se como ferramentas valiosas para a otimização da produção aquícola, com implicações diretas na sustentabilidade e rentabilidade da indústria.

Palavras-chave: *Penaeus vannamei*, Mananoligossacarídeos, Nucleotídeos, Simbiótico, Desempenho zootécnico, morfologia intestinal, nitrogenados, Índices econômicos

ABSTRACT

Shrimp farming, with an emphasis on *Penaeus vannamei* production, has become one of the most important sectors in global aquaculture, especially due to the increasing demand for marine-origin proteins. However, the intensification of this production has brought about critical challenges, such as the increased incidence of diseases, which result in significant losses in productivity. For sustainable production, it is crucial to develop practices and technologies that mitigate these challenges, such as intensive symbiotic systems and dietary additives that benefit cultured shrimp. The present study aimed to evaluate the effects of dietary supplementation with nucleotides (NT) and mannanoligosaccharides (MOS) on the zootechnical performance, health status, and economic indices of Pacific white shrimp (*P. vannamei*) reared in an intensive symbiotic system. In the first experiment, three levels of NT supplementation (75 mg kg^{-1}, 150 mg kg^{-1}, and 300 mg kg^{-1}) were tested, compared to a control diet without nucleotides. The shrimp, with an initial weight of 1.78 ± 0.02 g, were stocked at a density of 100 shrimp m^{-2} and fed four times a day for 60 days. The results indicated that NT supplementation promoted superior growth and improved the protein composition of the shrimp ($p < 0.05$), in addition to optimizing intestinal morphology, with an increase in villi height and enterocyte count. These improvements directly reflect on the zootechnical performance and health of the shrimp, although no significant differences were observed in intestinal microbiology (*Vibrio* spp. and *Bacillus* spp.) or resistance to ammonia and nitrite stress among the treatments. The 150 mg kg^{-1} NT treatment showed the highest return on investment (ROI), suggesting that this dosage is the most cost-effective. In the second experiment, MOS supplementation was evaluated at three concentrations (0.6 g kg^{-1}, 1.2 g kg^{-1}, and 2.4 g kg^{-1}). The shrimp, with an initial weight of 3.00 ± 0.04 g, were also stocked at 100 shrimp m^{-2} and fed four times a day for 60 days. The 0.6 g kg^{-1} MOS supplementation provided the best results in terms of growth, feed conversion ratio, and weekly growth rate, in addition to promoting improvements in both intestinal and gill morphology. An increase in villi height and enterocyte count was observed, as well as a lower incidence of gill lesions after chemical stress tests in the treatments with lower MOS concentrations. These results suggest that moderate MOS supplementation not only improves zootechnical performance but also contributes to the morphological integrity of critical tissues, increasing the resilience of shrimp under adverse conditions, such as ammonia and nitrite stress. Despite these significant improvements, no differences in intestinal microbiology were observed among the treatments. Net income was highest in the 0.6 g kg^{-1} MOS treatment (8,751 USD ha^{-1}), while higher dosages did not result

in additional benefits and, in some cases, reduced profitability. The results obtained from the two experiments demonstrate that NT and MOS administration has the potential to significantly improve growth, intestinal health, and economic returns in *P. vannamei* farming in intensive symbiotic systems. By promoting better zootechnical performance and greater shrimp resilience, these dietary additives are valuable tools for optimizing aquaculture production, with direct implications for the sustainability and profitability of the industry.

Keywords: *Penaeus vannamei*, Mannanoligosaccharides, Nucleotides, Synbiotic, Zootechnical performance, Gut morphology, Nitrogenous compounds, Economic indicators.

LISTA DE FIGURAS

ARTIGO I

ARTIGO II

LISTA DE TABELAS

ARTIGO I

ARTIGO II

Consta

SUMÁRIO

1. INTRODUÇÃO

A carcinicultura exerce um papel crucial na produção aquícola global, com 12,75 milhões de toneladas (FAO, 2024). A principal espécie cultivada é *Penaeus vannamei* com 7,93 milhões de toneladas produzidas em 2022, principalmente devido ao sabor agradável, alto valor nutricional, tecnologias bem desenvolvidas e adaptabilidade as diferentes condições de cultivo (FAO, 2024; Amelia et al., 2021; De Tailly et al., 2021; Wu et al., 2021).

No Brasil, a carcinicultura é uma atividade econômicamente importante, particularmente nos estados do Nordeste. As condições climáticas favoráveis na Região fazem com que ela responda por 99,6% da produção nacional. Em 2022, a produção atingiu 113,3 mil toneladas, registrando o quinto ano consecutivo de crescimento, com uma taxa de 5,9% em relação ao ano anterior (IBGE, 2023). Esse crescimento reflete uma recuperação significativa após perdas causadas por doenças como vírus da mionecrose infecciosa (IMNV) e doença da síndrome do vírus da mancha branca (WSSV). Para enfrentar esses desafios, estão sendo adotadas novas tecnologias e melhores práticas de manejo. Entre essas tecnologias, os sistemas com mínima a troca de água, controle de nitrogenados e sólidos sedimentáveis e reutilização de água, reduzindo o risco de entrada de patógenos (Samocha, 2019).

Nos últimos anos, o sistema simbiótico tem se destacado como uma abordagem promissora na aquicultura. Este sistema integra de forma sinérgica prebióticos e probióticos (Kolida e Gibson, 2011). Prebióticos são compostos que servem como substrato para o crescimento microrganismos probióticos, promovendo a saúde e redução de patógenos (Tran & Li, 2022; Sanders et al., 2019; Rohani et al., 2021). Por outro lado, os probióticos são microrganismos que contribuem para reduzir compostos tóxicos, colonizar trato digestório dos animais cultivados, promovendo benefícios à saúde, como a melhoria do sistema imunológico (Kerry et al., 2018; Guo et al., 2022).

No sistema simbiótico, durante a fertilização, utiliza-se fontes de carbono como farelo de trigo, arroz e soja como prebióticos. Esses são submetidos a processos de fermentação e respiração microbiana com microorganimos probióticos, para aumentar a biodisponibilidade de nutrientes essenciais, como proteínas e lipídios dos farelos (Santos et al., 2022; Pimentel et al., 2022). Essa abordagem visa otimizar os índices de desempenho zootécnico e reduzir a prevalência de *Vibrio* spp., favorecendo a proliferação de bactérias probióticas como *Bacillus* spp. (Santos et al., 2021).

Além da manutenção da qualidade da água, práticas de manejo alimentar estão sendo utilizadas para melhorar a saúde dos camarões. Tradicionalmente, antibióticos e quimioterápicos foram empregados para controlar os surtos bacterianos, mas essas práticas têm sido reduzidas ou abolidas, devido ao desenvolvimento de resistência bacteriana e as preocupações ambientais (Lulijwa et al., 2020). Desta forma, se faz necessário alternativas de controle desses surtos bacterianos. A suplementação com aditivos alimentares, vem se tornando uma dessas alternativas para várias espécies de camarões. Essas estratégias incluem o uso de ácidos orgânicos, extratos de plantas/algas, nucleotídeos, aminoácidos funcionais, vitaminas e compostos imunostimulantes naturais, que atuam como estimulantes de crescimento, reforço imunológico e abordagens alternativas para resistência a doenças e estresses ambientais (Dawood et al., 2018).

Os prebióticos são aditivos alimentares que não são digeríveis e servem como substrato específico para o crescimento de microrganismos benéficos, como *Bifidobacterium* spp. e *Lactobacillus* spp., no organismo hospedeiro, impedindo a proliferação de bactérias patogênicas (Gibson et al., 2017). A microbiota intestinal é essencial para o desenvolvimento saudável do hospedeiro, contribuindo para a regulação e fortalecimento dos sistemas imunológico e digestivo (Holt et al., 2020; Talwar et al., 2018), pois o trato intestinal é a principal interface de interação entre o ambiente externo e interno dos organismos, tornando essa microbiota crucial para a saúde e bem-estar dos animais (Egerton et al., 2018; Ochoa-Romo et al., 2022). *Saccharomyces cerevisiae*, conhecida como levedura, é um microrganismo unicelular eucariótico de grande importância prebiótica. Recentemente, a adição de levedura seca à alimentação animal tem sido reconhecida como um suplemento dietético valioso do ponto de vista nutricional e de saúde (Adel et al., 2017). A levedura é rica em nutrientes, como proteínas, lipídios, vitaminas do complexo B, quitina, nucleotídeos, mananoligossacarídeos (MOS) e β-glucana, entre outros (Pongpet et al., 2016).

Os mananoligossacarídeos (MOS) são conhecidos por melhorar significativamente as funções imunológicas e gastrointestinais (Gainza e Romero, 2020; Dawood et al., 2020; Yilmaz et al., 2022). Diversos estudos destacam os benefícios de sua aplicação na espécie *P. vannamei*. Zhang et al. (2012) relataram que a utilização de MOS promoveu o crescimento e a sobrevivência dos juvenis. Hamsah et al. (2020) demonstraram que a suplementação de 1% de MOS pode aumentar significativamente o desempenho de crescimento. Prastiti et al. (2018) também confirmaram que a administração de prebióticos na dose de 0,8% melhora a resposta imunológica e o

crescimento de forma eficaz. Os MOS também aumentam a resistência ao estresse e às infecções bacterianas, devido à sua capacidade de impedir a fixação e colonização de patógenos no trato digestivo (Torrecillas et al., 2014). Os benefícios dos MOS dietéticos também foram observados em várias espécies da aquicultura, incluindo melhorias no desempenho zootécnico (Chen et al., 2020; Akter et al., 2021), resposta imunológica (Ren et al., 2020; Harikrishnan et al., 2023), microbiota intestinal (Wang et al., 2022; Do Huu et al., 2023), nutrição e alimentação (Akter et al., 2021), e resiliência a fatores estressantes e doenças (Mameloco et al., 2020; Lu et al., 2022; Chen et al., 2024).

Uma nova geração de prebióticos à base de MOS, desenvolvida com solubilização da camada de mananas e exposição parcial da camada de glucanas, tem mostrado eficácia sinérgica. Estudos de Koch et al. (2020) revelaram que este MOS de segunda geração aumenta a sobrevivência de pós-larvas de *P. vannamei* em quase 50% ao enfrentar a Síndrome da Necrose Hepatopancreática Aguda (AHPNS) e reduz significativamente a presença de *Vibrio* spp.. Os β-glucanos expostos funcionam como imunoestimulantes, desencadeando respostas que ajudam a neutralizar patógenos (Gonçalves et al. 2014; Dawood et al., 2017). Eles também melhoram a imunidade e resistência a doenças em crustáceos, como o camarão-tigre (*Penaeus monodon*) (Luan et al., 2021) e o camarão-banana (*Penaeus merguiensis*) (Pooljun et al., 2022). A administração de 0,2% de β-glucano a *P. vannamei* mostrou efeitos positivos no crescimento (Murthy et al., 2009), e dietas com 0,05-0,20% podem aumentar a tolerância do Pompano-dourado (*Trachinotus ovatus*) ao estresse por baixa salinidade (Do Huu et al., 2016). Além disso, β-glucanos dietéticos podem alterar a microbiota intestinal em *P. vannamei* (da Silva et al., 2013; Wongsasak et al., 2015) e no linguado (*Scophthalmus maximus*) (Miest et al., 2016).

Os nucleotídeos são moléculas intracelulares de baixa massa molecular fundamentais para diversos processos fisiológicos e bioquímicos, incluindo a geração de energia (como no caso do ATP), o metabolismo e a comunicação celular (Biswas et al., 2012). Eles constituem os componentes básicos do DNA e RNA, além de serem cofatores indispensáveis na síntese de proteínas e enzimas, como o NAD+ (Krüger e Werf, 2018; Andrino et al., 2012). A farinha de peixe, uma das principais fontes proteicas em dietas comerciais, rica em nucleotídeos (Kanashiro, 2015), enfrenta desafios de escassez e alto custo para a indústria de rações (Hossain et al., 2018; Tantikitti, 2014). Embora os animais possam sintetizar nucleotídeos internamente, em situações de crescimento rápido, trauma ou estresse imunológico, a síntese endógena pode não ser suficiente. Nesses casos, a suplementação com nucleotídeos exógenos é crucial para garantir o crescimento rápido e

a função fisiológica adequada (Xiong et al., 2018). Pesquisas indicam que essa suplementação melhora o crescimento, a imunidade e a resistência a doenças em crustáceos (Li et al., 2007; Guo et al., 2016; Yong et al., 2020), particularmente aumentando as respostas imunológicas celulares e humorais (Manoppo et al., 2011). A eficácia dos nucleotídeos tem sido investigada em várias espécies aquáticas, como tilápia (Shiau et al., 2015), salmão do Atlântico (Burrells et al., 2001), camarão-branco-do-pacífico (Cao et al., 2011), garoupa (Lin et al., 2009) e truta arco-íris (Tahmasebi-Kohyani et al., 2011).

Há um grande interesse na suplementação de nucleotídeos e MOS na formulação de dietas para camarões e peixes, o que tem proporcionado um crescimento no uso desses aditivos nos últimos anos. Embora existam algumas linhas de dietas importadas com inclusão desses compostos, elas ainda são escassas. Em grande parte das fazendas comerciais, esses aditivos são frequentemente misturados nas rações comerciais, o que, devido à falta de mão de obra qualificada para a aplicação e aglutinação desses insumos, pode reduzir a eficiência dos mesmos. Uma possível estratégia para resolver essa problemática seria oferecer esses aditivos já adicionados na formulação de uma ração comercial e com dosagem adequada, facilitando o manejo alimentar durante o cultivo. Essa abordagem pode trazer benefícios significativos para o desempenho zootécnico e a resistência dos camarões cultivados, impactando diretamente na lucratividade. No entanto, o aumento do custo da ração deve ser avaliado em termos de custo de produção, levando em consideração o custo por quilograma de camarão produzido.

2. OBJETIVO GERAL

Avaliar a suplementação de nucleotídeos (NT) e mannanoligossacarideos (MOS) em dietas de *Penaeus vannamei* cultivado em sistema intensivo.

2.1 Objetivos Específicos

- Avaliar o desempenho zootécnico: sobrevivência, peso final, fator de conversão alimentar, produtividade de *P. vannamei* alimentados com dietas suplementadas com NT e MOS;
- Avaliar o melhor nível de suplementação de NT e MOS em dietas para *Penaeus vannamei* cultivado em sistema intensivo;
- Analisar a microbiota intestinal de *P. vannamei* alimentados com dietas suplementadas com NT e MOS;
- Analisar a morfologia intestinal de *P. vannamei* alimentados com dietas suplementadas com NT e MOS;
- Avaliar a resistência de *P. vannamei* a exposição de NH_3 e NO_2 após 60 dias de alimentação com dietas suplementadas com NT e MOS;
- Avaliar o retorno ao investimento da suplementação de NT e MOS em dietas para *Penaeus vannamei* cultivado em sistema intensivo.

3. HIPÓTESE

- A suplementação de NT e MOS melhora o desempenho zootécnico de *P. vannamei* cultivados em sistema intensivo.
- A suplementação de NT e MOS melhora os índices econômicos de *P. vannamei* cultivados em sistema intensivo.

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Effects of dietary nucleotides on performance, proximate composition, health status, nitrogen stress resistance and return on investment for Pacific white shrimp, *Penaeus vannamei* raised in an intensive synbiotic system

Danielle Alves da Silva[a*], Katharine Batista Santos de Souza [b], Gisely Karla de Almeida Costa[b]; Suzianny Maria Bezerra Cabral da Silva[b], Fernando Leandro dos Santos[c], Rodrigo Antônio Ponce de Leon Ferreira de Carvalho[d], Giovanni Sampaio Gonçalves[e], João Fernando Albers Koch[f], Luis Otavio Brito[a]

[a]Laboratório de Carcinicultura. Universidade Federal Rural de Pernambuco. Rua Dom Manoel de Medeiros, s/n, Dois Irmãos – CEP: 52171-900 – Recife, Pernambuco, Brazil. Email: doutorado.danielle2020@gmail.com ORCID: 0000-0002-5397-9510; luis.obsilva@ufrpe.br, ORCID: 0000-0002-6971-3020

[b]Laboratório de Sanidade de Animais Aquáticos. Universidade Federal Rural de Pernambuco. Rua Dom Manoel de Medeiros, s/n, Dois Irmãos – CEP: 52171-900 – Recife, Pernambuco, Brazil. Email: katharinesouza.pe@gmail.com; gisely.costa01@gmail.com, ORCID: 0000-0001-8207-1643; suzianny.silva@ufrpe.br, ORCID: 0000-0002-7571-3465.

[c]Laboratório de Histopatologia Maria Ignez Calvalcante. Universidade Federal Rural de Pernambuco. Rua Dom Manoel de Medeiros, s/n, Dois Irmãos – CEP: 52171-900 – Recife, Pernambuco, Brazil. Email: fernando.leandro@ufrpe.br

[d]Laboratório de Nutrição de Organismos Aquáticos. Universidade Federal do Rio Grande do Norte. Campus Universitário Lagoa Nova. CEP: 59078-900 – Natal, Rio Grande do Norte, Brazil. Email: rodrigoplfc@gmail.com

[e]Laboratório de Nutrição em Aquicultura do Instituto de Pesca. Av. Abelardo Menezes, S/N acesso Rod. Washington Luís km 444– CEP: 15025620 –, São José do Rio Preto, São Paulo, Brazil. Email: giovani.goncalves@sp.gov.br

[f]Gerente Técnico Global - Aquicultura. Biorigin, Rua XV de novembro, 865, CEP: 18680-900, Lençóis Paulista, São Paulo - Brazil, Email: joao.koch@biorigin.net, ORCID: 0009-0009-2780-1944.

*Corresponding author: Laboratório de Carcinicultura. Universidade Federal Rural de Pernambuco. Rua Dom Manoel de Medeiros, s/n, Dois Irmãos – CEP: 52171-900 – Recife, Pernambuco, Brazil. Email: doutorado.danielle2020@gmail.com Telephone: +55 81 995652648

ABSTRACT

Shrimp production has adapted to recent disease outbreaks that have caused significant economic losses. Recognizing the importance of optimal shrimp nutrition is crucial for the sustainability of the industry. With increasing demand for high quality feeds that promote shrimp health, this study aimed to evaluate the effects of dietary nucleotide addition on performance, health status, nitrogen stress resistance and ROI of Pacific white shrimp in an intensive synbiotic system. Experimental diets included a control group with no added nucleotides and diets with 75 mg kg^{-1}, 150 mg kg^{-1} and 300 mg kg^{-1}. Shrimp, initially weighing 1.78 ± 0.02 g at a density of 100 shrimp m^{-2}, were randomly distributed and fed four times a day for 60 days. At the end of the trial, the nucleotide-addition diets had higher shrimp performance and protein composition than the control ($p < 0.05$). Notably, nucleotide supplementation improved gut morphology, evidenced by increased fold height, villus height, and enterocyte height. While *Vibrio* sp. and *Bacillus* sp. counts decreased across all treatments, yeast (fungi) showed an insignificant increase. Besides, no significant differences in survival rates, hemocyte counts, or gill histology were observed among treatments in ammonia and nitrite stress tests. Revenue generated from shrimp sales minus feed costs ranged from US$ 29,244 to US$ 30,542 ha^{-1}, with the N150 treatment exhibiting the highest return on investment. Consistently, nucleotide addition enhanced shrimp performance, protein composition, gut morphology, and return on investment. These results highlight the potential of nucleotide supplementation in optimizing shrimp feeding practices.

