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SUPLEMENTAÇÃO DE MINERAIS ORGÂNICOS A PARTIR DO *Lithothaminium* PARA PRODUÇÃO DE *Penaeus vannamei* EM ÁGUA OLIGOHALINA

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Resumo

A produção do camarão marinho em águas de baixa é possível quando a dureza e alcalinidade estão em concentrações proporcionais ao ambiente marinho e as principais relações iônicas (Na:K, Cl:Na; Mg:Ca e Ca:K), estão adequadas. Esses íons são importantes para o desenvolvimento do camarão uma vez que participam das reações metabólicas e contribuem para melhoria do sistema imune. Para manter as concentrações de íons adequadas, frequentemente utilizam sais agrícolas diretamente na água de cultivo. Entretanto, existem diversas interações destes íons com o solo e compostos dissolvidos na coluna d'água, tornando questionável a quantidade absorvida pelos camarões cultivados. Desta forma, uma estratégia de fornecer esses íons é pela via nutricional. A forma comumente utilizada para adicionar íons nas rações para camarões marinhos, são os minerais inorgânicos, entretanto estes possuem baixa taxa de absorção e causam irritabilidade nas paredes do trato digestório. Uma alternativa para aumentar a taxa de absorção desses minerais para os camarões cultivados em baixa salinidade, é a utilização da suplementação com minerais orgânicos. Os minerais orgânicos estão ligados covalentemente à um composto orgânico (por exemplo aminoácidos e carboidratos), deixando o complexo estável e com maior biodisponibilidade, aumentando sua taxa de absorção pelos camarões. O *Lithothaminium* é uma fonte orgânica de micro e macrominerais e outros compostos orgânicos como os aminoácidos, tornando-se uma possível alternativa de suplementação de minerais para rações de camarões em baixa salinidade. Portanto, esse estudo teve por objetivo avaliar a substituição parcial de minerais inorgânicos pelo *Lithothaminium* na nutrição do camarão marinho *Penaeus vannamei* em sistema simbiótico. As dietas foram formuladas para *P. vannamei*, de forma a serem isoproteica, 350 g kg^{-1} e isoenergética, 3.800 Kcal kg^{-1}. Duas dietas foram formuladas com a inclusão de uma fonte comercial de *Lithothaminium* (100% natural e orgânico, composto de aproximadamente 93% de minerais e 7% de aminoácidos), nos níveis de 2 e 4% de *Lithothaminium* por kg de dieta (LT2 e LT4) para substituir parcialmente minerais inorgânicos da dieta controle. Além disso, mais duas dietas foram preparadas a partir da dieta controle com inclusão de 2 e 4% de *Lithothaminium* por kg de dieta (CTLT2 e CTLT4) fixados sobre os pellets da ração com um binder comercial O experimento foi conduzido por 50 dias com densidade de 50 camarões m^{-2}, com os

seguintes tratamentos: Controle – dieta com minerais inorgânicos; CTLT2- dieta controle com a adição de 2% de *Lithothaminium* com Binder comercial; CTLT4: dieta controle com a adição de 4% de *Lithothaminium* com Binder comercial; LT2 – adição de 2% de *Lithothaminium* na dieta em substituição aos minerais inorgânicos e LT4 – adição de 4% de *Lithothaminium* na dieta em substituição aos minerais inorgânicos. O uso de diferentes doses e formas de aplicação afetou as atividades das enzimas digestivas (tripsina, quimotripsina, leucina aminopeptidase, amilase e lipase) no hepatopâncreas de camarões cultivados. As enzimas do estresse oxidativo não diferiram significativamente entre os tempos analisados, mas o MDA aos 25 dias de cultivo apresentou valores diferentes entre os tratamentos LT2 e LT4 em relação ao CTLT4. Em relação a composição centesimal e mineral dos camarões, apenas a concentração de cálcio apresentou diferença significativa, sendo maior no tratamento CTLT4 quando comparado aos demais tratamentos. Ao final do experimento o tratamento LT2 apresentou diferença significativa no desempenho zootécnico quando comparado aos demais tratamentos e controle. Além disso, apresentou um maior ROI (Retorno sobre o Investimento) (34,16), comparado aos demais tratamentos. Com base nesses resultados, recomenda-se a utilização de 2% de *Lithothaminium* (LT2) em substituição aos minerais inorgânicos na dieta de *P. vannamei* cultivados em sistema simbiótico.