KEYWORDS: shrimp, nucleotides, growth, gut morphology, net benefit

1. INTRODUCTION

The enhancement of shrimp growth and immunity represents a crucial aspect of the prevention of economic losses. Consequently, bioproducts and microbial cells are regarded as superior alternatives for the management of animal health, facilitating accelerated growth and enhanced disease resistance. The administration of chemotherapeutics and antibiotics at the farm level is frequently impractical or prohibited (Biswas et al., 2012). In alignment with the initiatives of UN members and Tripartite organizations (FAO, WHO, and WOAH), there is an imperative need to regulate the dissemination of antimicrobial resistance (AMR) in global aquaculture. This will guarantee the essential function of aquaculture in the food supply chain and its incorporation into the "One Health" framework (Thornber et al., 2020; Caputo et al., 2023). *Saccharomyces cerevisiae* (either in single cell protein or whole cell form) has been reported to contain various immunostimulatory compounds such as β-glucans, nucleic acids, nucleotides and chitin, as well as mannan oligosaccharides (MOS) (Navarrete and Tovar-Ramrez 2014). In addition, its inclusion in the diet has been reported to improve *P. vannamei* growth (Jin et al. 2018; Yong et al. 2020; Segarra et al. 2023) and intestinal health (Ayiku et al. 2020).

Nucleotides are low molecular weight intracellular and immunomodulatory compounds that serve as the basic building blocks of DNA and RNA. They play a crucial role in various physiological processes in living organisms, including cell division, modulation of cell growth, regulation of the immune system and maintenance of gut health (Biswas et al. 2012; Novriadi et al. 2021; 2022). Nucleotides are considered semi-essential nutrients, meaning that under conditions of rapid growth, stress and disease, an animal's endogenous synthesis capacity may not be sufficient to meet demand. This process also requires significant energy expenditure, and certain immune and intestinal cells lack the ability to synthesis nucleotides, thereby hindering growth (Hess and Greenberg 2012; Chen et al. 2022). The addition of exogenous nucleotides in the diet contributes to energy conservation and promotes homeostasis, particularly in crustaceans (Manoppo et al. 2013; Guo et al. 2016).

This is achieved by meeting physiological needs, new synthesis and a process known as the 'salvage pathway', which involves the recycling of nucleotides from dead cells (Hoffmann 2007). By introducing an additional source of exogenous nucleotides into the diet, they can be rapidly optimized, particularly during periods of rapid growth, stress or disease (Burrells et al. 2001; Hossain et al. 2020). These gains in shrimp physiology may prove to be an important factor in the development of enhanced production systems. The ability of shrimp to resist the effects of nitrogen compounds (ammonia and nitrate) may contribute to more productive yields, as these compounds can reach critical levels in intensive systems, leading to increased mortality or even increased disease problems (Chen and Chen, 1992; Lin and Chen, 2001; Abakari et al., 2021).

From a biological point of view, the inclusion of nucleotides in diets seems to be most appropriate, especially during the early developmental stages, as rapid growth occurs during the juvenile stage (Xiong et al. 2018). In order to develop sustainable and economically viable feed formulations, nucleotides can be incorporated into shrimp feed formulations to mitigate the adverse effects of reduction in fishmeal (Ding et al. 2021). Consequently, there is a growing need for research focusing on the benefits, dosages and feeding duration of nucleotide inclusion in aquatic species (Huu 2016) to help aquaculture find alternatives to reduce fishmeal in diets (Ding et al. 2021).

Therefore, this study evaluated different levels of nucleotide addition on growth performance, health status (gut bacterial and fungal counts, ammonia and nitrite stress, histological changes in gills and gut morphology) and return on investment (ROI) of Pacific white shrimp, *Penaeus vannamei*, raised in an intensive synbiotic system.

2. MATERIAL AND METHODS

2.1 Ingredients and experimental diets

A control diet (C) containing commercial ingredients from the Brazilian feed industry was formulated to meet the recommended nutritional requirements for juvenile *Penaeus vannamei*. This diet was designed to be isoproteic, 360 g kg^{-1}, and isoenergetic, 4400 Kcal kg^{-1}. Furthermore, three test diets were formulated with the inclusion of a commercial nucleotide source to replace wheat flour in the control diet at levels of 75, 150 and 300 mg nucleotides per kg $feed^{-1}$, designated N75, N150 and N300, respectively. (Table 1).

The commercial nucleotide source consists of 15% RNA (nucleotides) from the yeast *Saccharomyces cerevisiae*, 50% crude protein, 15% ash and 8% water (Biotide, Biorigin, São Paulo, Brazil). The four experimental diets were processed at the Centro Avançado do Pescado Continental - Instituto de Pesca (São José do Rio Preto, São Paulo, Brazil) using standard shrimp feed production procedures (Table 1). The ingredients were ground in a hammer mill (model M300, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil) to a particle size of less than 600 µm, thoroughly mixed for 15 min (model M2200, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil), extruded at 80 to 90ºC (model E62, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil) into 1.2 mm pellets, and dried at 90 to 100ºC to reach a moisture level of 120 g kg^{-1}, crumbled, packed in sealed bags and stored frozen until used. The pellets were then crumbled, packed in sealed bags, and stored frozen until use.

Table 1. Ingredients, formulation and nutrient composition of the control and test diets containing nucleotides to evaluate the performance of *P. vannamei* juveniles reared in a synbiotic system for 60 days.

Ingredients (g kg^{-1})	**Diet**			
	Control	**N75**	**N150**	**N300**
Wheat flour[a]	180	179.5	179	178
Soybean meal[b]	150	150	150	150
Soy Protein Concentrate[c]	120	120	120	120
Poultry by-product[d]	120	120	120	120
Broken rice[e]	80	80	80	80
Fish meal[f]	60	60	60	60
Hemoglobin[g]	50	50	50	50
Wheat meal[e]	50	50	50	50
Sorghum[h]	45	45	45	45
Dicalcium phosphate[i]	23.5	23.5	23.5	23.5
Krill meal[j]	20	20	20	20

Soy lecithin[k]	22	22	22	22
Potassium chloride[l]	10	10	10	10
Fish oil[f]	10	10	10	10
Soybean oil[b]	10	10	10	10
Salt	10	10	10	10
Nucleotide[m]	0	0.5	1	2
Kaolin[n]	5.6	5.6	5.6	5.6
Magnesium oxide[o]	5	5	5	5
Vitamin and Mineral Supplement[p]	5	5	5	5
DL-Methionine[q]	5	5	5	5
L-Threonine[r]	5	5	5	5
L-Lysine[s]	5	5	5	5
Nutribinder[t]	5	5	5	5
Fylax (Antifungal)[u]	3	3	3	3
Vitamin C (35%)[v]	0.9	0.9	0.9	0.9
Proximate (g kg^{-1})				
Crude protein	366	362	368	366
Crude fat	67	64	64	63
Crude fiber	46	39	46	44
Ash	135	159	160	185
Gross Energy (kcal kg^{-1})	4446	4455	4442	4436

[a]Cidade Bella Moinho / Ponta Grossa-PR; [b]Cooperativa Comigo – Rio Verde-GO; [c]CJ Selecta/Araguari-MG; [d]Frango Rico / Votuporanga-SP; [e]Dallas / Nova Alvorada do Sul-MS; [F]BFP bioprodutos depescado LTDA / ITAJAÍ-SC; [g]Hemoprot – Lins-SP; [h]Raguife / Santa Fé do Sul-SP Raguife / Santa Fé do Sul-SP; [i]Ecophos-Formiga-MG ; [j]Aker Biomarine Antarctic AS (Lysaker, Norway); [k]Adicel Indústria e Comércio - Ingredientes para Indústrias de Alimentos – Belo Horizonte-MG; [l]Brasil Química Ind. e Com. LTDA / Batatais-SP; [m]Biotide/Biorigin/ Lençóis Paulista, SP; [n]CaO do Brasil Ltda / Iguatama-MG; [o]Magnesium do Brasil AS / Fortaleza-CE; [p]De Heus nutrição animal– Rio Claro-SP; [q]Rhodimet® NP99 Adisseo a bluestar company; [r]L-Threonine 98% Ajinomoto do Brasil Indústria e Comércio de Alimentos Ltda; [s]L-Lysine 78% Ajinomoto do Brasil Indústria e Comércio de Alimentos Ltda; [t]Nutri-Bind Aqua Adisseo a bluestar company; [u]Selko Feed Aditives, [v]Heilongjiang NHU Biotechnology CO. Ltd / China.

2.2 Experimental design and system

The study was conducted over 60 days at the Laboratório de Carcinicultura (LACAR), of the Departamento de Pesca e Aquicultura (DEPAq) at the Universidade Federal Rural de Pernambuco (UFRPE), Brazil. Four treatments, each in triplicate, were established using a completely randomized design: a control diet without added nucleotides (C); and diets with nucleotides at 75 mg kg $feed^{-1}$ (N75); feed with nucleotides at 150 mg kg $feed^{-1}$ (N150) and feed with nucleotides at 300 mg kg $feed^{-1}$ (N300) (Table 1).

The system was prepared in twelve fiberglass tanks (800L, 1.0 m^2), each filled with 600 L of seawater (32 gL^{-1}) filtered to 250 µm and chlorinated with 30 ppm of active chlorine using sodium hypochlorite. The water was then dechlorinated by aeration for 48 hours. Subsequently, 200L water from the synbiotic shrimp nursery, characterized by the following parameters: total ammonia nitrogen - TAN = 0.0 mg L^{-1}, NO_2^--N = 1.20 mg L^{-1}, NO_3^--N = 56.3 mg L^{-1}, total alkalinity = 125 mg $CaCO_3$ L^{-1}, pH 7.6, settleable solids - SS = 3 ml L^{-1} and salinity = 29, was added to each tank. The synbiotic was prepared through sequential anaerobic and aerobic processes, each lasting 24 hours. The synbiotic was composed of 5 g m^{-3} of rice bran (< 200 µm), 0.5 g m^{-3} of raw sugar, 0.04 g m^{-3} of commercial bacterial mixture (*Bacillus subtilis* [2.1 × 10^7 CFU g^{-1}], *B. licheniformis* [3.7 × 10^7 CFU g^{-1}], *Bacillus* sp. (2.8 × 10^7 CFU g^{-1}) (Kayros Agrícola e Ambiental, SP, Brazil) mixed with 250ppm dechlorinated seawater. During the experiment, the synbiotic was added to the experimental units every three days (n=15), except when the settleable solids reached 10 mL L^{-1}.

Calcium and magnesium hydroxide were added at 10% (w/w) of the daily feed lot throughout the study to maintain alkalinity above 150 mg $CaCO_3$ L^{-1} and pH above 7.5. No water changes were performed during the experiment, only evaporated water was replaced with freshwater. The experimental units had a working volume of 800 L and bottom area of 1 m^2, and were covered with a black mesh (70% UV protection) to prevent shrimp escape. The aeration system was maintained by a 1.5 HP radial blower, allowing individual control of each experimental unit, through microperforated tubes (0.70 m per unit).

2.3 Water quality

Water quality were monitored twice daily at 8:00 a.m. and 4:00 p.m. for dissolved oxygen - DO and temperature - T (AT-160, AlfaKit, Brazil). Salinity (AZ86031), pH (Asko AK90, Brazil) and settleable solids were monitored three times per week (Avnimelech 2012). Total ammonia nitrogen - TAN (APHA 2012), nitrite-nitrogen - NO_2-N (Fries 1971), nitrate nitrogen - NO_3-N (APHA 2012), orthophosphate - PO_4^{-3} (APHA 2012) and total alkalinity - TA (APHA 2012) were monitored every 10 days. All water samples were filtered through a 45-µm paper filter prior to analysis.

2.4 Shrimp nursery

Postlarvae of *P. vannamei* (PL10, 0.003 g) were obtained from a commercial shrimp hatchery (Aquatec, Canguaretama, Rio Grande do Norte, Brazil) and reared in four fiberglass tanks (working volume 800 L) at a stocking density of 5 PL L^{-1} until they reached a weight of 1.78 ± 0.02 g for the trial. The shrimps were fed with a commercial shrimp feed containing 45% crude protein (0.6 and 0.8 mm, Wean, ADM Animal Nutrition Company, Brazil). The feeding frequency in the shrimp nursery was four times a daily (8:00 am, 11:00 am, 2:00 pm and 5:00 pm). Initially, the daily feeding rate was 35% of body weight, gradually reduced to 8.0%. The feeding rate was adjusted daily based on estimated shrimp feed consumption and mortality (Van Wyk et al. 1999). After nursing, the shrimp were counted, weighed and transferred to culture tanks at a density of 100 shrimp m^{-2} (125 shrimp m^{-3}).

2.5 Shrimp feeding trial

During the feeding trial, the shrimps were fed three times daily (8.00 am, 01:00 pm and 5:00 pm) with the experimental diets detailed in Table 1. Initially, the daily feeding rate was 8.0% of body weight,

gradually reduced to 4.7% over 60 days. The feeding rate was adjusted daily based on estimated shrimp feed consumption and mortality (Van Wyk et al. 1999). Shrimp weight (n=20) was monitored every 10 days to determine growth and to adjust the amount of feed amount. At the 30 days and at the end of the experimental period, all shrimp were counted and final weight, feed conversion ratio (FCR), survival and yield were determined using the following equations:

$$Final\ weight\ (g)\ =\ final\ biomass\ (g)/number\ of\ individuals\ at\ the\ end\ of\ evaluation\ period$$

$$FCR\ =\ feed\ supplied/\ (final\ biomass\ -\ initial\ biomass)$$

$$Growth\ week-1\ (g)\ =\ ((Final\ weight\ -\ initial\ weight)\ /\ days\ between\ the\ actual\ and\ the\ previous\ weight\ measurement))\ x\ 7$$

$$Survival\ (\%)\ =\ (final\ number\ of\ individuals/initial\ number\ of\ individuals)\ \times\ 100$$

$$Yield\ (Kg\ m-3)\ =\ final\ biomass\ (Kg)/volume\ of\ experimental\ unit\ (m3).$$

$$Yield\ (Kg\ ha{-}1)\ =\ final\ biomass\ (Kg)\ x\ 10.000.$$

2.6 Proximate Composition

Analyses of crude protein, lipids, moisture, ash and fiber contents were performed in triplicate using standard methods (AOAC 2016) at the Departamento de Zootecnia, Universidade Federal Rural de Pernambuco, Recife, Brazil. Ten animals per treatment were collected at the beginning and end of the experiment. For moisture content, samples were oven-dried at 105°C to constant weight (model 315 SE, Fanem). The weight difference before and after drying was recorded and expressed as a percentage. Protein content was determined by measuring nitrogen (N x 6.25) using the Kjeldahl method (model TE 0363; Tecnal, São Paulo, Brazil). Total lipid content was determined by the Soxhlet extraction method using pure (98%) hexane solvent (model Ma 044/8/50, Marconi, São Paulo, Brazil). Crude fiber content was determined according to Detmann et al. (2012), and ash was determined by oven combustion at 550 °C (Q318 D24 model; Quimis, São Paulo, Brazil).

2.7 Presumptive total count on TCBS (Vibrio spp.), MYP (Bacillus spp.) and Sabouraud dextrose agar (fungi).

Presumptive total counts were performed at the Laboratório de Sanidade de Organismos Aquáticos (LASAq), DEPAq, UFRPE, Brazil, to quantify colony forming units (CFU g^{-1}) of *Vibrio* sp., *Bacillus* sp. and fungi. For the analysis, pooled gut samples of 50 mg were collected from shrimp in each experimental unit at the beginning, middle, and end of the experimental growth trial (Vandenberghe et al. 1999). The shrimp were euthanized by severing the nerve cord, and their external body surface was disinfected by immersion in 70% ethanol for 15 seconds, followed by a solution of 1.5% sodium hypochlorite with 0.1% Tween-80 for 15 minutes, and then washed three times in sterile distilled water. The gut samples were placed in sterile crucibles, and 600 µL of sterile alkaline peptone water solution was added, followed by maceration and homogenization of the samples.

The samples were then serially diluted from 10^{-1} to 10^{-5} and 100 µL of each dilution were plated onto thiosulfate citrate bile sucrose (TCBS, incubated in triplicate 30 °C for 24 hours), mannitol egg yolk polymyxin agar (MYP, incubated in triplicate 30 °C for 24 hours) and Sabouraud dextrose agar plates

(incubated in triplicate 36°C for 72 hours) for total *Vibrio* count, total *Bacillus* count and fungi count, respectively. After the incubation period, colonies of sucrose-fermenting and non-sucrose-fermenting *Vibrio* spp., colonies of *Bacillus* spp. (CFU g^{-1}), and yeast and/or filamentous fungi based on Trabulsi and Alterthum (2015).

2.8 Ammonia and nitrite nitrogen stress tests

At the end of the growth trial, a total of 30 shrimps (n=10 per replicate) were randomly collected from each experimental treatment, and subjected to a stress test for ammonia nitrogen (NH_3-N) levels in the rearing water (Oliveira et al. 2022; Pimentel et al. 2022). Subsequently, 24 shrimps (n=8 per replicate) were used for the NO_2-N stress test. In each test, experimental units containing 10 L of water (salinity 30 ± 0.6 g L^{-1}, water temperature 28 ± 0.4 °C, and pH 7.5 ± 0.2) were used. The NH_3-N concentration was achieved by applying a stock solution of 10 g L^{-1} of NH_4Cl (PA) and the NO_2-N concentration was achieved by applying a stock solution of 49.25 g L^{-1} $NaNO_2$ (PA) (Table 2). Each stress test was conducted for 96 hours, and survival was assessed every 24 hours (Zhang et al. 2012).

Table 2. Nitrogen concentration in the experimental units for ammonia and nitrite stress tests.

	TAN (mgL^{-1})	NH_3-N (mgL^{-1})	NO_2-N (mgL^{-1})
Initial	29.5	0.60	12.9
24 hrs	35.2	0.61	16.0
48 hrs	32.9	0.66	21.0
72 hrs	33.7	0.77	23.1
96 hrs	37.9	0.77	25.2

Data correspond to the mean (n=3). TAN- Total ammonia nitrogen

Hemolymph (10 shrimps per treatment) was collected for blood analysis before and after the ammonia and nitrite nitrogen concentration stress tests, following the protocol described by Guertler et al. (2013). For this purpose, aliquots of hemolymph (200 µL) were collected from the hemocoel located in the ventral region of the surviving shrimp using a 1 ml syringe containing anticoagulant (modified Alsever's solution [MAS: 336 mmol L^{-1} of NaCl, 115 mmol L^{-1} of glucose, 27 mmol L^{-1} of sodium citrate, and 9 mmol L^{-1} of EDTA, pH 7.2]) at a ratio of 1:2 (v:v). An aliquot of hemolymph was separated and stored in MAS and 4% formaldehyde (1:3). Total hemocyte count (THC) was performed using 100 µL of hemolymph with anticoagulant. A drop of this suspension was added to a Neubauer chamber for cell counting (Cheng and Chen, 2001). The differential hemocyte count (hyaline, semi-granular and granular cells) was performed using a protocol adapted from Celi et al. (2013). The hemolymph was placed on a slide, dried, and fixed by immersion in absolute methanol for 6 minutes. The slide with the fixed material

was then stained with Giemsa solution (dilution: 1:10) for 10 minutes, dehydrated with 70% ethanol for 1 minute, and immersed in xylol for 6 min, followed by counting under the microscope in triplicate.

2.9 Histological analysis

Following the nitrogen stress tests, 10 guts and gills from the shrimps in each treatment were collected and fixed in Davidson AFA solution for 70 hours. The samples were then transferred to a 70% ethanol solution and dehydrated in an ascending series of alcohols (70 to 100%), cleared in xylene, and embedded in histological paraffin, for sectioning (2µm) on a microtome (Leica, RM2255). Slides were stained with haematoxylin and eosin (HE), according to the procedure described by Lightner (1996). An optical microscope (BX 41 OLYMPUS, Olympus Corporation, Tokyo, Japan) coupled to a Leica camera (K5) with 40x and 100x objectives was used to analyses and obtain images of the histological sections.