Abstract

Shrimp production in low-salinity waters is possible when hardness and alkalinity are in concentrations proportional to the seawalter, and the main ionic relationships (Na, Cl, Mg, and Ca) are adequate. These ions are important for shrimp development as they participate in metabolic reactions and contribute to improving the immune system. To maintain adequate ion concentrations, agricultural salts are frequently used directly in the culture water. However, there are various interactions between these ions with the soil and dissolved compounds in the water column, making the amount absorbed by the shrimp culture questionable. Therefore, a strategy to provide these ions is through nutritional means. The commonly used form to add ions to shrimp feed is inorganic minerals; however, these have a low absorption rate and cause irritability in the digestive tract. An alternative to increase the absorption rate of these minerals for shrimp culture in low salinity is using organic mineral supplementation. Organic minerals are covalently bound to an organic compound (example amino acids and carbohydrates), making the complex stable and with greater bioavailability, increasing its absorption rate. *Lithothaminium*is an organic source of micro and macrominerals and other organic compounds such as amino acids, making it a possible alternative for mineral supplementation in shrimp feed in low salinity. Therefore, this study aimed to evaluate the partial replacement of inorganic minerals with *Lithothaminium*in in the nutrition of the shrimp *Penaeus vannamei* in a synbiotic system. The diets were formulated for *P. vannamei* to be isoproteic, 350 g kg^{-1}, and isoenergetic, 3,800 Kcal kg^{-1}. Two diets were formulated with the inclusion of a commercial *Lithothaminium* source (100% natural and organic, composed of approximately 93% minerals and 7% amino acids) at levels of 2% and 4% *Lithothaminium* per kg of diet to replace inorganic minerals in the control diet partially. Additionally, two more diets were prepared from the control diet with the inclusion of 2% and 4% *Lithothaminium* per kg of diet fixed onto the feed pellets with a commercial binder (BC). The experiment was conducted for 50 days with a density of 50 shrimp per m^2, with the following treatments: Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2%

Lithothamnium in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals. The use of different doses and forms of application affected the activities of digestive enzymes (trypsin, chymotrypsin, leucine aminopeptidase, amylase and lipase) in the hepatopancreas of farmed shrimp. The oxidative stress enzymes did not differ significantly between the times analyzed, but MDA at 25 days of culture showed different values between LT2 and LT4 treatments compared to CTLT4. Regarding the proximate and mineral composition of the shrimp, only the calcium concentration showed a significant difference, being higher in the On Top 4% treatment compared to the other treatments. At the end of the experiment, the On Coat 2% treatment showed a significant difference in zootechnical performance compared to the other treatments and control. Additionally, it presented a higher ROI (34.16) compared to the other treatments. Based on these results, the use of 2% *Lithothaminium* (LT2) is recommended to replace inorganic minerals in the diet of *P. vannamei* cultured in a synbiotic system.

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1. Introdução

A produção proveniente da aquicultura em 2022 foi de 94,4 milhões de toneladas, um aumento de 3,6% de em relação ao ano anterior (FAO, 2024). Apesar desse crescimento substancial nos últimos anos, é necessário aumentar a produção aquícola, já que o volume proveniente da pesca, está em nível de estabilidade e o consumo de proteína do pescado vem sendo incrementado. Portanto, a aquicultura precisará desenvolver novas estratégias para aumentar a produção, com sistemas mais eficientes, incremento de tecnologia para aumentar a produção verticalmente (ex. aumento da densidade de estocagem) como horizontalmente (expansão da área de produção).

A interiorização da carcinicultura é uma dessas estratégias, proporcionando a produção de crustáceos longe da zona costeira. Em 2022, a produção em águas interiores foi de 5,16 milhões de toneladas, representando 40,46% da produção total (FAO, 2024). Esse crescimento está atrelado a onerosidade das terras próximo a zona costeira e a disponibilidade de terra em regiões interioranas com boas condições de solo e água (NUNES, 2001; AMORIM e FARIAS, 2023; PIMENTEL et al., 2023).

Em 2022, a produção brasileira de camarão foi de 113,3 mil toneladas, incremento de 14% quando comparada ao ano anterior, demonstrando assim, uma recuperação da atividade após os surtos do vírus da Mancha Branca (WSSV) e vírus da Mionecrose Infecciosa (IMNV). Além do aumento da produção, foi observado que diversos municípios dos estados da região nordeste, distantes da zona costeira, passaram a produzir e contribuir para retomada da produção da carcinicultura brasileira (IBGE, 2024).

A principal espécie cultivada nas águas interiores é o *Penaeus vannamei*, devido sua capacidade de osmorregulação, o que proporciona desenvolver-se em uma ampla variação de salinidade (0,5 - 60 g/L) (ATWOOD et al. 2003; SAOUD et al. 2003; PRANGNELL et al.,

2019). A produção do camarão marinho em águas de baixa salinidade (salinidade menores que 5g/L) é possível quando a dureza e alcalinidade estão em concentrações maiores que 150 mg $CaCO_3$/L e 80 mg $CaCO_3$/L, respectivamente, além das relações iônicas Na:K (28:1), Cl:Na (1,8:1); Mg:Ca (3:1) e Ca:K (1:1) (ROY et al., 2010: MOURA et al., 2021; De OLIVEIRA et al., 2022; OLIVEIRA et al., 2022; PIMENTEL et al., 2022; SILVA et al., 2023).

Os íons participam em reações metabólicas, como cofatores enzimáticos, compondo o exoesqueleto, e têm participação ativa na osmorregulação e no fortalecimento do sistema imune dos camarões (CHENG et al., 2005; TRUONG et al., 2023). O íon Ca^{+2} também participa da formação do exoesqueleto e atua como cofator no processo enzimático juntamente com a osmorregulação. O Mg^{+2} possui papel importante na respiração celular e na atividade metabólica, sendo um dos componentes do exoesqueleto e um cofator da enzima Na^{+}/K^{+}-ATPase, responsável pela osmorregulação no transporte ativo de íons. Os íons potássio (K^+) e sódio (Na^+) participam na ativação da Na^{+}/K^{+}-ATPase e na absorção celular de aminoácidos (ROY *et al.*, 2009; GALKANDA-ARACHCHIGE et al., 2021; NESAPRIYAM et al., 2022; TRUONG et al., 2023). Estes íons estão associados ao crescimento e metabolismo básicos dos camarões, sendo assim, as quantidades e as relações iônicas na água entre eles são importantes para a sobrevivência e produtividade na carcinicultura (DJUWITO et al., 2014).

Além disso, quando cultivados em águas oligohalinas, os camarões tendem a perder íons através das brânquias e da urina, para manter o equilíbrio osmótico, absorvendo íons do meio aquático através do transporte ativo (ROMANO e ZENG, 2012). Esse gasto energético na tentativa do equilíbrio osmótico, causa efeitos negativos na eficiência alimentar, sobrevivência e ganho de peso (LI et al, 2007). Portanto, nestas condições quando os minerais estão desequilibrados na água e na dieta podem provocar problemas de doenças relacionadas à ecdise, no endurecimento da carapaça e mortalidade (TRUONG et al., 2023). Sendo assim,

fazem-se necessárias adequadas concentrações minerais na água ou na dieta para um bom desempenho dos camarões cultivados em águas oligohalinas.

Frequentemente produtores de camarões marinhos em águas de baixa salinidade utilizam sais agrícolas para correção dos íons na água, entretanto a quantidade absorvida pelos organismos é questionável, tendo em vista a interação destes íons com o solo e compostos dissolvidos na coluna d'água (BOYD, 2007). A utilização desses sais agrícolas é onerosa e pode elevar o custo de produção, fato que leva aos os produtores adicionam quantidades insuficientes para a manutenção das relações iônicas recomendadas para espécie, fazendo com que o camarão absorva mais um íon do que outro. Esse desequilíbrio pode acarretar efeitos negativos na saúde do camarão (TRUONG et al., 2023). Portanto é de suma importância a busca por estratégias alternativas para incorporação dos minerais essenciais para os camarões que não seja onerosa para a atividade econômica e torne a absorção desses íons mais eficaz (ROY et al., 2009)

Nas rações utilizadas no cultivo de camarão marinho comumente são incorporados minerais inorgânicos (CHENG et al., 2005; ROY et al., 2007; HONGYU et al., 2014; DJUWITO et al., 2014), porém estes elementos apresentam uma baixa taxa de absorção e estão presentes em quantidades insuficientes na água dos cultivos de baixa salinidade para a manutenção do equilíbrio osmótico dos camarões cultivados (SAOUD et al., 2007; ROY et al. 2009). Com o objetivo de aumentar a eficiência da absorção dos minerais pelos camarões, tem sido utilizado minerais quelados ou orgânicos adicionados à dieta, para que os íons suplementados sejam absorvidos no trato digestório dos camarões (SAOUD et al., 2007; ROY et al. 2009; LAINING et al., 2015). Os minerais orgânicos estão ligados covalentemente a um composto orgânico, geralmente os aminoácidos formando um complexo de carga considerada nula, deixando o complexo estável.

Diante do exposto o *Lithothaminium spp.* apresenta-se como uma alternativa para a suplementação de minerais orgânicos nas dietas do camarão marinho *P. vannamei* cultivados em águas de baixa salinidade. O *Lithothaminium* comercial é um produto proveniente dos fósseis das algas vermelhas pluricelulares do filo Rhofophyta, família Corallinaceae, subfamília Lithophylloideae, que crescem em profundidades variadas no ambiente marinho. Dentre os minerais orgânicos que são encontrados no produto comercial estão: Potássio (K), Cálcio (Ca), Magnésio (Mg) e Ferro (Fe), além de conter nutrientes (Nitrogênio e Fósforo) e alguns aminoácidos (PRIMASEA, 2024), elementos indispensáveis para um bom desempenho zootécnico dos camarões (NATES, 2016; AYISI et al., 2017; CARVALHO et al., 2021; NUNES et al., 2021; TRUONG et al., 2023).