ImageJ software (1.46r) was used for electronic measurements in the midgut. All villi found in the tissue were measured. The following parameters were measured at 400x magnification: 1) the number of villi in relation to the total number of villi (VN); 2) the height of the villus (µm) in relation to the length from the basement membrane to the top of the villus (VH); 3) the width of the villus (µm) in relation to the width of the villus (VW); 4) the height of the gut fold (µm) from the basement membrane to enterocyte layer (FH); 5) the height of the enterocytes in relation to the thickness of the enterocyte layer (EH) (Figure 1).

Histological changes in the gills (primary and secondary gill filaments) were assessed semi quantitatively by calculating the Histological Alteration Index (HAI), and damage was classified into three progressive stages of tissue damage: stage I (no effect on organ function), stage II (severe damage and impaired organ function), and stage III (very severe and irreversible changes in structure and function) (Figure 2, Table 3) (Fregoso-López et al. 2017). The HAI score was calculated using the following formula (Poleksic and Mitrovic-Tutundzic, 1994):

$$HAI = [1\ x\ \Sigma\ (stage\ I)] + [10\ x\ \Sigma\ (stage\ II)] + [100\ x\ \Sigma\ (stage\ III)]$$

The mean HAI was divided into five categories: 0-10 (normal tissue function), 11-20 (mild to moderate change), 21-50 (moderate to severe change), 51-100 (severe change) and > 100 (irreparable change).

2.10 Return on investment (ROI)

Nucleotide costs and feed processing were considered alongside shrimp performance to determine the return on investment (ROI) according to Phillips and Phillips (2019). Feed production costs in this trial included feed at US$1.03 per kilogram, nucleotide at US$6.50 per kilogram, and shrimp priced at US$3.50 per kilogram, with an estimated production for 1ha.

$$ROI = (Net\ benefit\ with\ nucleotide\ addition - Net\ benefit\ in\ diet\ control) \\ / difference\ in\ total\ expenses\ Nucleotide\ x\ control$$

2.11 Data analysis

Statistical analyses were performed using BioEstat Statistic 5.3 software (Brazil). Data were tested for homogeneity of variances using the Cochran test ($p \leq 0.05$) and for normality using the Lilliefors test ($P \leq 0.05$). For normally distributed and homogeneous data, parametric one-way ANOVA (shrimp performance, and proximate composition) and repeated measures ANOVA (temperature, salinity, pH) were used, followed by Tukey's means comparison test ($p \leq 0.05$). Non-parametric data were analysed using Friedman's test ($p \leq 0.05$) with Conover's multiple comparison test with Holm-Bonferroni correction for dissolved oxygen, total alkalinity, TAN, N-NO_2, N-NO_3 and settleable solids. TCBS, MYP, Sabouraud dextrose agar counts, gut morphology, survival (ammonia and nitrite nitrogen stress tests) and total differential hemocytes counts were analyses using the non-parametric Kruskal-Wallis test ($p \leq 0.05$) followed by Dunn's post-hoc test with Holm-Bonferroni correction.

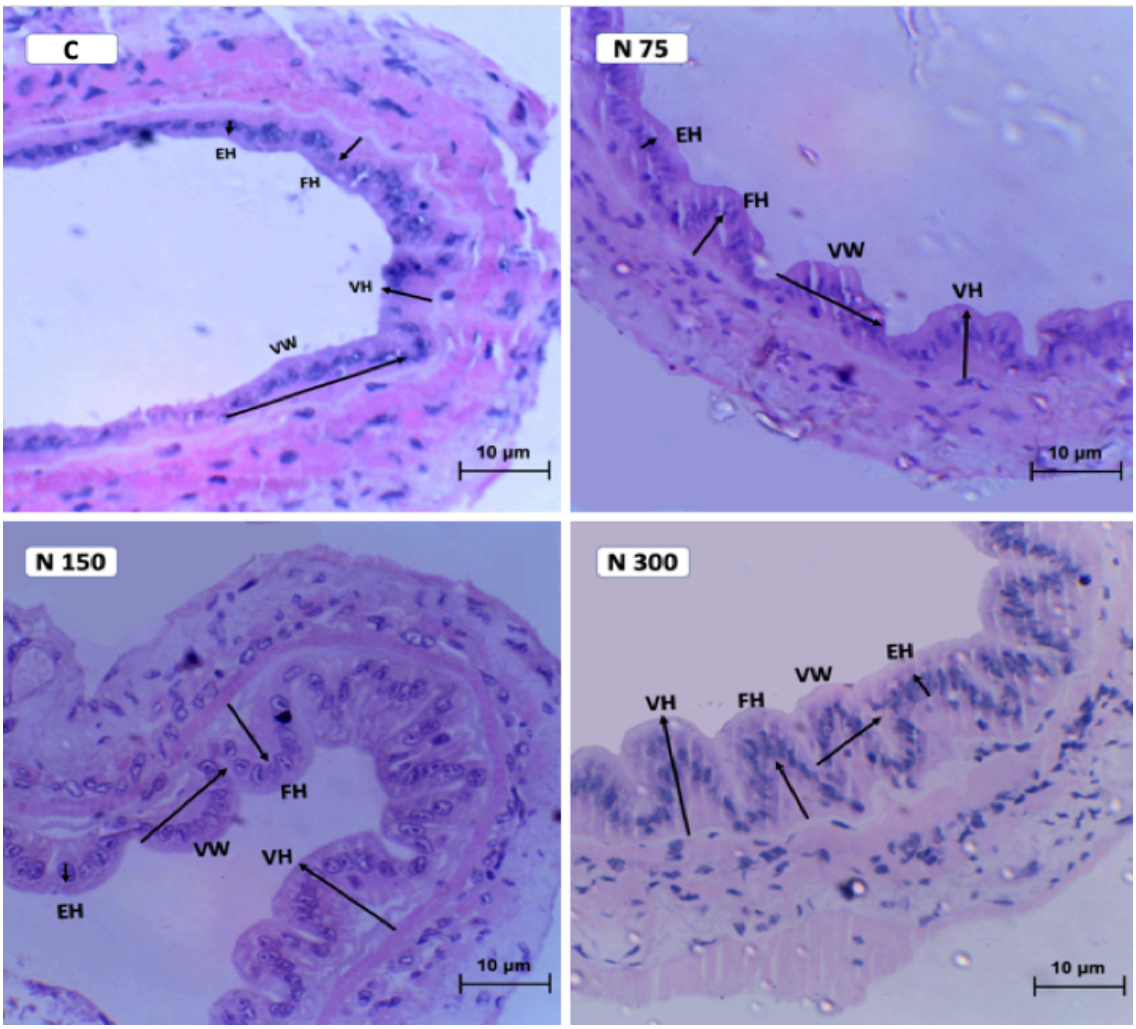

Figure 1. Details of midgut morphology examination of *Penaeus vannamei* fed with the experimental diets. From left to right are C, N75, N150 and N300, respectively. EH, FH, VH and VM. Scale bar=10 µm; original magnification × 400.

Table 3. Classification of the histological changes found in primary and secondary gill filaments of *Penaeus vannamei* examined after ammonia and nitrite nitrogen stress tests.

Stage 1	Stage 2	Stage 3
Melanization	Deformation	Necrosis
Hemolymph infiltration	Atrophy	
Dilation of hemolymph vessels (mild and moderate)	Dilation of hemolymph vessels (severe)	Separation of the epithelium
This stage represents a challenge in progress and the organism's response to it.	At this stage, there is less area available for gas exchange to carry out haematosis. As a result, from a physiological point of view, the animal's performance is reduced.	At this stage, the gills are not working efficiently to oxygenate the blood.

3. RESULTS

3.1 Water quality

The data for the water quality variables are summarized in Table 4. No significant differences ($P < 0.05$) were observed for water quality.

3.2 Shrimp performance

The shrimp performance data after 30 and 60 days of experimental culture are summarized in Table 5. Significant differences between treatments were found for final weight, growth (g $week^{-1}$), yield kg m^{-3} and kg ha^{-1}, with higher values observed in the nucleotide addition treatments. However, FCR was lower in all nucleotide treatments than in the control. Significant differences between treatments were also found for survival at day 60 (Table 5).

3.3 Proximate composition

Analysis of the body composition of the shrimps fed the experimental diets at the end of the trial showed that the crude protein levels were significantly higher in the shrimps fed the nucleotide treatments (N75, N150 and N300), whereas the lipid levels in the N150 and N300 treatments were significantly lower than those recorded in the shrimps fed the control (C) and N75 treatment (Table 6).

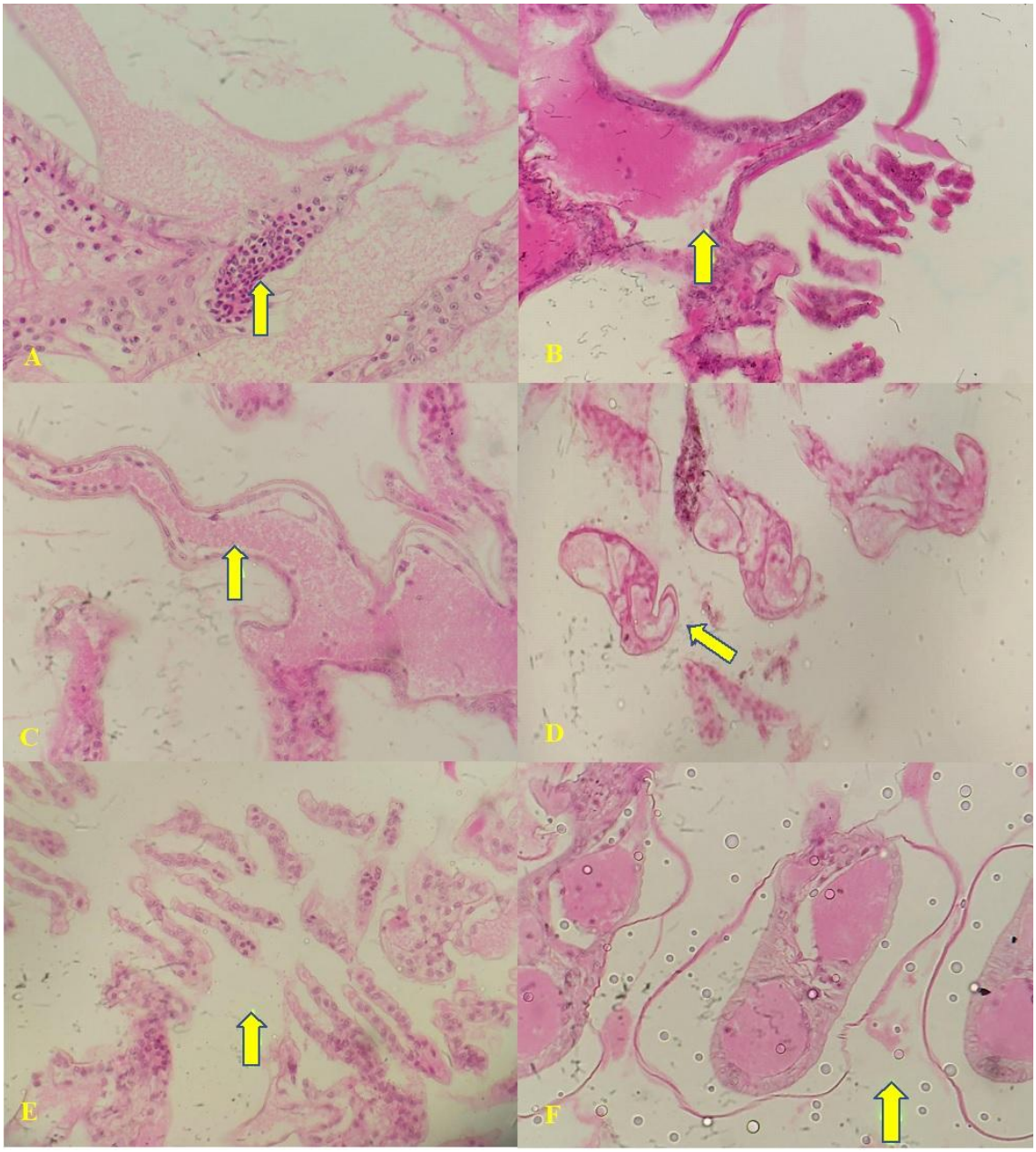

Figure 2. Histological changes in the primary and secondary gill filaments of *Penaeus vannamei*. A - Intense haemolymph infiltration; B - Intense melanisation; C - Branchial atrophy; D - Deformation; E - Necrosis; F - Epithelial separation.

Table 4 Water quality variables in the *P. vannamei* grow-out feed with dietary nucleotides in an intensive synbiotic system.

Variables	Treatments				*P value*
	C	N75	N150	N300	
Temperature in morning (°C)	28.68 ± 0.37	28.74 ± 0.34	28.79 ± 0.36	28.59 ± 0.35	0.3241
Temperature in afternoon (°C)	28.54 ± 0.36	28.60 ± 0.33	28.72 ± 0.37	28.49 ± 0.33	0.1832
DO 8:00 am (mgL^{-1})	5.86 ± 0.45	5.76 ± 0.30	5.74 ± 0.22	5.70 ± 0.22	0.7599
DO 4:00 pm (mgL^{-1})	5.95 ± 0.42	5.88 ± 0.33	5.84 ± 0.25	5.77 ± 0.29	0.5071
Salinity (gL^{-1})	30.20 ± 1.63	31.66 ± 1.23	30.14 ± 1.85	32.21 ± 1.04	0.9966
pH 8:00 am	7.84 ± 0.10	7.80 ± 0.09	7.80 ± 0.09	7.82 ± 0.11	0.5430
pH 4:00 pm	7.81 ± 0.11	7.78 ± 0.11	7.78 ± 0.08	7.78 ± 0.15	0.7036
Total alkalinity (mg $CaCO_3L^{-1}$)	163.61 ± 16.23	162.22 ± 24.29	166.67 ± 25.57	165.83 ± 16.96	0.6369
TAN (mgL^{-1})	0.22 ± 0.26	0.24 ± 0.25	0.20 ± 0.24	0.22 ± 0.27	0.3639
Nitrite-N (mgL^{-1})	0.25 ± 0.21	0.32 ± 0.20	0.29 ± 0.20	0.25 ± 0.21	0.1771
Nitrate-N (mgL^{-1})	233.49± 129.01	198.25± 115.44	187.20± 92.67	142.97± 84.21	0.0594

Orthophosphate (mgL^{-1})	47.39 ± 26.39	44.62 ± 26.12	46.67 ± 26.83	41.16 ± 22.53	0.8610
SS (mLL^{-1})	3.03 ± 1.76	2.49 ± 0.90	2.39 ± 1.16	2.67 ± 0.99	0.0617

Data are expressed as mean (n=21) ± standard deviation. Results were analyzed by repeated measures ANOVA ($p \leq 0.05$) for parametric data and Friedman's test ($p \leq 0.05$) for non-parametric data. Control - without added nucleotides (C); feed with nucleotides at 75 mg kg $feed^{-1}$ (N75); feed with nucleotides at 150 mg kg $feed^{-1}$ (N150) and feed with nucleotides at 300 mg kg $feed^{-1}$ (N300). TAN - Total ammonia nitrogen, DO – Dissolved Oxygen, SS - settleable solids.

Table 5. Performance of *P. vannamei* fed with the experimental diets with different addition levels of nucleotides during the grow-out in an intensive synbiotic system.

Variables	Treatments				*P value*
	C	N75	N150	N300	
	30 days				
Final weight (g)	4.57±0.09^{b}	5.45±0.23^{a}	5.48±0.33^{a}	5.68±0.51^{a}	0.0137
Survival (%)	98.67±1.53^{a}	98.67±1.53^{a}	98.67±1.53^{a}	98.67±1.53^{a}	1.0000
Growth (g week^{-1})	0.65±0.02^{b}	0.85±0.06^{a}	0.86±0.07^{a}	0.92±0.12^{a}	0.0081
Yield (kg m^{-3})	0.56±0.02^{b}	0.67±0.03^{a}	0.68±0.04^{a}	0.70±0.05^{a}	0.0118
Yield (kg ha^{-1})	4,513±153^{b}	5,378±266^{a}	5,405±285^{a}	5.596±418^{a}	0.0091
FCR	1.94±0.09^{a}	1.52±0.08^{b}	1.51±0.16^{b}	1.41±0.15^{b}	0.0036
	60 days				
Final weight (g)	8.20±0.28^{b}	8.92±0.16^{a}	9.09±0.12^{a}	9.09±0.22^{a}	0.0027
Survival (%)	89.33±1.53^{b}	93.67±0.57^{a}	93.00±1,00^{a}	96.00±1.73^{a}	0.0021
Growth (g week^{-1})	0.75±0.03^{b}	0.83±0.02^{a}	0.85±0.^{01a}	0.86±0.02^{a}	0.0044
Yield (kg m^{-3})	0.92±0.02^{c}	1.04±0.02^{b}	1.06±0.01^{ab}	1.09±0.01^{a}	<0.0001
Yield (kg ha^{-1})	7,323±194^{b}	8,335±202^{a}	8,453±81^{a}	8,727±113^{a}	<0.0001
FCR	1.95±0.07a	1.79±0.06^{b}	1.77±0.01^{b}	1.74±0.02^{b}	0.0056

Data are expressed as mean (n=3) ± standard deviation. The results were analyzed by means of ANOVA ($p \leq 0.05$) followed by the Tukey test. Mean values on the same line with different superscripts differ significantly. Control - without added nucleotides (C); feed with nucleotides at 75 mg kg feed^{-1} (N75); feed with nucleotides at 150 mg kg feed^{-1} (N150) and feed with nucleotides at 300 mg kg feed^{-1} (N300).

Table 6. Body composition of *P. vannamei* after 60 days of feeding with increasing levels of dietary nucleotides in an intensive synbiotic system.

% Dry matter	Treatments				*P value*
	Initial	C	N75	N150	N300	
Moisture	76.81 ± 1.41[a]	76.33 ± 1.09[a]	76.04 ± 0.72[a]	75.25 ± 0.46[a]	76.32 ± 0.59[a]	0.3323
Protein	61.89 ± 1.80[c]	69.46 ± 1.74[b]	74.47 ± 0.52[a]	72.92 ± 0.97[a]	73.49 ± 1.57a	<0.0001
Lipids	3.25 ± 0.95[b]	3.88 ± 0.63[a]	2.89 ± 0.28[b]	2.73 ± 0.23[b]	3.06 ± 0.23[ab]	0.0239
Fiber	8.29 ± 0.75[a]	5.02 ± 1.26[b]	5.67 ± 0.37[b]	5.95 ± 0.60[b]	5.72 ± 0.42[b]	0.0001
Ash	11.85 ± 0.79[a]	12.20 ± 1.05[a]	10.95 ± 0.16[b]	12.16 ± 0.33[a]	12.75 ± 0.16[a]	0.0332

Values in dry weight, g $100g^{-10}$, expressed as mean (n=4) ± standard deviation. The results were analyzed by means of ANOVA ($p \leq 0.05$) followed by the Tukey test. Mean values on the same line with different superscripts differ significantly. Control - without added nucleotides (C); feed with nucleotides at 75 mg kg $feed^{-1}$ (N75); feed with nucleotides at 150 mg kg $feed^{-1}$ (N150) and feed with nucleotides at 300 mg kg $feed^{-1}$ (N300).

3.4 Presumptive total count of TCBS (Vibrio spp.), MYP (Bacillus spp.) and Sabouraud dextrose agar (fungi) in the shrimp gut.