Na nutrição animal, o *Lithothaminium* têm sido utilizados como suplemento mineral e tem apresentado bons resultados zootécnicos. Em aves, o uso do mineral orgânico como fonte de cálcio para nutrição de frango de corte, resultando em uma melhor conversão alimentar (CARLOS et al., 2011), já em ruminantes, a utilização de 10% na dieta, promoveu um aumento de 26% no peso final dos animais (MELO e MOURA, 2009). Entretanto, estudos com *Lithothaminium* como fonte de macro e micronutrientes orgânicos para dietas de camarões marinhos cultivados em águas de baixa salinidade não foram relatados até o presente momento.

1.1. Objetivo Geral

Avaliar o efeito do *Lithothaminium* na alimentação de *Penaeus vannamei* em água oligohalina.

1.2. Objetivos Específicos

- Avaliar o melhor nível e estratégia de inclusão do *Lithothaminium* na alimentação de *P. vannamei* em relação ao desempenho zootécnico;
- Avaliar a atividade enzimática no hepatopâncreas dos camarões alimentados com diferentes níveis e estratégias de inclusão do *Lithothaminium*;
- Avaliar a atividade das enzimas do sistema antioxidante no hepatopâncreas dos camarões alimentados com diferentes níveis e estratégias de inclusão do *Lithothaminium*;
- Avaliar a composição centesimal dos camarões alimentados com diferentes níveis e estratégias de inclusão do *Lithothaminium*;
- Avaliar o retorno do investimento em relação aos diferentes níveis e estratégias de inclusão do *Lithothaminium*;

1.3. Hipótese

- A suplementação de *Lithothaminium* na alimentação melhora o desempenho zootécnico de *P. vannamei* cultivados em águas oligohalinas;
- A suplementação de *Lithothaminium* na alimentação melhora os índices econômicos de *P. vannamei* cultivados em águas oligohalinas

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3. Artigo Científico

Os resultados obtidos durante o trabalho experimental desta dissertação estão apresentados no artigo intitulado "**The partial replacement of inorganic minerals by *Lithothamnium* in diets for *Penaeus vannamei* reared in low salinity water in a synbiotic system"** (manuscrito), que se encontra anexado.

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The partial replacement of inorganic minerals by *Lithothamnium* in diets for *Penaeus vannamei* reared in low salinity water in a synbiotic system

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ABSTRACT

In shrimp feed, inorganic minerals have low absorption rates and can cause digestive tract irritation. Organic minerals, like *Lithothamnium*, offer a better alternative. *Lithothamnium* is an organic source of microminerals, macrominerals, and amino acids, making it an ideal supplementation alternative for shrimp feed. This study aimed to evaluate the partial replacement of inorganic minerals by *Lithothamnium* in the nutrition of the *Penaeus vannamei* in low salinity water with synbiotic system. Four diets were formulated by partially replacing inorganic minerals with a commercial source of *Lithothamnium* at levels of 2% (LT2) and 4% (LT4) per kg of diet. Two more diets were prepared using the same levels: 2% (CTLT2) and 4% (CTLT4) of *Lithothamnium* per kg of diet, fixed onto the pellets using a commercial binder. The experiment was carried out for 50 days with a stocking density of 50 shrimps m^{-2} in a low salinity synbiotic system. The use of different doses and forms of application affected the activities of digestive enzymes (trypsin, chymotrypsin, leucine aminopeptidase, amylase and lipase) in the hepatopancreas of farmed shrimp. The oxidative stress enzymes did not differ significantly between the times analyzed, but MDA at 25 days of culture showed different values between LT2 and LT4 treatments compared to CTLT4. The shrimp's proximate and mineral composition showed that the concentration of lipids was lower in the CTLT2 and CTLT4 treatments, while the concentration of calcium was higher in the CTLT4 treatment. The LT2 treatment demonstrated a significant difference in shrimp performance (final weight, weekly growth, yield, and FCR) and a higher return on investment (ROI: 34.26) compared to the other treatments and the control. Based on these results, the use of 2% *Lithothamnium p*er kg $diet^{-1}$ (LT2) is the best substitute for inorganic minerals in the diet of *P. vannamei* reared in low salinity water in a synbiotic system.

Keywords: organic minerals; growth; proximal composition; enzymes; antioxidants.

1.**Introduction**

In 2022, 12.7 million tons of crustaceans were produced, 40% of which came from inland waters. The main cultured species is *Penaeus vannamei*, which accounts for 7.9 million tons (62.2%) (FAO, 2024). The advancement in *P. vannamei* production for inland waters (oligohaline and mesohaline) is a direct result of the species' osmoregulatory capacity and tolerance to a wide salinity range (Jaffer et al., 2020). However, the survival and growth results of the species vary significantly between regions where production is carried out in inland waters (Li et al., 2007; Li et al., 2017; Pimentel et al., 2022).

The different performances of shrimp under low salinity conditions can be attributed to nitrogen compounds (ammonia and nitrite) (Esparza-Leal et al., 2016; Pimentel et al., 2023a). The use of a synbiotic system allows us to exert control over these compounds. This system uses fermented vegetable meal as an organic substrate for probiotic microorganisms, which successfully control nitrogen compounds and reduce the concentrations of *Vibrios* spp. in production units (De Andrade et al., 2021; Silva et al., 2021; Khanjani et al., 2023).

The ionic composition of the waters is another factor that contributes to variations in shrimp performance. This is especially true of the imbalance between the main ions: potassium, magnesium, sodium, calcium, sulfate, chloride, bicarbonate, and carbonate. This is part of the challenge that shrimp farming in inland waters must overcome (Roy et al., 2010). The imbalance of ions provides an inadequate environment for the development of shrimp, especially when cultured at higher stocking densities. Ions are essential for the composition of the exoskeleton, maintenance of physiology, and participation in various biochemical reactions as cofactors and enzyme activators (Roy et al., 2010; Li et al., 2017; Truong et al., 2023).

The scarcity or low ionic concentration in oligohaline waters makes the organism hyperosmotic in relation to the environment. This results in the loss of ions through the gills and the release of water into the environment (Romano and Zheng, 2012). This loss of ions unbalances the

ionic relationships in the hemolymph and, consequently, in the intracellular medium, causing deleterious effects on crustacean physiology (Buckle et al., 2006; Pan et al., 2007). Shrimp recover the minerals released through active transport carried out by specialized cells, located mainly in the gills. This mechanism requires a high energy expenditure (Saoud et al., 2003; Davis et al., 2005; Li et al., 2007; Hou et al., 2012).

To compensate for the lack of minerals, agricultural salts or mineral mixes are added directly to the aquatic environment in shrimp farms in inland waters. However, the maintenance of these ions and the economic effectiveness of this management are questionable. When added directly to the water, they interact with other elements (Roy et al., 2009; De Oliveira et al., 2022; Pimentel et al., 2022). An alternative is nutritional application, as shrimp absorb part of these ions in the intestinal tract (Gong et al., 2004).

Peneid shrimps require seven minerals (calcium, copper, phosphorus, potassium, magnesium, selenium, and zinc) to be included in their diet (Nates, 2016). Calcium is essential for blood clotting, muscle function, nerve transmission, membrane permeability, and the process of osmoregulation (Nates, 2016). Copper plays a vital role in biochemical functions, acting as a cofactor for enzymes including lysyl oxidase, cytochrome C oxidase (CCO), ferroxidase, tyrosinase, and superoxide dismutase (Dawood, 2022). Phosphorus is a component of ADP, ATP, and phosphocreatine molecules, the cell membrane, genetic material (DNA, RNA), and coenzymes (Nates, 2016). Potassium, along with sodium, plays a crucial role in osmoregulation and cell division. It is also essential for supporting the development of tissues and organs (Roy et al., 2010). Magnesium plays a vital role in numerous metabolic processes, including enzyme activity, protein synthesis, and energy production. It also helps maintain the osmotic balance in cells (Davis & Gatlin, 1996). Selenium is essential for the enzyme glutathione peroxidase, which works with vitamin E to protect cells, tissues, and membranes from oxidative damage (McDowell, 2003). Zinc is an integral constituent and active central ion of zinc-dependent

metalloenzymes, including alkaline phosphatase, superoxide dismutase, alcohol dehydrogenase, carbonic anhydrase, and insulin (Dawood et al., 2022).

However, when inorganic minerals are added to the feed, the majority are leached out and only a small portion is absorbed in the gut. Furthermore, some inorganic minerals cause damage to the gut, irritating the walls and reducing microvilli (Panmei et al., 2023). Organic minerals could be solution to this deficiency and ensure the nutritional requirements of shrimp reared in low salinity waters are met. Organic minerals are more stable and absorb better than inorganic minerals. They are associated with organic molecules like amino acids, which helps them to be absorbed more efficiently by the gut tract (Bharadwaj et al., 2014; Laining et al., 2015; De Oliveira et al., 2022; Takiya et al., 2023).