At the beginning, the presumptive *Vibrio* counts were 6.15 × 10^6 CFU g^{-1}, but the sucrose-fermenting bacteria were higher. In the middle and at the end, a decrease in non-sucrose fermenting bacteria and sucrose fermenting bacteria counts was observed in all treatments. The presumptive *Vibrio* counts at the end ranged from 0.81 × 10^5 CFU g^{-1} (N300) to 5.99 × 10^5 CFU g^{-1} (N150), but there were no significant differences (*P*= 0.0569) between treatments (Figure 3). Similarly, the higher levels of *Bacillus* sp. in the shrimp gut (267.68 × 10^5 CFU g^{-1}) were recorded at the beginning of the experiment and decreased during the experiment to reach their lower levels on day 60 (0.01-0.03 × 10^4 CFU g^{-1}), with no significant difference (*P*= 0.0819) between treatments. For yeast, the higher concentrations (8.86-14.25 × 10^5 CFU g^{-1}) at the end were observed in N150 and N300, but there were no significant differences (*P*= 0.0994) between treatments (Figure 3).

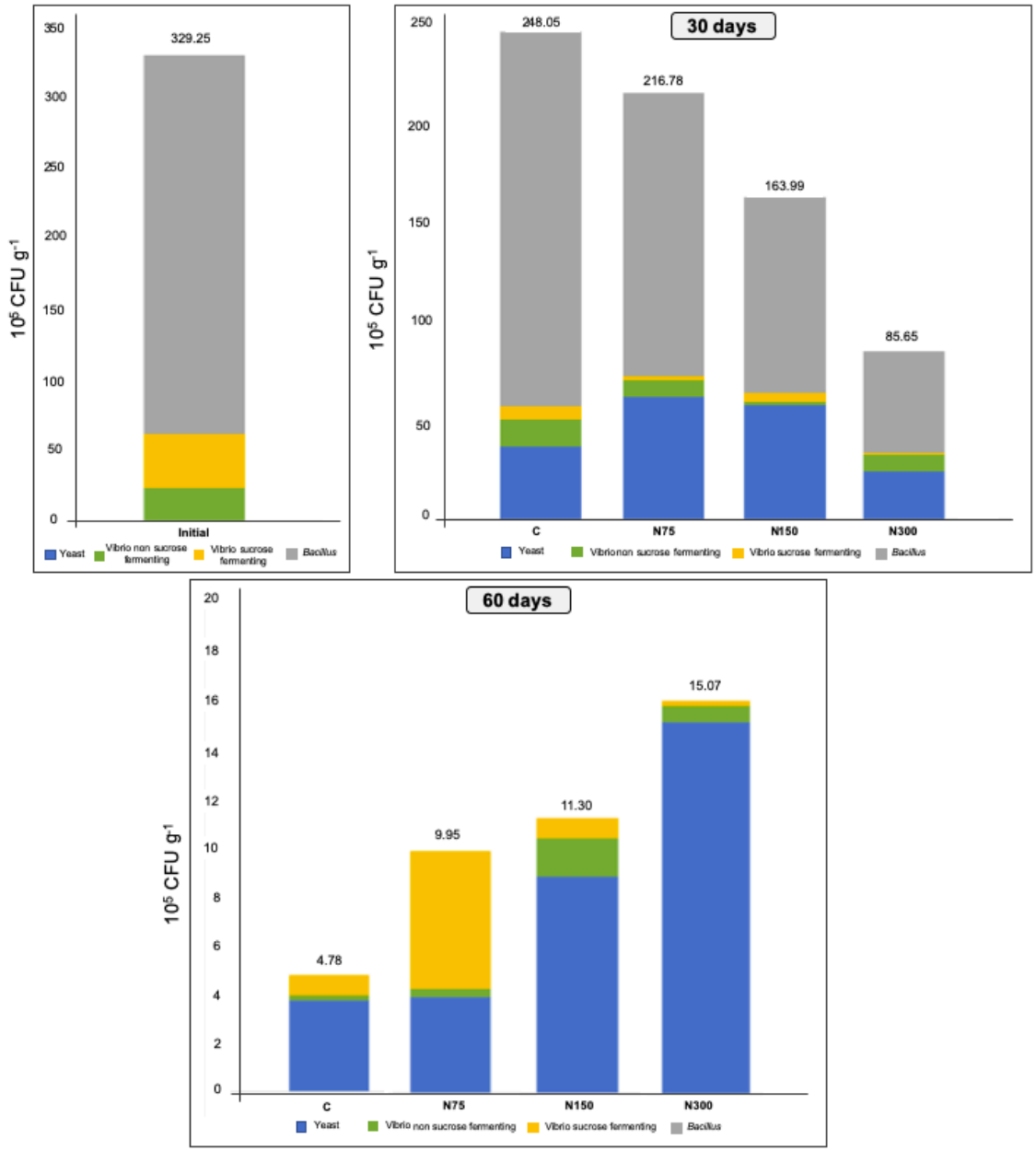

Figure 3. Presumptive total count of TCBS (*Vibrio* spp.) non-sucrose-fermenting bacteria and sucrose-fermenting bacteria, MYP (*Bacillus* spp.) and Sabouraud dextrose agar (yeast) in shrimp gut. Control - without added nucleotides (C); feed with nucleotides at 75 mg kg feed^{-1} (N75); feed with nucleotides at 150 mg kg feed^{-1} (N150) and feed with nucleotides at 300 mg kg feed^{-1} (N300).

3.5 Ammonia and nitrite nitrogen stress tests

With regard to ammonia nitrogen stress tests, the survival of *P. vannamei* with nucleotide addition was between 77.8 and 80.6% and the control 75%, although without significant differences (Table 7). Similarly, for nitrite nitrogen stress tests, survival was between 79.2 and 83.3% for nucleotide treatments and 70.8% for control, although without significant differences (Table 8).

Total hemocyte counts (THC) before and after the stress tests are shown in Table 9. There were no significant differences (P= 0.9058) in THC between treatments after the stress tests. A decrease in hemocyte counts was observed in all treatments after the stress tests. The proportions of hyaline (H), semi-granular (SG) and granular (G) cells are shown in Figure 4. No significant differences in differential hemocyte count (DHC) were observed between treatments. In all treatments, hyaline cells were consistently predominant throughout the experimental period.

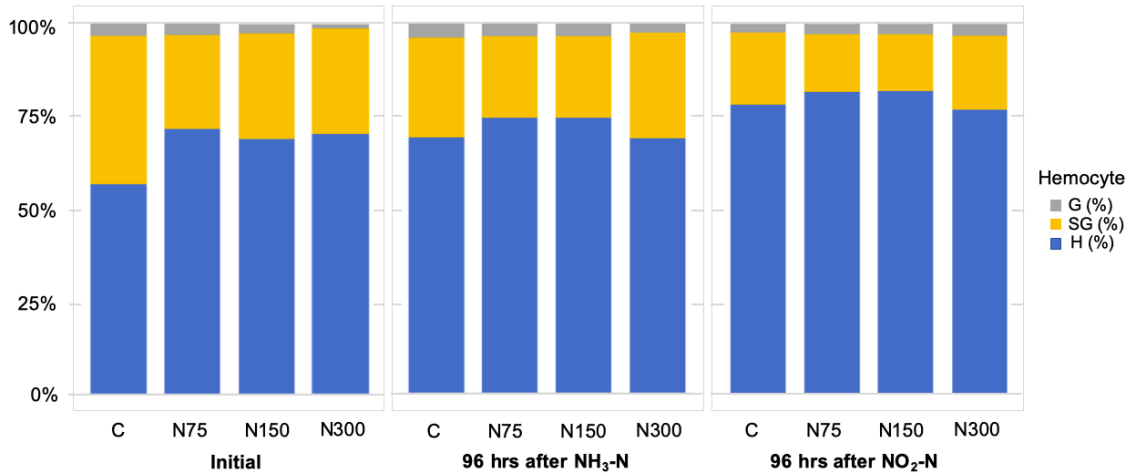

Figure 4. Differential hemocyte counts at first and after ammonia and nitrite stress tests, feeding with dietary nucleotides. H = Hyaline; SG = Semi-granular; G = Granular. Control - without added nucleotides (C); feed with nucleotides at 75 mg kg feed^{-1} (N75); feed with nucleotides at 150 mg kg feed^{-1} (N150) and feed with nucleotides at 300 mg kg feed^{-1} (N300).

3.6 Shrimp Gut and gill morphology

Shrimp midgut morphology is summarized in Table 10. The FH, VH and EH of shrimp fed the nucleotide diet were significantly higher than those fed the control diet, but the VW was higher in those fed the control treatment (Table 10). Histological changes in the gills of shrimps after stress tests with ammonia and nitrite resulted in irreparable changes in the control, N150 and N300, and severe changes in N75 (Table 11).

3.7 Return on investment (ROI)

For the dietary nucleotide treatments, the income from shrimp sales minus the cost of feed ranged from US$ 29,244 ha^{-1} to US$ 30,542 ha^{-1} for N75 and N300, respectively (Table 12). The ROI was estimated using a feed cost of US$1.03 kg^{-1}, a cost of nucleotides of US$6.50 kg^{-1} (Biotide, Biorigin, Brazil) and a shrimp selling price of US$3.50 kg^{-1} (in Brazil). The control had the lower ROI, with the highest in the N150 (3.96).

Table 7. Survival of *P. vannamei* fed with increasing levels of dietary nucleotides in an intensive synbiotic system along the 96 hours of the ammonia nitrogen stress test.

Exposure time	Treatments				*P value*
	C	N75	N150	N300	
24 hrs	94.4 ± 9.6	94.4 ± 4.8	94.4 ± 4.8	94.4 ± 4.8	0.9924
48 hrs	83.3 ± 14.4	88.9 ± 4.8	91.7 ± 8.3	88.9 ± 4.8	0.7299
72 hrs	77.8 ± 12.7	83.3 ± 8.3	91.7 ± 8.3	80.6 ± 9.6	0.3786
96 hrs	75.0 ± 8.3	80.6 ± 12.7	80.6 ± 12.7	77.8 ± 12.7	0.9048

Data are expressed as mean (n=3 per exposure time) ± standard deviation. The results were analyzed by non-parametric Kruskal-Wallis test ($P \leq 0.05$). Control - without added nucleotides (C); feed with nucleotides at 75 mg kg feed^{-1} (N75); feed with nucleotides at 150 mg kg feed^{-1} (N150) and feed with nucleotides at 300 mg kg feed^{-1} (N300).

Table 8. Survival of *P. vannamei* fed with increasing levels of dietary nucleotides in an intensive synbiotic system along the 96 hours of the nitrite nitrogen stress test.

Exposure time	Treatments				*P value*
	C	N75	N150	N300	
24hrs	95.8 ± 7.2	91.7 ± 7.2	100.00 ± 0.0	100.00 ± 0.0	0.2139
48hrs	87.5 ± 0.0	83.3 ± 7.2	87.50 ± 0.0	83.33 ± 7.2	0.5319
72hrs	79.2 ± 7.2	83.3 ± 7.2	83.3 ± 7.2	83.33 ± 7.2	0.8151
96hrs	70.8 ± 7.2	79.2 ± 7.2	83.3 ± 7.2	83.33 ± 7.2	0.2159

Data are expressed as mean (n=3 per exposure time) ± standard deviation. The results were analyzed by non-parametric Kruskal-Wallis test ($P \leq 0.05$). Control - without added nucleotides (C); feed with nucleotides at 75 mg kg feed^{-1} (N75); feed with nucleotides at 150 mg kg feed^{-1} (N150) and feed with nucleotides at 300 mg kg feed^{-1} (N300).

Table 9. Total hemocyte count (THC) (10^6 cells mL^{-1}) of the *P. vannamei* fed with increasing levels of dietary nucleotides in an intensive synbiotic system before and after a chemical stress test.

Exposure time	Treatments				*P value*
	C	N75	N150	N300	
Initial	6.68 ± 3.18	5.69 ± 2.97	5.72 ± 3.73	5.73 ± 3.18	0.6337
96hrs after NH_3-N	6.71 ± 3.97	5.53 ± 5.13	5.28 ± 1.83	6.32 ± 3.50	0.3287
96hrs after NO_2-N	4.17 ± 3.66	5.48 ± 5.97	3.91 ± 1.77	5.64 ± 5.08	0.9058

Data are expressed as mean (n =24 per exposure time) ± standard deviation. The results were analyzed by non-parametric Kruskal-Wallis test ($P \leq 0.05$). Control - without added nucleotides (C); feed with nucleotides at 75 mg kg $feed^{-1}$ (N75); feed with nucleotides at 150 mg kg $feed^{-1}$ (N150) and feed with nucleotides at 300 mg kg $feed^{-1}$ (N300).

Table 10. Midgut morphology of *P. vannamei* fed experimental diets containing a nucleotide addition for 60 days.

Morphology	Treatments				*P value*
	C	N75	N150	N300	
Villus number (VN)	12.5±5.10^{b}	18.00±6.86^{b}	23.50±4.27^{a}	19.70±7.16^{b}	0.0053
Fold height (FH, µm)	2.76±0.81^{c}	4.42±1.48^{b}	7.35±2.36^{a}	6.65±1.63^{ab}	<0.0001
Villus width (VW, µm)	20.77±4.00^{a}	14.24±3.19^{b}	12.92±3.12^{b}	13.80±2.84^{b}	<0.0001
Villus height (VH, µm)	5.85±1.24^{d}	10.06±1.53^{c}	13.67±2.56^{a}	12.60±2.60^{b}	<0.0001
Enterocyte height (EH, µm)	1.71±0.36^{c}	2.11±0.41^{b}	2.98±0.53^{a}	2.81±0.92^{a}	<0.0001

Data are expressed as mean (n=10 gut by treatment) ± standard deviation. The results were analyzed by non-parametric Kruskal-Wallis test ($P \leq 0.05$) followed by Dunn's post-hoc test with Holm-Bonferroni correction. Mean values on the same line with different superscripts differ significantly. Control - without added nucleotides (C); feed with nucleotides at 75 mg kg feed^{-1} (N75); feed with nucleotides at 150 mg kg feed^{-1} (N150) and feed with nucleotides at 300 mg kg feed^{-1} (N300).

Table 11. Histological alteration index values of gills (primary and secondary gill filaments) of *P. vannamei* challenged with N-NH_3 and N-NO_2 stress test.

Stage		Treatments			
		C	N75	N150	N300
Stage I	Normal tissue function	0.0 %	0.0 %	0.0 %	0.0 %
Stage II	Mild to moderate alteration	11.1%	0.0 %	0.0 %	0.0 %
	Moderate to severe alteration	22.2%	55.6%	0.0%	0.0%
	Severe alteration	0.0 %	0.0 %	0.0 %	0.0 %
Stage III	Irreparable alteration	66.7%	44.4%	100.00%	100.00%
	HAI	115	94	212	202

Data are expressed as mean (n=10 gill by treatment) ± standard deviation. Control - without added nucleotides (C); feed with nucleotides at 75 mg kg $feed^{-1}$ (N75); feed with nucleotides at 150 mg kg $feed^{-1}$ (N150) and feed with nucleotides at 300 mg kg $feed^{-1}$ (N300).

Table 12. Return on investment (ROI) of *P. vannamei* culture fed with dietary nucleotides in an intensive synbiotic system.

			Treatments			
			C	N75	N150	N300
Revenues	Stocking	shrimp ha^{-1}	1,000,000	1,000,000	1,000,000	1,000,000
	Survival	%	89	94	93	96
	Harvested shrimp	shrimp ha^{-1}	893,300	936,700	930,000	960,000
	Shrimp final weight	g	8.20	8.90	9.10	9.10
	Harvested shrimp	kg ha^{-1}	7,325	8,355	8,454	8,726
	Revenues	US$ ha^{-1}	25,638	29,244	29,588	30,542
Expenses	FCR		1.95	1.79	1.77	1.74
	Feed supplied	kg ha^{-1}	14,284	14,956	14,963	15,184
	Feed cost (U$$ 1.03/kg feed x FCR)	USD kg^{-1}	2.01	1.84	1.82	1.79
	Investment with the Application of nucleotides	USD kg^{-1}	0.00	48.61	97.26	197.39
	Total cost (feed + nucleotides)	USD ha^{-1}	14,712	15,453	15,509	15,837
ROI	Net Revenue	USD ha^{-1}	10,925	13,790	14.079	14,706
				3.87	3.96	3.36

4. DISCUSSION

The water quality variables found in this study were within an acceptable range for intensive farming, according to Samocha (2019). The pH and alkalinity levels were around 7.8 and 168 mg $CaCO_3$ L^{-1}, respectively, due to the weekly addition of calcium and magnesium hydroxide at 10% (w/w) of the daily feed, which contributed to nitrification and minimized the effect of nitrogen on shrimp growth. Nucleotide addition levels do not appear to affect water quality as concentrations were similar between treatments.

In this study, dietary nucleotides added (75, 150 and 300 mg kg^{-1}) to *P. vannamei* improved their growth, yield and FCR compared to shrimp in the control treatment. Similar improvements in shrimp performance with dietary nucleotides ranging from 50 to 750 mg kg^{-1} have been reported for Pacific white shrimp (Murthy et al.

2009; Andrino et al. 2012; Novriadi et al. 2021; Rairat et al. 2022). Murthy et al. (2009) found that at a shrimp biomass stocking of 0.53 kg m^{-3} (180 shrimp m^{-3}) and 36% fish meal in the shrimp feed, growth was significantly higher than the control with the addition of nucleotides (200 and 500 mg kg $feed^{-1}$). Novriadi et al. (2021) showed that decreasing fish meal from 10% to 3% in the feed and adding nucleotides (50 or 100 mg kg^{-1}) did not reduce shrimp performance. In this study, with low inclusion of fish meal (6%) in the feed, the shrimp performance was higher in treatments with the addition of nucleotides (75, 150 and 300 mg kg $feed^{-1}$), confirming the importance of adding nucleotides when feed has a low percentage of fish meal.

According to Rairat et al. (2022), a nursery system study (400 shrimp m^{-3}, 3mg to 2.0g) with shrimp diets containing 21% fish meal, 4.5% fish hydrolysate and 2.33% krill meal, and the addition of 250, 500 and 750 mg nucleotides kg^{-1}, showed no significant effect on shrimp growth, but an effect on survival was observed after 45 days. Therefore, the results suggest that the levels of nucleotides added depends on the stocking density and the substitution of ingredients in the feed, such as fish meal and other marine meal components. In addition to dosage, the purity of the nucleotides in the yeast extracts or other nucleotide products must be considered when comparing the results of different studies. Nucleotides are traditionally not considered essential nutrients, as they can be produced endogenously under normal conditions via salvage. However, when fishmeal is reduced and stocking densities are increased, nucleotide supplementation contributes to greater shrimp growth. This is due to lower energy costs and the improvement of functions in tissues that divide rapidly, especially during periods of rapid growth (Burrells et al. 2001; Ceseña et al. 2021).

The present study revealed that the crude protein content of shrimp in the nucleotide addition treatments was higher than that in the control, a finding that is consistent with the results reported by Novriadi et al. (2022), who observed a protein content of 340 mg NT kg $feed^{-1}$. Nucleotides are formed from molecules comprising a phosphate, ribose, and a nitrogen base. They serve as the fundamental building blocks for RNA and DNA, which are responsible for energy transfer in the form of ATP and protein synthesis. This latter process is subsequently transcribed and translated into protein within the body of fish and shrimp (Hossain et al., 2020).