Lithothamnium is the alternative for supplementing organic minerals in the diets of *P. vannamei* reared in low salinity waters. *Lithothamnium* is a pluricellular red algae of the phylum Rhodophyta, family Corallinaceae, subfamily Lithophylloideae, which grows at varying depths in the marine environment. *Lithothamnium* contains a number of organic minerals, including: It contains potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), copper (Cu), selenium (Se), as well as nutrients (nitrogen and phosphorus) and amino acids (PRIMASEA, Brazil, 2024). These elements are indispensable for shrimp good performance (Nates, 2016; Ayisi et al., 2017; Truong et al., 2023). *Lithothamnium* is a source of organic minerals in the nutrition of terrestrial animals (Melo and Moura, 2009; Carlos et al., 2011). However, it has not been reported in the literature as a source of organic minerals in the diet of shrimp. This study aimed to evaluate the use of different concentrations and ways of adding *Lithothamnium* to the diet of juvenile *P. vannamei* reared in a low-salinity water in a synbiotic system.

2. Materials and Methods

2.1 Ingredients and experimental diets

A control diet 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 isoprotein, 350 g kg^{-1}, and isoenergetic, 3800 Kcal kg^{-1}. Furthermore, two test diets were formulated with the inclusion of a commercial *Lithothamnium* source (100% natural and organic, composed of approximately 93% minerals and 7% amino acids, 74, Lothar, PRIMASEA, Brazil, Table 1), to replace inorganic mineral in the control diet at levels of 2% (20g) and 4% (40g) per kg $feed^{-1}$ (Table 2). Furthermore, two additional diets were created by modifying the control diet with the inclusion of 2 and 4% *Lithothamnium* per kg of $diet^{-1}$, fixed onto the feed pellets with a commercial binder (BC) (Table 2). To fix the organic mineral, mix 400ml of distilled water with 3.0g of BC. Then, use 30ml of this mixture for one kilogram of diet. The pellets were then fixed with the organic mineral and left to dry at room temperature for 24 hours. To ensure accurate data interpretation, all other diets received the same proportion of commercial binder without the additive.

The experimental diets were processed at the Advanced Continental Fish Center - Fisheries Institute (São José do Rio Preto, São Paulo, Brazil) using standard shrimp feed production procedures (Table 2). 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 1.4 mm (diameter) pellets, and dried at 90 to 100ºC to reach a moisture level of 120 g kg^{-1}. The pellets were then crumbled, packed in sealed bags, and stored frozen until use.

The treatments were: Control (CT) - diet with inorganic minerals -100% inorganic mineral; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder -30% organic mineral; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder – 47% organic mineral; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals – 44% organic mineral and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals – 88% organic mineral.

Table 1. Nutritional composition of *Lithothamnium* used in shrimp diets cultured in low salinity water.

* Amino Acid Composition (g kg^{-1})		*Mineral Composition (g kg^{-1})	
Tryptophan	1.53	Calcium	320-340
Tyrosine	0.56	Magnesium	20-35
Aspartic acid	0.24	Potassium	0.32
Glutamic acid	0.15	Sodium	3.00
Glycine	0.14	Phosphorus	0.41
Cystine	0.13	Iodine	3.90
Lysine	0.13	Zinc	0.02
Phenylalanine	0.11	Iron	3.00
Serine	0.08	Fluoride	0.17
Valine	0.07	Copper	0.01
Methionine	0.04	Chrome	0.01
Proline	0,03	Boron	0.02

* Information provided by the manufacturer (PRIMASEA, Brazil)

Table 2. Formulation and composition of control diets and diets with *Lithothamnium* to *P. vannamei* in low salinity water.

Ingredients (g kg^{-1})	CT	LT2	LT4	CTL2	CTL4
Soybean meal[1]	220	220	220	220	220
Broken rice[2]	135	135	135	135	135
Meat and bone meal[3]	135.3	135.3	135.3	135.3	135.3
Wheat flour[4]	120	120	120	120	120
Poultry by-product[5]	120	120	120	120	120
Hemoglobin[6]	60	60	60	60	60
Fish meal[7]	60	60	60	60	60
Rice bran meal[2]	40	40	40	40	40
Fish oil[7]	15	15	15	15	15
Soybean oil[5]	15	15	15	15	15
Nutribinder [8]	6	6	6	6	6
Soy lecithin[9]	5	5	5	5	5
Vitamin and Mineral Supplement[10]	5	5	5	5	5
Lignobond[11]	5	5	5	5	5