In addition to the effects on shrimp performance, there was a decrease in the *Vibrio* count in the shrimp gut throughout the experiment, but yeast levels increased in both treatments, with a tendency to increase in the nucleotide treatments, although without significant differences. Rairat et al. (2022) showed that the addition of nucleotides (750 mg kg $feed^{-1}$) to the diet reduced *Vibrio* counts in the shrimp gut and hepatopancreas compared to those without nucleotide. In this study, no differences were observed with or without nucleotide addition, which may be related to the use of the synbiotic system (fermentation and respiration of rice bran with probiotic microorganisms) and nucleotide addition (75, 150 and 300 mg kg $feed^{-1}$). This synbiotic system aims to provide eubiosis in the shrimp gut and hepatopancreas (Andrade et al. 2021; Silva et al. 2023). In addition, most research on the addition of nucleotides and or yeast-derived to improve gut health has been carried out in clean water or RAS (recirculating aquaculture systems) without the use of probiotics (Ayiku et al. 2020; Rairat et al. 2022).

The use of yeast cell wall derivatives has become more prominent in the animal feed industry, improving immune responses, survival and growth performance (Andrino et al. 2012; Biswas et al. 2012; Shankar et al. 2012; Novriadi et al. 2021) and survival after environmental stress such as high ammonia (Yong et al. 2020). This is probably due to two main factors: the modulation of the gut microflora in a probiotic-like manner (Rairat et al. 2022) and the signaling pathways involved in immunomodulation, in particular purinergic signaling mediated by purine NT (ATP, ADP and AMP) and purine nucleosides (adenosine) (Hossain et al. 2020). However, this study

showed that nucleotide addition did not improve resistance to ammonia and nitrite stress. After the ammonia and nitrite challenge test, shrimp fed nucleotide addition diets had survival rates between 77% and 80% ($N\text{-}NH_3$) and 79% and 83% ($N\text{-}NO_2$), while the control treatment had survival rates between 75% and 70%, no significant differences. Similar results were observed by Yong et al. (2020) using different nucleotide sources to feed *P. vannamei* for 10 days. Research with *Bacillus* and prebiotic (fermented rice bran - synbiotic) can improve growth, enhance the metabolism of beneficial bacteria and increase the resistance of *P. vannamei* to ammonia (Chen et al. 2020), a strategy similar to the control and nucleotides treatments in this study and commonly used in shrimp farms in Latin America and Asia, with the application of bran fermented by probiotic microorganisms in the ponds.

In this study, THC levels in the hemolymph of shrimp fed nucleotides at the end of the culture period and during ammonia and nitrite stress were not significantly different from those fed the control diet. This result was similar to that observed by Novriadi et al. (2022) in open pond systems, but different from previous studies by Murthy et al. (2009) and Guo et al. (2016) in RAS. The microbial-based system (synbiotic) may contribute to an improved immune status of shrimp in both treatments, reducing the effect of nucleotide addition on THC levels compared to RAS.

Alterations such as an increase in villus height result in an expansion of the surface area available for nutrient absorption, which can consequently increase the efficiency of digestion and nutrient absorption in aquatic animals (Chen and Wang 2013; Torrecillas et al. 2015; Sun et al. 2018). In this study, it was observed that the height of villi, gut folds and enterocytes was significantly higher in the nucleotide addition treatments than in the control treatment, which may indicate that the shrimp fed with nucleotide had a higher nutrient absorption capacity. These observations of improved gut morphology are consistent with the increased growth performance resulting from nucleotide supplementation. Xiong et al. (2018) used diets containing 30 and 50 g kg^{-1} of nucleotide-rich yeast, while Guo et al. (2016) used 60, 90 and 120 mg kg^{-1} and observed improvements in gut morphology of juvenile *P. vannamei* fed for 8 and 10 weeks, respectively.

In this study, histological changes were observed in the gills of *P. vannamei* after exposure to ammonia and nitrite stress, with and without nucleotide addition. High histological changes index values were observed during the final stage of nitrogen stress, with greater damage observed in all treatments. Previous studies with high concentrations of nitrogen compounds have demonstrated histological changes in the gills of *P. vannamei* at different concentrations and exposure times (Fregoso-López et al. 2017). The simultaneous presence of these compounds, especially ammonia and nitrite, significantly increases their toxicity in penaeids due to the synergistic effects of the compounds (Alcaraz, Chiappa-Carrara, Espinoza and Vanegas 1999; Schuler, Boardman, Kuhn and Flick 2010).

Many novel ingredients and feed additives are currently being researched to improve shrimp growth, health and disease resistance (Segarra et al. 2023). However, production costs are also a limiting factor for shrimp producers. It is important to strive for higher profits and competitive advantage, which requires minimizing production costs while maintaining profitability and sustainable development. The feed cost was US$ 1.05 kg $feed^{-1}$ and the FCR values in the treatments were US$ 2.01 (control), US$ 1.84 (N75), US$ 1.82 (N150) and US$ 1.78 (N300). The percentage of improved ROI over the control group ranged from 3.36 (N300) to 3.96 (N150), demonstrating that the addition of nucleotides to shrimp feed improved profitability. These results suggest that nucleotides may be a promising additive for shrimp feed. However, more research is needed to fully understand the mechanisms by which nucleotides benefit shrimp health and nutrition. Investigating the specific nutritional

needs met by these nucleotides in shrimp metabolism will contribute to a better understanding of their potential applications in aquaculture.

5. CONCLUSION

The addition of nucleotides (75, 150 and 300 mg kg feed^{-1}) to the diet of *P. vannamei* reared in an intensive synbiotic system improved growth, survival and yield compared to the control treatment. The addition also increased whole body protein levels in shrimp and improved gut health (FH - fold height, VH - villus height and EH - enterocyte height). The addition of nucleotides to the shrimp feed also improved the return on investment. The most recommended dose is 150 mg nucleotide feed kg^{-1}, as it gave the highest ROI. Overall, this study opens new avenues for improving shrimp feed formulations and improving shrimp production efficiency in intensive synbiotic systems.

AUTHOR CONTRIBUTION

Danielle Alves da Silva: Conceptualization, Methodology, Investigation, Data Curation, Formal analysis, and Writing - Original Draft. **Katharine Batista Santos de Souza:** Data Curation, and Writing. **Gisely Karla de Almeida Costa:** Data Curation, and Writing. **Suzianny Maria Bezerra Cabral da Silva:** Conceptualization, Methodology, Resources, Project administration, Funding acquisition, and Writing - Review & Editing. **Fernando Leandro dos Santos:** Data Curation, and Writing - Review & Editing. **Rodrigo Antônio Ponce de Leon Ferreira de Carvalho:** Conceptualization, Writing - Review & Editing. **Giovanni Sampaio Gonçalves:** Methodology, Writing - Review & Editing. **João Fernando Albers Koch:** Conceptualization, Resources, Funding acquisition, and Writing - Review & Editing. **Luis Otavio Brito:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, and Writing - Review & Editing.

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DATA AVAILABILITY

The findings of this study are supported by data, which can be made available upon request from the corresponding author.

DECLARATIONS

Ethics approval and consent to participate

The research conducted adheres to the current animal welfare regulations in Brazil. The use of *Penaeus vannamei* in this experimental study does not require approval from the Brazilian Ethics Committee for Animal Use. All authors consented to participate in this research.

Human and animal ethics

The authors adhered to both international and institutional guidelines for the management of animals during the experiments.

Consent for publication

All the authors of this article agree to the publication.

Competing interests

The authors declare no competing interests.

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5. ARTIGO CIENTÍFICO II

Artigo científico a ser encaminhado à Revista Aquaculture International (ISSN:1365-2109)

Todas as normas de redação e citação, deste artigo, atendem aquelas estabelecidas pela referida revista.

Effects of dietary solubilized mannanoligosaccharides on performance, health status, nitrogen stress resistance, and economic benefits in Pacific white shrimp, *Penaeus vannamei*, raised in an intensive synbiotic system

ABSTRACT

This study was conducted to evaluate the effects of dietary supplementation with commercially available mannanoligosaccharides (MOS) on performance, health status (gut bacterial and fungal counts, ammonia and nitrite stress, gut histology), and economic benefits of Pacific white shrimp, *Penaeus vannamei*. Experimental diets included a control group with no added MOS and diets containing 0.6 g kg^{-1}, 1.2 g kg^{-1} and 2.4 g kg^{-1}. Shrimp, initially weighing 3.00 ± 0.04 g at a density of 100 shrimp m^{-2}, were randomly distributed and fed four times a day for 60 days. At the end of the trial, higher performance was observed in shrimp fed the diet supplemented with 0.6g MOS kg^{-1} ($p < 0.05$). MOS supplementation resulted in a significant improvement in gut morphology as evidenced by increased villus and enterocyte heights. No significant differences were observed between treatments for *Bacillus* spp, *Vibrio* spp and yeast. In the ammonia and nitrite stress tests, shrimp fed the MOS supplement showed improved resistance as evidenced by higher survival rates during stress periods. The economic benefit in the M1 treatment was the most favorable. The results show that MOS supplementation improves shrimp performance, shrimp gut health and economic benefits. To optimize economic and health benefits on *P. vannamei* in intensive synbiotic systems, it is recommended to supplement with MOS at a level of 0.6 kg^{-1} feed.

Keywords: shrimp, MOS, growth, gut morphology, net benefit

1.INTRODUCTION

As shrimp farming techniques have evolved and intensified, concerns have arisen regarding the physiological stress and immune status of farmed shrimp. Historically, chemotherapy, such as the use of antibiotics, has been used to control disease outbreaks in the shrimp farming industry. This has resulted in the widespread spread of antibiotic-resistant pathogens and significant environmental contamination (Song et al., 2014; Zullhisyam et al., 2020; Kari et al., 2021). As a natural alternative to chemotherapy, prebiotics provide several benefits to the host, including promoting the growth of beneficial bacteria in the gastrointestinal tract, enhancing zootechnical performance, improving crop productivity, and exerting immunostimulatory effects (Davani-Davari et al., 2019; Rohani et al., 2021; Mountzouris, 2022).

The most utilized prebiotics in aquaculture are naturally occurring compounds found in yeast cell walls, including mannanoligosaccharides (MOS) and β-glucans (Dawood et al., 2020; Yilmaz et al., 2022). Yeasts are highly valued in aquaculture as they serve as an alternative ingredient due to their high nutritional value and the presence of bioactive compounds (Goh et al., 2022). MOS is primarily derived from the cell walls of *Saccharomyces cerevisiae* by lysis (Gu et al., 2011; Sang et al., 2014) and is known to improve the health of aquatic animals. These compounds have been shown to increase the length of intestinal microvilli, thereby enhancing nutrient absorption and consequently improving growth performance, feed conversion ratio, and weight gain (Gainza and Romero, 2020; Faustino et al., 2021; Akter et al., 2021). In addition, MOS act as immunostimulants, increasing resistance to stress and bacterial infection in crustaceans (Sang and Fotedar, 2010; Zhang et al., 2012; Ren et al., 2020; Harikrishnan et al., 2023).

This is achieved through their agglutinating potential, which prevents the adhesion and colonization of pathogens in the digestive tract, facilitating their excretion (Torrecillas et al., 2014). In species such as the *Penaeus semisulcatus* and *P. vannamei*, MOS increase the population of beneficial bacteria while reducing the presence of opportunistic pathogens like *Vibrio* and *Aeromonas*, leading to improved survival rates (Rungrassamee et al., 2014; Gainza and Romero, 2020). Furthermore, MOS have been demonstrated to enhance intestinal microbiota and resilience to stressors (Wang et al., 2022; Lu et al., 2022).

A new generation of MOS-based prebiotics is emerging that achieves solubilization of the mannan layer within the yeast cell wall, making it more accessible and partially exposing the glucan layer. This synergistic process serves to enhance the overall efficacy of these

prebiotics. β-Glucan is a complex polysaccharide that has been shown to have remarkable immunostimulatory, anti-inflammatory and antioxidant properties in aquatic animals (Wang et al., 2008; Zhao et al., 2012; Yamamoto et al., 2020; Xu et al., 2021). Despite the growing evidence of the effects of MOS on growth, survival, and immune response in various aquaculture species, only a limited number of studies have been conducted to investigate the potential synergistic effects of these solubilized MOS in shrimp farming. The objective of this study is to evaluate the efficacy of solubilized MOS at different levels in the diet of *P. vannamei*, with particular focus on shrimp performance, gut health (including bacterial and fungal counts and gut histological changes), ammonia and nitrite resistance, and economic benefits in an intensive synbiotic system.

2.MATERIAL AND METHODS

2.1 Ingredients and experimental diets

A control diet (C) containing commercial ingredients from the Brazilian feed industry was formulated to meet the recommended nutritional requirements for juvenile *Penaeus vannamei*. This diet was designed to be isoproteic, 360 g kg^{-1}, and isoenergetic, 4400 Kcal kg^{-1}. Furthermore, three test diets were formulated with the inclusion of a commercial nucleotide source to replace wheat flour in the control diet at levels of 0.6, 1.2 and 2.4 g solubilized mannanoligosaccharides (MOS) per kg $feed^{-1}$, designated M0.6, M1.2 and M2.4, respectively. (Table 1).

The commercial nucleotide source consists of 60.2% solubilized mannanoligosaccharides (comprising 33.0% glucans and 27.2% mannans), 17% crude protein, 3.4% ash, and 8% water (Hypergen, Biorigin, São Paulo, Brazil). The four experimental diets were processed at the Centro Avançado do Pescado Continental - Instituto de Pesca (São José do Rio Preto, São Paulo, Brazil) using standard shrimp feed production procedures (Table 1). The ingredients were ground in a hammer mill (model M300, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil) to a particle size of less than 600 µm, thoroughly mixed for 15 min (model M2200, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil), extruded at 80 to 90ºC (model E62, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil) into 2.0 mm (diameter) pellets, and dried at 90 to 100ºC to reach a moisture level of 120 g kg^{-1}, crumbled, packed in sealed bags and stored frozen until used. The pellets were then crumbled, packed in sealed bags, and stored frozen until use.

Table 1. Ingredients, formulation and nutrient composition of the control and test diets containing solubilized mannanoligosaccharides to evaluate the performance of *P. vannamei* juveniles reared in a synbiotic system for 60 days.

Ingredients (g kg^{-1})	**Diet**			
	Control	**M0.6**	**M1.2**	**M2.4**
Wheat flour[a]	180	179	178	176
Soybean meal[b]	150	150	150	150
Soy Protein Concentrate[c]	120	120	120	120
Poultry by-product[d]	120	120	120	120
Broken rice[e]	80	80	80	80
Fish meal[f]	60	60	60	60
Hemoglobin[g]	50	50	50	50
Wheat meal[e]	50	50	50	50
Sorghum[h]	45	45	45	45
Dicalcium phosphate[i]	23.5	23.5	23.5	23.5
Krill meal[j]	20	20	20	20
Soy lecithin[k]	22	22	22	22
Potassium chloride[l]	10	10	10	10
Fish oil[f]	10	10	10	10
Soybean oil[b]	10	10	10	10
Salt	10	10	10	10
MOS[m]	0	1.00	2.00	4.00
Kaolin[n]	5.6	5.6	5.6	5.6
Magnesium oxide[o]	5	5	5	5
Vitamin and Mineral Supplement[p]	5	5	5	5
DL-Methionine[q]	5	5	5	5
L-Threonine[r]	5	5	5	5
L-Lysine[s]	5	5	5	5
Nutribinder[t]	5	5	5	5
Fylax (Antifungal)[u]	3	3	3	3
Vitamin C (35%)[v]	0.9	0.9	0.9	0.9

Proximate composition (g kg^{-1})				
Crude protein	385	380	385	380
Crude fat	69	66	71	69
Crude fiber	48	43	48	44
Ash	148	143	147	149
Gross Energy (kcal kg^{-1})	4446	4441	4458	4451

[a]Cidade Bella Moinho / Ponta Grossa-PR; [b]Cooperativa Comigo – Rio Verde-GO; [c]CJ Selecta/Araguari-MG; [d]Frango Rico / Votuporanga-SP; [e]Dallas / Nova Alvorada do Sul-MS; [F]BFP bioprodutos de pescado LTDA / ITAJAÍ-SC; [g]Hemoprot – Lins-SP; [h]Raguife / Santa Fé do Sul-SP Raguife / Santa Fé do Sul-SP; [i]Ecophos-Formiga-MG ; [j]Aker Biomarine Antarctic AS (Lysaker, Norway); [k]Adicel Indústria e Comércio - Ingredientes para Indústrias de Alimentos – Belo Horizonte-MG; [l]Brasil Química Ind. e Com. LTDA / Batatais-SP; [m]Hypergen/Biorigin/ Lençóis Paulista, SP; [n]CaO do Brasil Ltda / Iguatama-MG; [o]Magnesium do Brasil AS / Fortaleza-CE; [p]De Heus nutrição animal– Rio Claro-SP; [q]Rhodimet® NP99 Adisseo a bluestar company; [r]L-Threonine 98% Ajinomoto do Brasil Indústria e Comércio de Alimentos Ltda; [s]L-Lysine 78% Ajinomoto do Brasil Indústria e Comércio de Alimentos Ltda; [t]Nutri-Bind Aqua Adisseo a bluestar company; [u]Selko Feed Aditives, [v]Heilongjiang NHU Biotechnology CO. Ltd / China.

2.2 Experimental design and system

The study was conducted over 60 days at the Laboratório de Carcinicultura (LACAR), of the Departamento de Pesca e Aquicultura (DEPAq) at the Universidade Federal Rural de Pernambuco (UFRPE), Brazil. Four treatments, each in triplicate, were established using a completely randomized design: a control diet without added mannanoligosaccharides (C); and diets with solubilized mannanoligosaccharides at 0.6g kg $feed^{-1}$ (M0.6); feed with solubilized mannanoligosaccharides at 1.2 g kg $feed^{-1}$ (M1.2) and feed with solubilized mannanoligosaccharides at 2.4 g kg $feed^{-1}$ (M2.4) (Table 1).

The system was prepared in twelve fiberglass tanks (800L, 1.0 m^2), each filled with 400 L of seawater (25 gL^{-1}) filtered to 250 μm and chlorinated with 30 ppm of active chlorine using sodium hypochlorite. The water was then dechlorinated by aeration for 48 hours. Subsequently, 400L water from the synbiotic shrimp nursery, characterized by the following parameters: Total ammonia nitrogen (TAN) = 0.05 mg L^{-1}, NO_2^--N = 0.2 mg L^{-1}, NO_3^--N = 83.3 mg L^{-1}, total

alkalinity = 140 mg $CaCO_3L^{-1}$, pH = 7.5, settleable solids (SS) = 5 ml L^{-1}, and salinity = 22.5 mg L^{-1} was added to each tank. For the preparation of the synbiotic 0.29g of commercial product ($UFCg^{-1}$) composed of *Bacillus subtilis* [2.1×10^7 CFU g^{-1}], *B. licheniformis* [3.7×10^7 CFU g^{-1}], *Bacillus* sp. (2.8×10^7 CFU g^{-1}) (Kayros Agrícola e Ambiental, SP, Brazil), 2.9 g of sugar and 0.2 L of dechlorinated seawater (25 g L^{-1}) were inserted in 2-liter buckets for activation for 2 hours. After activation, 28.8 g of rice bran (< 200 μm) and 0.29 L of dechlorinated seawater (25 g L-1) were added and the anaerobic (24 h) and aerobic (24 h) processes started. At the end of the process, 40 ml of the symbiotic was added to each experimental tank. During the experiment, the synbiotic was added to the experimental units every three days (n=15), except when the settleable solids reached 10 mL L-1. Calcium and magnesium hydroxide were added at 25 g m^{-3} every five days throughout the study to maintain alkalinity above 150 mg $CaCO_3$ L^{-1} and pH above 7.5. No water changes were performed during the experiment, only evaporated water was replaced with freshwater. The experimental units had a working volume of 800 L and bottom area of 1 m^2, and were covered with a black mesh (70% UV protection) to prevent shrimp escape. The aeration system was maintained by a 1.5 HP radial blower, allowing individual control of each experimental unit, through microperforated tubes (0.70 m per unit).

2.3 Water quality

Water quality were monitored twice daily at 8:00 a.m. and 4:00 p.m. for dissolved oxygen - DO and temperature - T (AT-160, AlfaKit, Brazil). Salinity (AZ86031), pH (Asko AK90, Brazil) and settleable solids were monitored three times per week (Avnimelech 2012). Total ammonia nitrogen - TAN (APHA 2012), nitrite-nitrogen - NO_2-N (Fries 1971), and total alkalinity - TA (APHA 2012) were monitored every 10 days. Nitrate nitrogen - NO_3-N (APHA 2012) and orthophosphate - PO_4^{-3} (APHA 2012) were monitored every 20 days. All water samples were filtered through a 45-μm paper filter prior to analysis.

2.4 Shrimp nursery

Postlarvae of *P. vannamei* (PL10, 0.003 g) were obtained from a commercial shrimp hatchery (Aquasul, Rio Grande do Norte, Brazil) and reared in four fiberglass tanks (working volume 800 L) at a stocking density of 675 PL m^{-3} until they reached a weight of 3.0 ± 0.04 g for the trial. The shrimps were fed with a commercial shrimp feed containing 45% crude protein (0.6 and 0.8 mm, Wean, ADM Animal Nutrition Company, Brazil). The feeding frequency in the shrimp nursery was four times a daily (8:00 am, 11:00 am, 2:00 pm and 5:00 pm). Initially, the daily feeding rate was 35% of body weight, gradually reduced to 6.0%. The feeding rate was adjusted daily based on estimated shrimp feed consumption and mortality (Van Wyk et al. 1999). After nursing, the shrimp were counted, weighed and transferred to experimental tanks at a density of 100 shrimp m^{-2} (125 shrimp m^{-3}).

2.5 Shrimp feeding trial

During the feeding trial, the shrimps were fed three times daily (8.00 am, 01:00 pm and 5:00 pm) with the experimental diets detailed in Table 1. Initially, the daily feeding rate was 6.0% of body weight, gradually reduced to 3.0% over 60 days. The feeding rate was adjusted daily based on estimated shrimp feed consumption and mortality (Van Wyk et al. 1999). Shrimp weight (n=20) was monitored every 10 days to determine growth and to adjust the amount of feed amount. At the 30 days and at the end of the experimental period, all shrimp were counted and final weight, feed conversion ratio (FCR), survival and yield were determined using the following equations:

Final weight (g) = final biomass (g)/number of individuals at the end
FCR = feed supplied/ (final biomass – initial biomass)
Growth rate (g week−1) = ((Final weight – initial weight) / time (days)) x 7
Survival (%) = (final number of individuals/initial number of individuals) × 100
Yield (Kg m^{-3}) = final biomass (Kg)/volume of experimental unit (m3)
Yield (Kg ha^{-1}) = final biomass (Kg) x 10.000.

2.6 Proximate Composition

Analyses of crude protein, lipids, moisture, ash and fiber contents (feed and shrimp) were performed in triplicate using standard methods (AOAC 2016) at the Departamento de Zootecnia, Universidade Federal Rural de Pernambuco, Recife, Brazil. Ten shrimps per treatment were collected at the beginning and end of the experiment. For moisture content,

samples were oven-dried at 105°C to constant weight (model 315 SE, Fanem). The weight difference before and after drying was recorded and expressed as a percentage. Protein content was determined by measuring nitrogen (N x 6.25) using the Kjeldahl method (model TE 0363; Tecnal, São Paulo, Brazil). Total lipid content was determined by the Soxhlet extraction method using pure (98%) hexane solvent (model Ma 044/8/50, Marconi, São Paulo, Brazil). Crude fiber content was determined according to Detmann et al. (2012), and ash was determined by oven combustion at 550 °C (Q318 D24 model; Quimis, São Paulo, Brazil).

2.7 Presumptive total count on TCBS (Vibrio spp.), MYP (Bacillus spp.) and Sabouraud dextrose agar (fungi).

Presumptive total counts were performed at the Laboratório de Sanidade de Organismos Aquáticos (LASAq), DEPAq, UFRPE, Brazil, to quantify colony forming units (CFU g^{-1}) of *Vibrio* sp., *Bacillus* sp. and fungi. For the analysis, pooled gut samples of 50 mg were collected from shrimp in each experimental unit at the beginning, middle, and end of the experimental growth trial (Vandenberghe et al. 1999). The shrimp were euthanized by severing the nerve cord, and their external body surface was disinfected by immersion in 70% ethanol for 15 seconds, followed by a solution of 1.5% sodium hypochlorite with 0.1% Tween-80 for 15 minutes, and then washed three times in sterile distilled water. The gut samples were placed in sterile crucibles, and 600 µL of sterile alkaline peptone water solution was added, followed by maceration and homogenization of the samples.

The samples were then serially diluted from 10^{-1} to 10^{-5} and 100 µL of each dilution were plated onto thiosulfate citrate bile sucrose (TCBS, incubated in triplicate 30 °C for 24 hours), mannitol egg yolk polymyxin agar (MYP, incubated in triplicate 30 °C for 24 hours) and Sabouraud dextrose agar plates (incubated in triplicate 36°C for 72 hours) for total *Vibrio* count, total *Bacillus* count and fungi count, respectively. After the incubation period, colonies of sucrose-fermenting and non-sucrose-fermenting *Vibrio* spp., colonies of *Bacillus* spp. (CFU g^{-1}), and yeast and/or filamentous fungi based on Trabulsi and Alterthum (2015).

2.7 Ammonia and nitrite nitrogen stress tests

At the end of the growth trial, a total of 30 shrimps (n=10 per replicate) were randomly collected from each experimental treatment, and subjected to a stress test for ammonia nitrogen (NH_3-N) levels in the rearing water (Oliveira et al. 2022; Pimentel et al. 2022). Subsequently, 24 shrimps (n=8 per replicate) were used for the NO_2-N stress test. In each test, experimental

units (15-liter buckets) were filled with seawater under the following conditions: salinity 22 ± 0.9 g L^{-1}, water temperature 27 ± 0.3 °C, and pH 8.0 ± 0.1. Subsequently, they were stocked at a density of 1 shrimp L^{-1}. The NH_3-N concentration was achieved by applying a stock solution of 10 g L^{-1} of NH_4Cl (PA) and the NO_2-N concentration was achieved by applying a stock solution of 49.25 g L^{-1} $NaNO_2$ (PA) (Table 2). Each stress test was conducted for 96 hours, and survival was assessed every 24 hours (Zhang et al. 2012).

Table 2. Nitrogen concentration in the experimental units for ammonia and nitrite stress tests.

	TAN (mgL^{-1})	NH_3-N (mgL^{-1})	NO_2-N (mgL^{-1})
Inicial	14.70	0.76	20.13
24hrs	13.17	0.68	21.20
48hrs	15.40	0.80	19.20
72hrs	16.20	0.84	22.77
96hrs	14.67	0.76	25.43

Data correspond to the mean (n=3). TAN- Total ammonia nitrogen

Hemolymph (10 shrimps per treatment) was collected for blood analysis before and after the ammonia and nitrite nitrogen concentration stress tests, following the protocol described by Guertler et al. (2013). For this purpose, aliquots of hemolymph (200 µL) were collected from the hemocoel located in the ventral region of the surviving shrimp using a 1 ml syringe containing anticoagulant (modified Alsever's solution [MAS: 336 mmol L^{-1} of NaCl, 115 mmol L^{-1} of glucose, 27 mmol L^{-1} of sodium citrate, and 9 mmol L^{-1} of EDTA, pH 7.2]) at a ratio of 1:2 (v:v). An aliquot of hemolymph was separated and stored in MAS and 4% formaldehyde (1:3). Total hemocyte count (THC) was performed using 100 µL of hemolymph with anticoagulant. A drop of this suspension was added to a Neubauer chamber for cell counting (Cheng and Chen, 2001). The differential hemocyte count (hyaline, semi-granular and granular cells) was performed using a protocol adapted from Celi et al. (2013). The hemolymph was placed on a slide, dried, and fixed by immersion in absolute methanol for 6 minutes. The slide with the fixed material was then stained with Giemsa solution (dilution: 1:10) for 10 minutes,

dehydrated with 70% ethanol for 1 minute, and immersed in xylol for 6 min, followed by counting under the microscope in triplicate.

2.8 Histological analysis

Following the nitrogen stress tests, 10 guts and 10 gills from the shrimps in each treatment were collected and fixed in Davidson AFA solution for 70 hours. The samples were then transferred to a 70% ethanol solution and dehydrated in an ascending series of alcohols (70 to 100%), cleared in xylene, and embedded in histological paraffin, for sectioning (2μm) on a microtome (Leica, RM2255). Slides were stained with haematoxylin and eosin (HE), according to the procedure described by Lightner (1996). An optical microscope (BX 41 OLYMPUS, Olympus Corporation, Tokyo, Japan) coupled to a Leica camera (K5) with 40x objectives was used to analyses and obtain images of the histological sections.

ImageJ software (1.46r) was used for electronic measurements in the midgut. All villi found in the tissue were measured. The following parameters were measured at 400x magnification: 1) Dimensions of intestinal villus number (VN); 2) the height of the villus (μm) in relation to the length from the basement membrane to the top of the villus (VH); 3) the width of the villus (μm) in relation to the width of the villus (VW); 4) the height of the gut fold (μm) from the basement membrane to enterocyte layer (FH); 5) the height of the enterocytes in relation to the thickness of the enterocyte layer (EH) (Figure 1).

Histological changes in the gills (primary and secondary gill filaments) were assessed semi quantitatively by calculating the Histological Alteration Index (HAI), and damage was classified into three progressive stages of tissue damage: stage I (no effect on organ function), stage II (severe damage and impaired organ function), and stage III (very severe and irreversible changes in structure and function) (Figure 2, Table 3) (Fregoso-López et al. 2017). The HAI score was calculated using the following formula (Poleksic and Mitrovic-Tutundzic, 1994):

$$HAI = [1\ x\ \Sigma\ (stage\ I)] + [10\ x\ \Sigma\ (stage\ II)] + [100\ x\ \Sigma\ (stage\ III)]$$

The mean HAI was divided into five categories: 0-10 (normal tissue function), 11-20 (mild to moderate change), 21-50 (moderate to severe change), 51-100 (severe change) and > 100 (irreparable change).

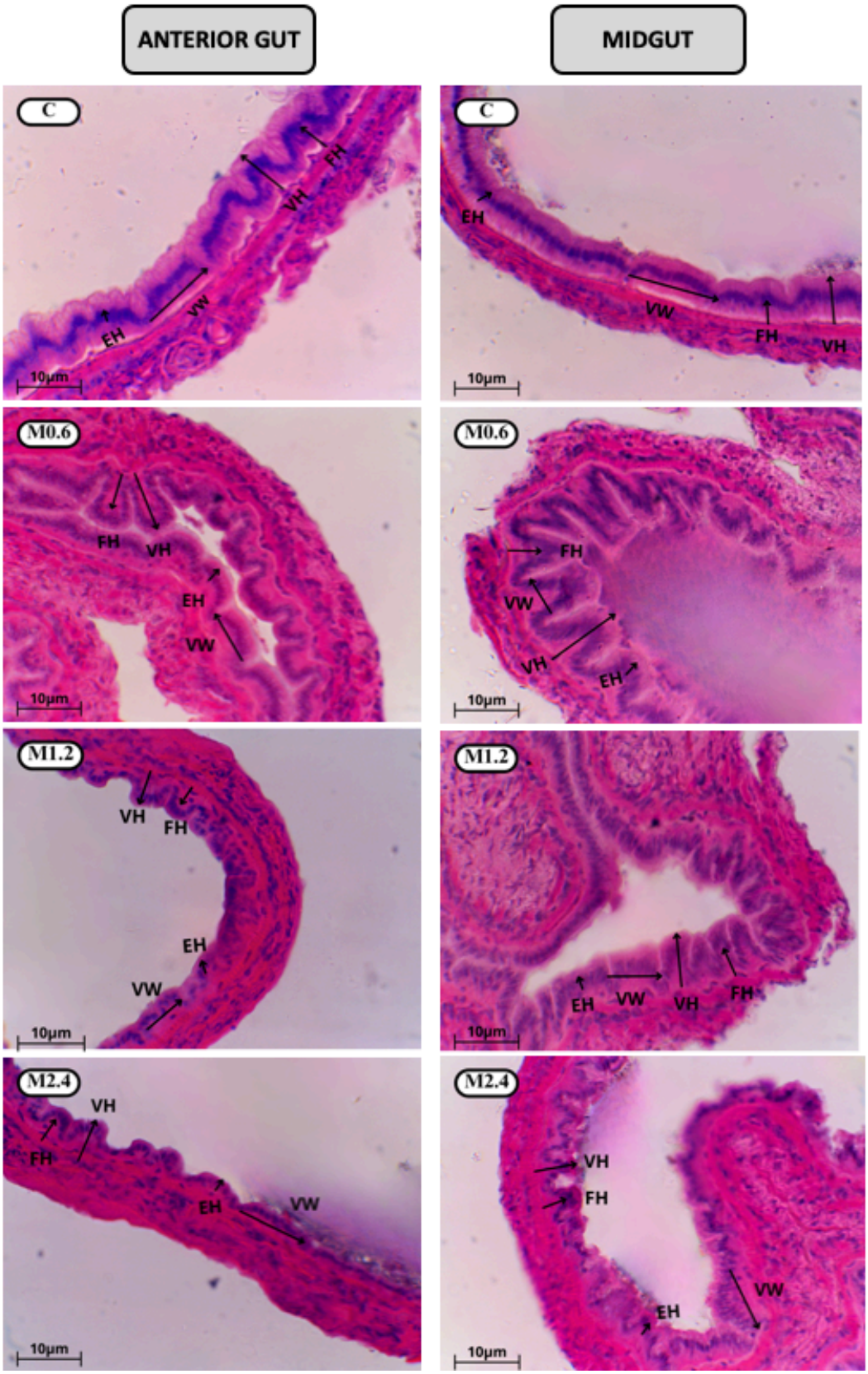

Figure 1. Details of gut morphology examination of *Penaeus vannamei* fed with the experimental diets. From left to right are C, M0,6, M1.2 and M2.4, respectively. EH, FH, VH and VM. Scale bar=10 µm; Original magnification × 400.

Table 3. Classification of the histological alterations found in gills of *Penaeus vannamei* examined after ammonia and nitrite nitrogen stress tests.

Stage 1	Stage 2	Stage 3
Melanization Dilation of hemolymph vessels (mild and moderate) Hemolymph infiltration	Deformation or Atrophy Dilation of hemolymph vessels (severe)	Necrosis Deformation and Atrophy
This stage represents the organism's initial response to stress, with lesions that do not yet significantly impair gill function, indicating an ongoing challenge.	At this stage, lesions become more severe, leading to a reduced capacity of the gills for gas exchange, which compromises the organism's physiological efficiency and overall performance.	At this stage, severe and irreversible damage to the gills is indicated, resulting in significant functional failure, where the gills can no longer effectively oxygenate the blood, endangering the organism's survival.

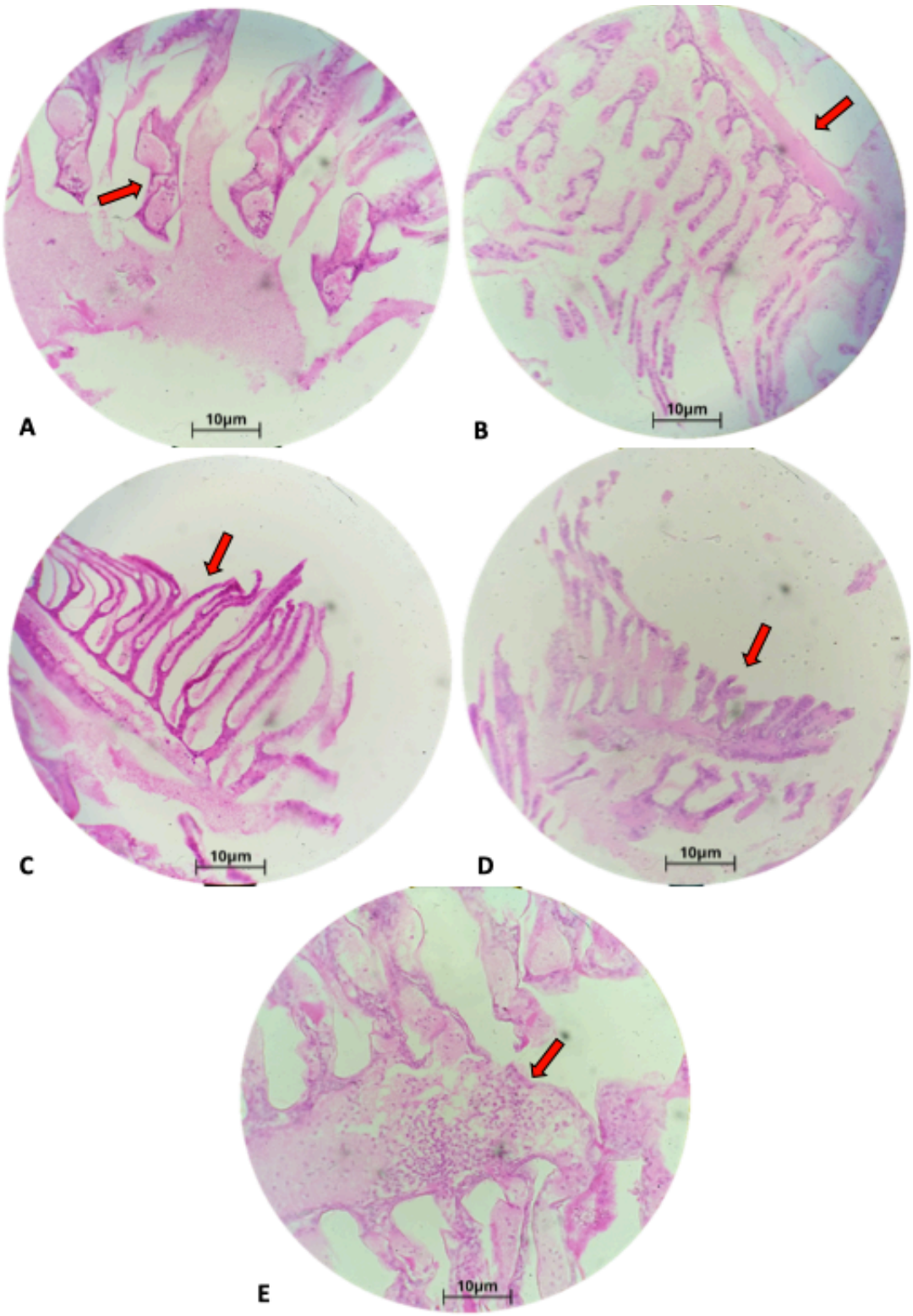

Figure 2. Histological alterations in the gills of *Penaeus vannamei* (400x magnification). A – Dilation of efferent and afferent vessels; B- Intense melanization; C- Atrophy of branchial; D- Deformation; E- Intense haemolymph infiltration. ections of tissue were stained using Hematoxylin and Eosin (H & E). Scale bar = 10 µm.

2.9 Economic benefits

Partial costs (feed x FCR, additive) and net profit (gross profit - costs) with an estimated production for 1ha. Feed production costs for this experiment included feed ($1.03 per kilogram), MOS (Hypergen $9.0 per kilogram), and shrimp price ($3.50 per kilogram). Labor costs, electricity, fertilizer, and alkalinity were not included in the comparison between treatments because the same amounts were used for both.