L-Threonine[12]	3.3	3.3	3.3	3.3	3.3
Salt	3	3	3	3	3
Fylax (Antifungal)[13]	3	3	3	3	3
Emulsifying agent [14]	2	2	2	2	2
DL-Methionine[15]	1.8	1.8	1.8	1.8	1.8
Vitamin C 35%[16]	0.6	0.6	0.6	0.6	0.6
Antioxidant Banox[17]	0.2	0.2	0.2	0.2	0.2
Dolomite limestone[18]	26.7	19.11	3.61	26.7	26.7
Lithothamnium[19]	0.0	20	40	0.0	0.0
Magnesium oxide[20]	12.4	0.0	0.0	12.4	12.4
Potassium chloride [21]	5.66	5.66	1.16	5.66	5.66
Total	100.00	100.00	100.00	100.00	100.00
Lithothamnium addition with commercial binder	-	-	-	20	40
% organic mineral	0	44	88	30	47

Proximate composition					
Dry matter (g kg^{-1})	896	883	883	897	891
Crude protein (g kg^{-1})	349	343	351	343	339
Crude fat (g kg^{-1})	42	40	41	43	40
Crude fiber (g kg^{-1})	33	51	52	50	40
Ash (g kg^{-1})	130	123	125	137	145
Calcium (g kg^{-1})	32	36	31	36	37
Magnesium (g kg^{-1})	8	3	4	9	10
Potassium (g kg^{-1})	13	12	11	13	13
Phosphorus (g kg^{-1})	13	13	14	15	14
Copper (mg kg^{-1})	8	10	11	10	10
Iron (mg kg^{-1})	819	960	780	702	928
Zinc (mg kg^{-1})	274	301	328	298	303

[1]Cooperativa Comigo – Rio Verde-GO; [2]Dallas / Nova Alvorada do Sul-MS;[3]Minerva S/A – Mirassol D'oeste/MT; [4]Cidade Bella Moinho / Ponta Grossa-PR; [5]Frango Rico / Votuporanga-SP; [6]Hemoprot – Lins-SP; [7]BFP bioprodutos depescado LTDA / ITAJAÍ-SC; [8]Nutri-Bind Aqua Adisseo a bluestar company;[9]Adicel – Belo Horizonte-MG; [10]De Heus nutrição animal– Rio Claro-SP;[11]Borregard -São Paulo/SP; [12]L-Threonine 98% Ajinomoto do Brasil Indústria e Comércio de Alimentos Ltda; [13]Selko Feed Aditives; [14]Duas Rodas, Jaraguá do Sul - SC;[15]Rhodimet® NP99

Adisseo a bluestar company;[16]Heilongjiang NHU Biotechnology CO. Ltd / China;[17]Alltech, São Pedro do Ivaí, PR;[18]Mineracao Joao Vaz Sobrinho LTDA, Arcos, MG; [19]Lothar, Primasea, BA, Brasil; [20]Magnesium do Brasil AS / Fortaleza-CE; [21]Brasil Química Ind. e Com. LTDA / Batatais-SP. Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals.

2.2 Production System

The experiment was conducted for 50 days at the Department of Fisheries and Aquaculture (DEPAq) of the Federal Rural University of Pernambuco in Brazil. Three tanks with a useful volume of 800 L (1.0 m²) were used for each treatment. Each tank was filled with 400 L of water inoculum, which had the following characteristics: TAN 0.49 mg L^{-1}, $N\text{-}NO_2$ 0.30 mg L^{-1}, $N\text{-}NO_3$ 45 mg L^{-1}, pH 7.8; salinity 4 g L^{-1}; total alkalinity 120 mg $CaCO_3$ L^{-1}; settleable solids 8 ml. L^{-1}; and 400 L of previously filtered, chlorinated, and dechlorinated oligohaline water (4 g L^{-1}) were added to complete the volume.

The synbiotic was applied seven times, with a two-day interval between applications. To prepare the synbiotic, take 0.36 g of a commercial product (CFU g^{-1}), which consists of *Bacillus subtilis* –8,5 x 10^8 CFU g^{-1}; *B. licheniformis* – 8,5 x 10^8 CFU g^{-1} e *B. amyloliquefaciens* – 8,5 x 10^8 CFU g^{-1}, *Lactobacillus acidophilus*-3,7 x 10^8 CFU g^{-1}, *L. plantarum* - 3,7 x 10^8 CFU g^{-1}, mannan oligosaccharide 396 g kg^{-1} (N-Pro, Kayros Ambiental e Agrícola, Brasil), 3.6 g of sugar and 0.42 L of sterilized clean water were placed in 2 L buckets for activation for 2 hours. After activation, rice bran (FR) (36 g) and another 1.1 L of clean water were added, which started fermentation (24 h) and then microbial respiration (24 h) processes. After preparation, 0.1 L of the synbiotic was added to each experimental tank. During the experiment, the synbiotic was added to the experimental units three times a week. Three applications of 32 g calcium magnesium hydroxide were also added to each experimental tank, with an interval of three days between applications before the shrimp were stocked.