2.10 Data analysis

Statistical analyses were performed using BioEstat Statistic 5.3 software. Data were tested for homogeneity of variance using the Cochran test ($p < 0.05$) and for normality using the Lilliefors test ($p < 0.05$). One-way parametric ANOVA was applied to normally distributed and homogeneous data (shrimp performance, proximate composition, and HAI), along with repeated measures ANOVA for temperature, salinity, and pH. Tukey's mean comparison test ($p < 0.05$) was then performed. Non-parametric data were subjected to Friedman's test ($p < 0.05$), followed by Conover's multiple comparison test with Holm-Bonferroni correction for TAN, $N\text{-}NO_2$, $N\text{-}NO_3$, settleable solids, and dissolved oxygen. The non-parametric Kruskal-Wallis test ($p < 0.05$) was used for TCBS, MYP, Sabouraud dextrose agar counts, gut morphology, ammonia and nitrite nitrogen stress tests, and total differential hemocyte counts, followed by Dunn's post hoc test with Holm-Bonferroni correction.

3.RESULTS

3.1 Water quality

During the 60-day culture period, there were no significant differences in water quality among the treatments (Table 4).

Table 4. Water quality variables in the grow-out feed *P. vannamei* with dietary mannanoligosaccharide in an intensive synbiotic system.

Variables	Treatments			
	C	M0.6	M1.2	M2.4
Temperature (°C)	27.82 ± 0.38	27.78 ± 0.31	27.81 ± 0.30	27.80 ± 0.32
DO (mgL^{-1})	6.32 ± 0.18	6.40 ± 0.13	6.42 ± 0.19	6.34 ± 0.15

Salinity (gL^{-1})	25.25 ± 2.57	25.51 ± 2.85	25.75 ± 2.64	25.62 ± 2.73
pH	7.70 ± 0.20	7.71 ± 0.17	7.67 ± 0.19	7.71 ± 0.17
TA (mg $CaCO_3L^{-1}$)	112.62 ± 19.17	118.09 ± 15.68	110.71 ± 15.66	106.90 ± 17.86
TAN (mgL^{-1})	0.26 ± 0.19	0.23 ± 0.19	0.28 ± 0.19	0.22 ± 0.18
Nitrite-N (mgL^{-1})	0.18 ± 0.05	0.18 ± 0.09	0.21 ± 0.09	0.19 ± 0.08
Nitrate-N (mgL^{-1})	190.95± 159.60	197.65± 176.30	182.93± 138.58	204.09± 175.61
Orthophosphate (mgL^{-1})	29.56 ± 9.86	25.72 ± 7.47	29.04 ± 10.00	27.86 ± 9.00
SS (mLL^{-1})	5.24 ± 1.15	5.50 ± 2.52	5.10 ± 2.19	4.60 ± 1.66

Data are expressed as mean ± standard deviation. Results were analyzed by repeated measures ANOVA ($p \leq 0.05$) for parametric data and Friedman's test ($p \leq 0.05$) for non-parametric data. A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg $feed^{-1}$ (M0.6); feed with mannanoligosaccharides at 1.2 g kg $feed^{-1}$ (M1.2) and feed with mannanoligosaccharides at 2.4 g kg $feed^{-1}$ (M2.4). DO – Dissolved Oxygen, TAN - Total ammonia nitrogen, TA - Total alkalinity, SS -settleable solids.

3.2 Shrimp performance

No significant differences were observed in the shrimp performance results at the 30-day period. However, at the end of the 60-day period, significant differences were observed between the treatment MOS (M0.6 and M1.2) which showed higher value as than to the Control. The final weight of the M0.6 (10.87 ± 0.26 g) was higher ($p < 0.05$) than to the control (8.86 ± 0.13 g). Similarly, the weekly growth rate (0.91±0.03 g) and yield (1.25±0.09 kg m^{-3}, 9,967±719 kg ha^{-1}) were also higher than the control. The feed conversion ratio (FCR) was lower in M0.6 (1.20±0.14) than in the control and other MOS treatments (Table 5).

Table 5. Performance of *P. vannamei* grow-out feeding with dietary mannanoligosaccharides in intensive synbiotic system.

Variables	Treatments			
	C	M0.6	M1.2	M2.4
	30 days			
Final weight (g)	6.04 ± 0.22[a]	6.19±0.33 [a]	5.93±0.18 [a]	5.72 ± 0.03 [a]
Survival (%)	100 ± 0.0 [a]	100 ± 0.0 [a]	100 ± 0.0 [a]	100 ± 0.0 [a]
Growth week g^{-1}	0.70 ± 0.05 [a]	073 ±0.08 [a]	0.67±0.04 [a]	0.63 ± 0.01 [a]

kg m^{-3}	0.75 ± 0.03 [a]	0.77 ± 0.04[a]	0.74 ± 0.2 [a]	0.70 ± 0.01 [a]
kg ha^{-1}	6,039 ± 218 [a]	6,188 ± 333[a]	5.927 ± 175 [a]	5.724 ± 32 [a]
FCR	1.56 ± 0.12[ab]	1.41 ± 0.15[a]	1.61 ± 0.10[ab]	1.76 ± 0.02[b]
		60 days		
Final weight (g)	8.86 ± 0.13[c]	10.87 ± 0.26[a]	9.30 ± 0.13[b]	8.39 ± 0.17[d]
Survival (%)	90.50 ± 1.50[a]	91.67 ± 5.51[a]	93.00 ± 3.00[a]	73.00 ± 2.00[b]
Growth week g^{-1}	0.68 ± 0.01[b]	0.91 ± 0.03[a]	0.73 ± 0.02[b]	0.63 ± 0.02[c]
kg m^{-3}	1.0 ± 0.01[c]	1.25 ± 0.09[a]	1.08 ± 0.02[b]	0.77 ± 0.02[d]
kg ha^{-1}	8,014 ± 131[c]	9,967 ± 719[a]	8,644 ± 161[b]	6,182 ± 51[d]
FCR	1.70 ± 0.01[b]	1.20 ± 0.14[d]	1.50 ± 0.02[c]	2.10 ± 0.10[a]

Data are expressed as mean (n=3) ± standard deviation. The results were analyzed by means of ANOVA ($p \leq 0.05$) followed by the Tukey test. Mean values on the same line with different superscripts differ significantly. A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg $feed^{-1}$ (M0.6); feed with mannanoligosaccharides at 1.2 g kg $feed^{-1}$ (M1.2) and feed with mannanoligosaccharides at 2.4 g kg $feed^{-1}$ (M2.4).

3.3 Proximal composition

Table 6 provides a summary of the proximal composition of *P. vannamei*. In comparison to the initial measurements, significant differences were observed in the proximal composition of crude protein, lipids, fiber, and ash. Nevertheless, only significant differences in ash were identified between the various treatments at the end.

Table 6. Proximal composition of *P. vannamei* (dry weight) in grow-out feeding with dietary MOS in an intensive synbiotic system.

% Dry matter	Treatments				
	Initial	C	M0.6	M1.2	M2.4
Moisture	76.81± 1.41[a]	76.44 ± 0.55[a]	75.91 ±1.58[a]	77.73 ± 1.56[a]	77.05 ±1.11[a]
Crude	61.89 ± 1.80[b]	70.17±1.36[a]	71.30±1.42[a]	70.69±0.98[a]	70.32± 0.78[a]

Protein					
Lipids	3.25 ±0.95^{b}	3.99 ±0.63^{a}	3.78 ± 0.64^{ab}	4.31 ±0.14^{ab}	4.95 ± 0.63^{a}
Fiber	8.14±0.84^{a}	5.62±0.35^{b}	6.01± 0.82^{b}	7.06 ± 0.72^{ab}	7.25 ±0.42^{ab}
Ash	11.85±0.79^{c}	12.43±0.49^{c}	12.83±0.32^{cb}	14.35±0.48^{a}	14.09±0.95^{ab}

Values in dry weight, g $100g^{-10}$, expressed as mean (n=4) ± standard deviation. The results were analyzed by means of ANOVA ($p \leq 0.05$) followed by the Tukey test. Mean values on the same line with different superscripts differ significantly. A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg $feed^{-1}$ (M0.6); feed with mannanoligosaccharides at 1.2 g kg $feed^{-1}$ (M1.2) and feed with mannanoligosaccharides at 2.4 g kg $feed^{-1}$ (M2.4).

3.4 Presumptive total count on TCBS (Vibrio spp.), MYP (Bacillus spp.) and Sabouraud dextrose agar (fungi) at the shrimp gut

The presumptive total counts on TCBS (*Vibrio* spp.), MYP (*Bacillus* spp.), and Sabouraud dextrose agar (fungi) in the shrimp gut are presented in Figure 2. At the beginning of the experiment, the presumptive Bacillus spp. counts were 17.81×10^{8} CFU g^{-1}, which was approximately ten times higher than the *Vibrios* spp. counts of 1.79×10^{8} CFU g^{-1}. In contrast, the fungal counts were 3.0×10^{6} CFU g^{-1}. Following a 30-day period, a decline was observed in the *Bacillus* spp. counts across all treatments, with a reduction ranging from 90.72% at the beginning to between 37.09% (M0.6) and 83.07% (M2.4). However, after 60 days, an increase occurred in all treatments except for M2.4, which exhibited a slight reduction.

The yeast count exhibited an increase over time in all treatments, with the M2.4 treatment demonstrating the most increase, from 0.14% to 2.85%. Filamentous fungi demonstrated minimal variation throughout the experimental period, with initial values of 0.01% remaining almost constant across all treatments. However, higher proportions were observed in the M2.4 treatment.

For sucrose-positive bacteria, there was a significant increase in all treatments at 30 days. The M0.6 and M1.2 treatments experienced the largest increases, from 4.30% to 17.94% and 33.15%, respectively. At 60 days, the M2.4 treatment and the control showed the lowest proportions, 9.55% and 5.78%, respectively. Sucrose-negative bacteria decreased over time in all treatments. At the end of the 60-day period, the M1.2 treatment and the control had the

lowest proportions, 3.73% and 1.92%, respectively. For sucrose-positive bacteria, a significant increase was observed in all treatments at the 30-day period. The higher increases observed in treatments M0.6 and M1.2, with values increasing from 4.30% to 17.94% and 33.15%, respectively. At the 60-day period, the lower levels of sucrose-positive bacteria were observed in treatments M2.4 and the Control, with proportions of 9.55% and 5.78%, respectively. The proportion of sucrose-negative bacteria exhibited a decline over time in all treatments. At the 60-day period, treatment M1.2 and the Control demonstrated the lower proportions, at 3.73% and 1.92%, respectively.

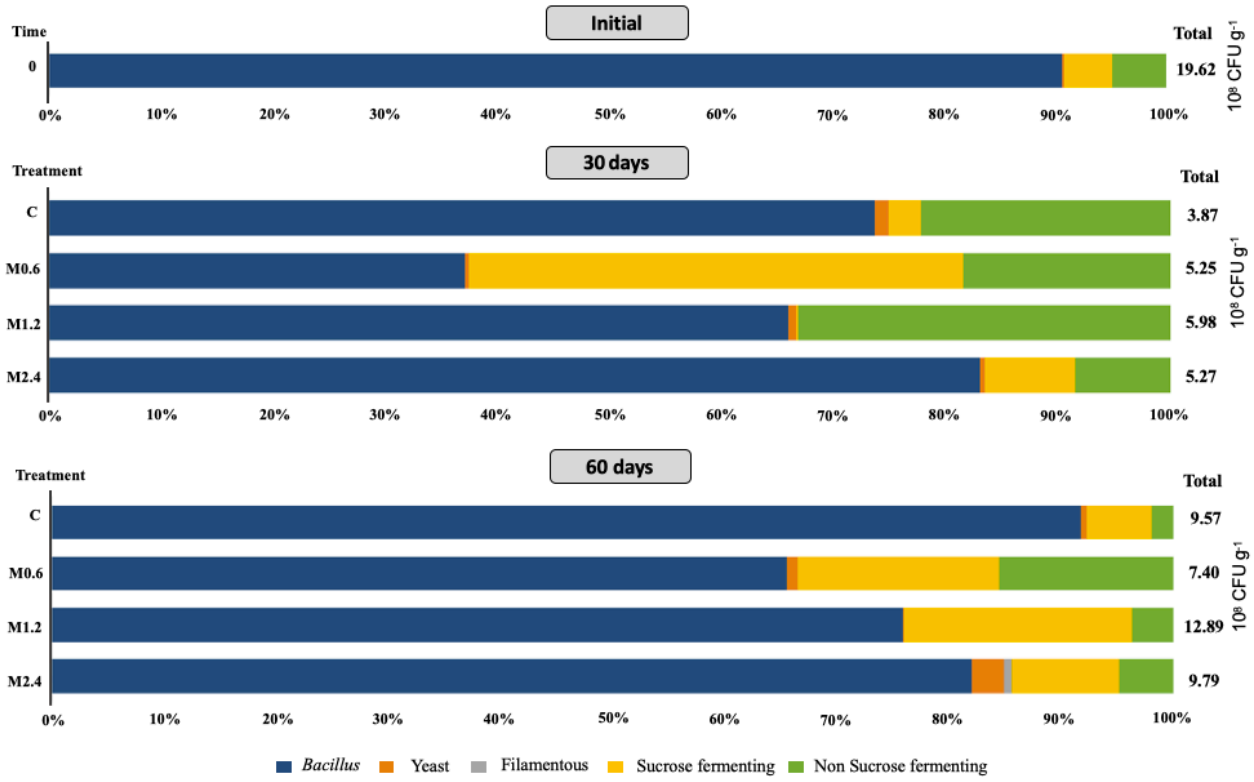

Figure 3. Presumptive total count on MYP (*Bacillus* spp x 10^8 CFU), Sabouraud dextrose agar (Filamentous Fungi and Yeast x 10^8 CFU) and TCBS (*Vibrio* spp. x 10^8 CFU) at the shrimp gut. A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

3.5 Ammonia and nitrite nitrogen stress tests

Significant differences were observed in the survival of the shrimp among the different treatments during the 96-hour ammonia stress tests (Table 7). The M0.6 and M1.2 treatments demonstrated the higher survival rates. Following a 96-hour period, the survival rate in the

M0.6 (80.6% ± 3.9) and M1.2 (69.4% ± 7.9) treatments was higher than that of the control treatment (47.2% ± 7.9). No significant differences were observed among the treatments in the nitrite stress tests after 96 hours (Table 8).

Table 7. Shrimp *P. vannamei* survival (%) after ammonia nitrogen stress tests feeding with dietary mannanoligosaccharides.

Exposure time	Treatments			
	C	M0.6	M1.2	M2.4
24hrs	100 ± 0.0^{a}	94.4 ± 3.9^{a}	97.2 ± 3.9^{a}	91.7 ± 6.8^{a}
48hrs	80.6 ± 10.4 ^{a}	91.7 ± 6.8^{a}	88.9 ± 3.9^{a}	83.3 ± 6.8^{a}
72hrs	61.1 ± 7.9^{b}	83.3 ± 0.0^{a}	83.3 ± 0.0^{a}	75.0 ± 6.8^{ab}
96hrs	47.2 ± 7.9^{c}	80.6 ± 3.9^{a}	69.4 ± 7.9^{ab}	55.5 ± 3.9^{bc}

Data are expressed as mean (n=3 per exposure time) ± standard deviation. The results were analyzed by non-parametric Kruskal-Wallis test ($p < 0.05$). A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

The total hemocyte counts in shrimp showed no significant differences between treatments (Table 9). However, following the ammonia stress test, a reduction in hemocyte counts was evident in the control, M0.6, and M1.2 treatments. Nevertheless, following the nitrite stress test, there was an increase in hemocyte counts as than to the initial values.

The differential hemocyte counts, including hyaline (H), semi-granular (SG), and granular (G) cells, from the ammonia and nitrite stress tests are presented in Figure 4. Initially, H values ranged from 66.58 ± 13.74% in the C treatment to 88.44 ± 3.98% in the M0.6. The SG values ranged from 9.31 ± 2.91% in the M0.6 to 23.74 ± 8.15% in the M2.4. G values ranged from 10.82 ± 6.03% in the C treatment to 6.04 ± 2.85% in the M1.2.

Following the ammonia stress test, the H count decline in the M0.6 and M1.2 treatments, while an increase was observed in the C and M2.4 treatments. The M0.6 and M1.2 treatments increase in both SG and G values, whereas the C and M2.4 treatments decrease in both

variables. Subsequent to the nitrite stress test, the H values decline across all treatments. The SG increase in all treatments with the exception of M2.4 and the G increase across all treatment.

Table 8. Shrimp *P. vannamei* survival (%) after nitrite nitrogen stress tests feeding with dietary mannanoligosaccharides.

Exposure time	Treatments			
	C	M1	M2	M4
24hrs	100 ± 0.0	94.4 ± 9.6	94.4 ± 9.6	88.9 ± 19.2
48hrs	77.8 ± 9.6	83.3 ± 16.7	88.9 ± 9.6	88.9 ± 19.2
72hrs	77.8 ± 9.6	83.3 ± 16.7	88.9 ± 9.6	77.8 ± 9.6
96hrs	77.8 ± 9.6	77.8 ± 19.2	77.8 ± 25.4	72.2 ± 9.6

Data are expressed as mean (n=3 per exposure time) ± standard deviation. The results were analyzed by non-parametric Kruskal-Wallis test ($p < 0.05$). A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

Table 9. Shrimp total hemocyte counts after ammonia and nitrite nitrogen stress tests (10^6 cells mL^{-1}).

Time	Treatments			
	C	M0.6	M1.2	M2.4
Initial N-NH_3	4.1 ± 3.3[a]	3.8 ± 2.0 [a]	3.8 ± 4.8 [a]	4.5± 3.5 [a]
96 hours N-NH_3/ Initial N-NO_2	2.1 ± 2.1 [a]	3.3 ± 2.2 [a]	3.1 ± 2.8 [a]	4.6± 3.0 [a]
96 hours N-NO_2	7.2 ± 4.6 [a]	5.6 ± 4.0 [a]	3.7 ± 2.5 [a]	2.2± 8.10 [a]

Data are expressed as mean ± standard deviation. The results were analyzed by non-parametric Kruskal-Wallis test ($P \leq 0.05$). A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

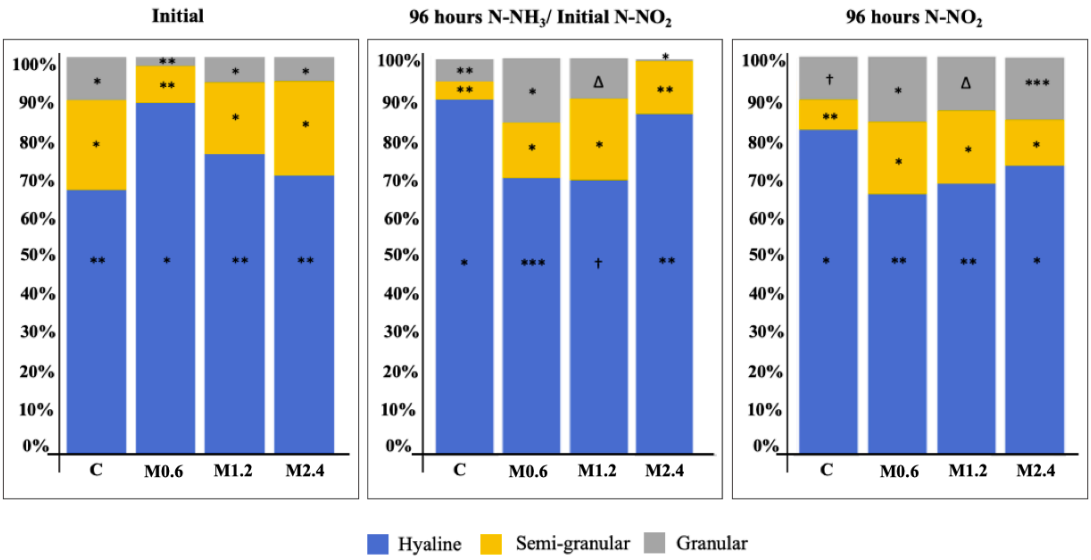

Figure 4. Differential hemocyte counts (Hyaline, Semi-granular, and Granular) in *P. vannamei* after ammonia and nitrite nitrogen stress tests.

Symbols indicate statistical significance: * = a; ** = b; *** = c; Δ = ab; † = bc.

Data correspond to the mean ± standard deviation. Different letters represent Kruskal-Wallis ($p < 0.05$), followed by Dunn test ($p< 0.05$).

A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

3.6 Histological analysis

The results for the shrimp gut samples (midgut and anterior) are presented in Table 10. The gut morphology showed significant differences between the MOS as than control. In the midgut, the M2.4 treatment showed the higher number and width of villi, while the M0.6 treatment higher villus height. The higher enterocyte height was observed in the M0.6 and M1.2 treatments. In the anterior, the M1.2 and M2.4 treatments showed the higher number and width of villi, while the M0.6 treatment higher villus height.

Table 10. The gut morphology of *P. vannamei* fed experimental diets with a mannanoligosaccharide additive for 60 days.

Histological	Treatments			
	Midgut			
	C	M0.6	M1.2	M2.4

Villus number (VN)	12.0±4.52[b]	23.00±6.60[a]	20.20±4.73[a]	21.40±4.43[a]
Fold height (FH, μm)	4.28±1.64[c]	8.04±1.18[a]	8.22±1.19[b]	6.23±1.21[c]
Villus width (VW, μm)	17.22±2.94[a]	9.15±2.98[c]	10.05±3.23[c]	13.06±2.26[b]
Villus height (VH, μm)	7.36±2.32[d]	22.85±3.17[a]	17.37±3.23[b]	12.99±1.83[c]
Enterocyte height (EH, μm)	1.81±0.11[c]	3.20±0.15[a]	2.79±0.17[b]	2.01±0.18[c]
	Anterior Gut			
	C	M0.6	M1.2	M2.4
Villus number (VN)	14.6±2.99[b]	24.20±5.39[a]	22.00±5.27[a]	22.60±4.22[a]
Fold height (FH, μm)	5.51±1.15[c]	7.84±0.99[a]	6.99±1.13[b]	5.01±1.23[c]
Villus width (VW, μm)	14.16±1.94[a]	9.42±2.07[b]	10.11±2.00[b]	14.05±2.11[a]
Villus height (VH, μm)	9.40±2.19[c]	17.57±1.99[a]	13.50±2.03[b]	9.92±1.97[c]
Enterocyte height (EH, μm)	1.90±0.11[c]	3.00±0.14[a]	2.50±0.11[b]	1.90±0.12[c]

The data correspond to mean ± standard deviation. Different letters indicate one-way ANOVA followed by Tukey's test ($p < 0.05$). A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

Histological alterations in the gills of shrimp after stress tests with ammonia and nitrite resulted in severe alteration in Control and M2.4., while M0.6 exhibited the highest index of normal tissue function (Table 11 and Figure 5).

Table 11. Histological alteration index values of gills of *P. vannamei* challenged with N-NH_3 e N-NO_2 stress test.

Stage		Treatments			
		C	M0.6	M1.2	M2.4
Stage I	Normal tissue function	30.8 %	48.3 %	32.4 %	31.4 %
Stage II	Mild to moderate alteration	7.7%	6.9 %	8.1 %	5.7 %
	Moderate to severe alteration	46.2%	44.8%	56.8%	42.9%
Stage III	Severe alteration	15.38 %	0.0 %	2.7 %	20.0 %

Irreparable alteration	0.0%	0.0%	0.0%	0.0%
HAI	111.3 ± 6.7	14.7±5.1	25.5±7.6	107.8±8.7

A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

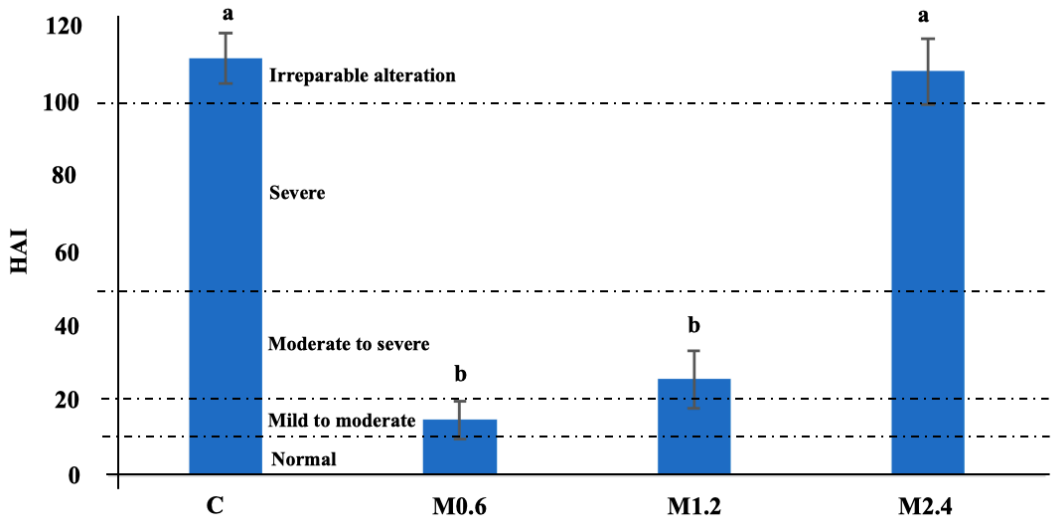

Figure 5. Histological alteration index (HAI ± SD) values of gills in *Penaeus vannamei* shrimp after stress tests with ammonia and nitrite. Different letters indicate significant differences ($p<0.05$) between treatments. A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg feed^{-1} (M0.6); feed with mannanoligosaccharides at 1.2 g kg feed^{-1} (M1.2) and feed with mannanoligosaccharides at 2.4 g kg feed^{-1} (M2.4).

3.7 Economic benefits

The results of the net income of *P. vannamei* grow-out feeding with dietary mannanoligosaccharides are presented in Table 12. The M0.6 (USD 34,621 ha^{-1}) and M1.2 (USD 30,272 ha^{-1}) treatments with dietary MOS showed higher revenues than the control (USD 27,090 ha^{-1}). Moreover, the net benefit, obtained by subtracting partial expenses (feed + MOS additive) from revenue, demonstrated that the M0.6 (USD 8,728 ha^{-1}) and M1.2 (USD 2,666 ha^{-1}) treatments exhibited the higher net profit compared to the control.

Table 12. Economic benefits of *P. vannamei* grow-out feeding with dietary mannanoligosaccharides in intensive synbiotic system.

			Treatments			
			C	M0.6	M1.2	M2.4
REVENUES	Stocking	shrimp ha^{-1}	1000000	1000000	1000000	1000000
	Survival	%	90	91	93	73
	Harvested shrimp	shrimp ha^{-1}	900000	910000	930000	730000
	Shrimp final weight	kg	0.0089	0.0109	0.0093	0.0084
	Harvested shrimp	kg ha^{-1}	8,010	9,892	8,649	6,125
	Revenues	US$ ha^{-1}	28,035	34,621	30,272	21,436
EXPENSES	FCR		1.70	1.20	1.50	2.10
	Feed supplied	kg	13,617	11,870	12,974	12,862
	Feed cost (U$$ 1.03/kg feed x FCR)	USD kg^{-1}	1.75	1.24	1.55	2.16
	Feed additive Cost	USD kg^{-1}	0.00	106.83	233.52	463.03
	Expenses total (feed + additive)	USD ha^{-1}	14,026	12,333	13,596	13,711
	Net income	USDha^{-1}	14,009	22,288	16,675	7,726
	Comparison MOS/kg feed x Control			8,728	2,666	-6,284

A control diet without added mannanoligosaccharides (C); and diets with mannanoligosaccharides at 0.6g kg $feed^{-1}$ (M0.6); feed with mannanoligosaccharides at 1.2 g kg $feed^{-1}$ (M1.2) and feed with mannanoligosaccharides at 2.4 g kg $feed^{-1}$ (M2.4).

4. DISCUSSION

In intensive systems, an increase in microbial biomass and alkalinity consumption results in a reduction in pH levels (Silva et al., 2013). To prevent fluctuations in pH levels, it is essential to maintain alkalinity above 100 mg $CaCO_3$ L^{-1}, as this serves as a buffer (Emerenciano et al., 2017). In this study, the pH was approximately 7.7, and alkalinity remained above 100 mg $CaCO_3$ L^{-1} due to the addition of 25 g m^{-3} of calcium and magnesium hydroxide every five days, which stabilized these variables. The synbiotic system demonstrated efficient nitrification, which was attributed to the equilibrium between heterotrophic and nitrifying bacteria (Romano et al., 2018). The observation of low levels of TAN and N-NO_2 during the course of the experiment indicates the effective transformation of nitrogen, with higher nitrate level. Water quality parameters remained within acceptable limits for shrimp farming (Zahraie et al., 2019), with no significant differences between treatments. These results show that the addition of MOS to *P. vannamei* diets does not compromise water quality.

The results indicate that adding commercial MOS at 0.6 g kg^{-1} to the diet improve the shrimp performance in intensive systems. However, was observed that the supplementation levels were inversely proportional to the positive effects on performance. Previous research has also demonstrated the beneficial effects of dietary MOS on growth performance in shrimp culture. Sang et al. (2014) observed a similar relationship between supplementation and performance effects at concentrations ranging from 1 to 8 g kg^{-1} in black tiger shrimp (*Penaeus monodon*); Li et al. (2018) reported positive performance effects in *P. vannamei* supplemented with 5 g MOS kg^{-1} for 28 days, while Zhang et al. (2012) observed beneficial effects at concentrations of 2 to 8 g MOS kg^{-1} for 56 days. However, the dosage of MOS supplementation in crustacean diets differs between studies. The variation in inclusion levels may be related to environmental conditions, such as stocking density, water quality and system. This is due to the fact that most studies have been carried out in clean water systems, which do not assess the potential for synergistic interactions with microorganisms present in aquaculture systems.

In contrast to the findings of Ayiku et al. (2020), who observed an improvement in crude protein and ash content in *P. vannamei* supplemented with 2% *S. cerevisiae* yeast, our study with the addition of MOS (0.6-2.4 g kg^{-1}) did not observe significant changes in the proximal composition of the shrimp (lipids and proteins). However, a significant increase in ash content was observed in the M1.2 and M2.4 treatment compared to the control. These discrepancies illustrate the variability of the effects of prebiotic dosages on proximal composition.

The presumptive analyses of the shrimp's gut microbiota indicated a higher prevalence of *Bacillus* spp. in comparison to yeast and *Vibrios* during the 60-day culture period. This was

likely due to the continuous addition of synbiotics throughout the culture cycle in all treatments. This suggests that the fermentation and respiration of rice bran with probiotic microorganisms contributes to an improvement in the gut microbiota. This is evidenced by the fact that in the control treatment, in which no MOS was added, the proportion of *Bacillus* spp. at the end of 60-day culture period was above 90%. The higher proportion of *Bacillus spp*. in relation to *Vibrios* spp. provides benefits to the hosts, as the reduction of pathogens contributes to enhanced growth and survival of the shrimp (Noor et al., 2015; Martínez et al., 2021). However, despite the high proportions of *Bacillus* in the Control and M2.4 treatments, the presence of filamentous fungi in these groups may have contributed to a reduction in the shrimp performance. These findings reinforce the necessity for further investigation into the utilization of prebiotics in conjunction with probiotics and other microbial community, rather than isolated studies in clear water no has evaluate synergistic interactions.

The structure of gut microvilli provides an extensive surface area for nutrient absorption, with increased height potentially enhancing this capacity (Sang & Fotedar, 2010; Daniels et al., 2010). The results of the histological analysis indicated that the height of the gut microvilli and enterocytes decreased with increasing levels of MOS supplementation in both the midgut and the anterior gut. The results of this study indicate a need for further investigation into the relationship between the level of MOS and the various environmental culture conditions, as well as the shrimp genetics in each production area. It is important to note that continued activation of the shrimp immune system has the potential to compromise the structural and functional gut integrity. Similar results were reported by Zhang et al. (2012) and Lu et al. (2019), who found that lower doses of MOS resulted in higher growth performance in juvenile *P. vannamei* and Chinese mitten crabs (*Eriocheir sinensis*) compared to higher doses. Additional studies have demonstrated that MOS can enhance intestinal morphology in various aquatic species, including the European sea bass (*Dicentrarchus labrax*) (Torrecillas et al., 2013), the black tiger prawn (*P. monodon*) (Sang et al., 2014), gibel carp juveniles (*Carassius auratus gibelio*) (Akrami et al., 2012), cobia (*Rachycentron canadum*) (Razeghi Mansour et al., 2012), juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) (Zhu et al., 2023).

In aquaculture systems, the accumulation of ammonia and nitrite can reach critical levels, which can affect aquatic organisms and increase mortality and susceptibility to pathogens (Duan et al., 2018; Guo et al., 2013; Tong et al., 2023). The results of our study indicated that dietary supplementation with MOS resulted in higher shrimp survival in the M0.6

and M1.2 treatments, suggesting a synergistic effect that enhances resistance to environmental stress (Zhang et al., 2012; Chen et al., 2020; Mameloco et al., 2020). However, the M2.4 treatment, which received a higher MOS addition level, was observed lower survival rates, possibly due to immune overload where high concentrations of β-glucan can be detrimental (Meena et al., 2013; Song et al., 2014). The subsequent nitrite test yielded no significant difference in shrimp survival between treatments, indicating that while MOS may provide initial protection against ammonia stress, its efficacy may not extend to other stressor or that adaptation to the first stressor depleted the shrimp's immune reserves, compromising their response to nitrite.

According by Chen et al. (2023), the shrimp hemocyte cells increase represents a defensive mechanism induced by environmental or biological alterations. Following the ammonia stress test, the hemocyte count in the control group exhibited a 50% reduction, whereas the nitrite stress test yielded an elevated hemocyte count in all groups with the exception of M2.4. The treatments M0.6 and M1.2 demonstrated greater stability in hemocyte counts. Andrino et al. (2014) propose that the combined supplementation of these prebiotics in *P. vannamei* can enhance the immunity and overall health of the shrimp, although high inclusions (5 g kg^{-1}) may potentially reduce the total hemocyte count, as observed in M2.4.

The differential analysis indicated a decrease in hyaline cells and an increase in semi-granular and granular cells following stress tests. This suggests a shift in the composition of shrimp immune cells towards more specialized cells. The M0.6 and M1.2 treatments exhibited more efficacious alterations, indicating enhanced resistance. At the end nitrogen test stress trial, the control and M2.4 treatments exhibited granular and semi-granular cell percentages that were below the optimal range for *P. vannamei* according by Darwantin and Sidik (2016).

Gills, due to their extreme sensitivity, are crucial for monitoring the impact of pathogens and environmental changes on aquatic organisms. Being in direct contact with water, they are often the first to exhibit signs of stress caused by toxic substances present in the environment (Ribeiro et al., 2005; Piontkivska et al., 2011). Exposure to compounds such as ammonia and nitrite has the potential to seriously compromise the structural integrity of gills, as demonstrated in previous studies with *Macrobrachium amazonicum* juveniles (Dutra et al., 2017). In this study, it was observed that all treatments resulted in some degree of gill injury after the application of stress; however, diets supplemented with moderate levels of MOS, particularly in the M0.6 and M1.2 treatments, showed lower indices of histological alterations. These

findings suggest that MOS supplementation may be beneficial in preserving gill function, thereby enhancing the shrimp's resistance to environmental stress.

In intensive aquaculture practices, the cost of feed represents the most significant expense among operational costs. Despite the elevated costs, the incorporation of functional ingredients into the diet can result in substantial financial benefits through enhanced feed efficiency and improved shrimp performance. The available evidence indicates that these ingredients can enhance growth, feed utilization, and disease tolerance (Segarra et al., 2023). The M0.6 (USD 34,621 ha^{-1}) and M2 (USD 30,272 ha^{-1}) treatments exhibited higher revenues than the control (USD 27,090 ha^{-1}) treatment. The elevated revenue can be attributed to the enhanced final weight of the shrimp and the improved survival rate observed in these treatments. The financial data demonstrate that, despite the additional expense of the MOS additive, the revenue benefits exceeded the costs, resulting in a positive net income. The M0.61 treatment, exhibited a remarkably high net income, indicating an optimal cost-benefit ratio.

5. CONCLUSION

The of 0.6 g MOS kg^{-1} in the diet of *P. vannamei* raised in an intensive synbiotic system significantly improved final weight, feed conversion ratio and weekly growth rate compared to the control. In addition, the study observed significant improvements in gut and gill morphology in the MOS supplemented treatments. Economically, the net income was highest for the 0.6 g MOS kg^{-1}, making it a viable option for shrimp nutrition. These results suggest that appropriate levels of MOS supplementation can improve shrimp production efficiency, health, and economic returns in intensive aquaculture systems.

AUTHOR CONTRIBUTION

Danielle Alves da Silva: Conceptualization, Methodology, Investigation, Data Curation, Formal analysis, and Writing - Original Draft. **Flávia Abreu Everton:** Data Curation, and Writing. **Gisely Karla de Almeida Costa:** Data Curation, and Writing. **Suzianny Maria Bezerra Cabral da Silva:** Conceptualization, Methodology, Resources, Project administration, Funding acquisition, and Writing - Review & Editing. **Fernando Leandro dos Santos:** Data Curation, and Writing - Review & Editing. **Rodrigo Antônio Ponce de Leon Ferreira de Carvalho:** Conceptualization, Writing - Review & Editing. **Giovanni Sampaio Gonçalves:** Methodology, Writing - Review & Editing. **João Fernando Albers Koch:** Conceptualization, Resources, Funding acquisition, and Writing - Review & Editing. **Luis Otavio Brito:**

Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, and Writing - Review & Editing.

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DATA AVAILABILITY

The findings of this study are supported by data, which can be made available upon request from the corresponding author.

DECLARATIONS

Ethics approval and consent to participate

The research conducted adheres to the current animal welfare regulations in Brazil. The use of *Penaeus vannamei* in this experimental study does not require approval from the Brazilian Ethics Committee for Animal Use. All authors consented to participate in this research.

Human and animal ethics

The authors adhered to both international and institutional guidelines for the management of animals during the experiments.

Consent for publication

All the authors of this article agree to the publication.

Competing interests

The authors declare no competing interests.

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6. CONSIDERAÇÕES FINAIS

Com os resultados obtidos durante as pesquisas realizadas para o desenvolvimento da tese, é possível concluir que o estudo contribuiu significativamente para o entendimento das práticas de suplementação dietética na carcinicultura, especificamente com nucleotídeos (NT) e mananoligossacarídeos (MOS), visando o desempenho e a eficiência econômica da produção de *P. vannamei*. Os resultados obtidos mostram que a suplementação com NT e MOS pode

melhorar o desempenho dos camarões, a composição proteica, e a morfologia intestinal, índices imunológicos e crescimento dos animais. Além disso, o estudo destacou a viabilidade econômica dessas práticas, com o tratamento N150 e M1 apresentando os melhores retornos sobre o investimento. Embora não tenham sido observadas diferenças significativas na microbiologia intestinal entre os tratamentos, a suplementação com NT e MOS demonstrou efeitos positivos na resistência ao estresse por amônia e nitrito, o que é crucial para o sistema de cultivo. Esses achados reforçam a importância de adotar estratégias nutricionais para maximizar a produtividade do cultivo de camarões.

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