P. vannamei juveniles were transported from commercial shrimp farming and acclimated to the experimental salinity for seven days. After system preparation and acclimation,

shrimp (mean weight 3.1 ± 0.2 g) were transferred to the experimental units and stocked at a density of 50 shrimp m^{-2}. During the evaluation of the diets, the synbiotic was applied every three days as described in section 2.2. Alkalinity corrections were made with calcium and magnesium hydroxide every ten days after chemical analysis of the water to maintain alkalinity ≥75 mg L^{-1}. Shrimp were fed the experimental rations according to the methodology of Van Wyk (1999), where the feed rate varied from 5% to 3.5%, with a feeding frequency of four times a day (8am, 11am, 1pm and 4pm).

2.3 Management and monitoring of water quality variables

Water quality variables were monitored daily: Dissolved oxygen (DO) and temperature (AT-160, microprocessor oximeter, ALFAKIT, Brazil) at 8 am and 4 pm; pH (AK90, ASKO, Brazil), salinity (salinity meter AZ 8371, China) and settleable solids (SS) were measured every five days using an Imhoff cone (Avnimelech, 2012), and total ammonia nitrogen was measured every ten days (APHA, 2012), nitrogen-nitrite (Fries, 1971), nitrogen nitrate (APHA, 2012), total alkalinity (APHA, 2012), total hardness (APHA, 2012), calcium (APHA, 2012), magnesium (APHA, 2012), sodium (APHA, 2012), potassium (Fries and Getrost, 1977), chloride (APHA, 2012), sulfate (APHA, 2012).

2.4. Enzymatic analyses

For digestive enzyme and antioxidant analyses, 12 shrimp were sampled per experimental unit, collected at 25 and 50 days for digestive enzymes and at 0, 25 and 50 days for antioxidant analyses. The sampled shrimp hepatopancreas was collected, weighed and then homogenized (40 mg mL^{-1} buffer Tris-HCl 0.1 M and NaCl 0.15 mM pH 8) and centrifuged at 8000 rpm for 15 min in a refrigerated centrifuge (4º C). The concentration of total protein was determined by the Bradford method (1976), using bovine serum albumin as a standard. Trypsin, chymotrypsin, and leucine aminopeptidase activities were

59 determined according to Buarque et al. (2009) using BApNA 8.0 mM (Nα-benzoyl-DL-
60 arginine-p-nitroanilide), SApNA 8.0 mM (Suc-Ala-Ala-Pro-Phe p-nitroanilide), and
61 leucine-p-nitroanilide 8.0 mM (leu-p-nan) as substrates, respectively. Activities were
62 determined in triplicate, with one enzyme activity unit (mU) defined as the amount of
63 enzyme required to release 1 μmol of p-nitroaniline per minute (Ɛ = 9100 M- 1cm $^{-1}$
64 calculated for the microplate). Specific activity was calculated in mU.mg^{-1} protein.

65 Total amylase activity was determined by the method of Bernfeld (1955) using
66 2% (w/v) soluble starch as substrate, using a maltose standard curve. One enzyme activity
67 unit (mU) was defined as the amount of enzyme capable of releasing 1 μg of maltose per
68 minute per milligram of protein. Lipase activity was determined using a modification of
69 the method of Arye et al. (2007) with 8.0 mM p-nitrophenyl palmitate (p-NPP) as
70 substrate. One enzyme activity unit (mU) was defined as the amount of enzyme that
71 catalyzes the hydrolysis of 1 μmol of p-nitrophenol (p-NP) per minute per milligram of
72 protein (Ɛ = 17,500 M-1cm^{-1}).

73

74 2.5. Antioxidant analysis

75 Catalase (CAT) activity was determined by measuring the decrease in hydrogen
76 peroxide concentration (Aebi, 1984). Reduced glutathione (GSH) levels were estimated
77 using the Sedlak and Lindsay (1968) method, which is based on the reaction between
78 Ellman's reagent (DTNB) and free thiol. Lipid peroxidation (malondialdehyde MDA/μM)
79 was quantified using the TCA (trichloroacetic acid) and TBA (thiobarbituric acid)
80 reaction, the results being expressed in μM/mg of sample (Wallin et al., 1993).

81

82 2.6 Proximate and mineral composition

For the proximate and mineral composition analysis, approximately 20 g of shrimp per experimental unit were sampled at the beginning (0 days) and end (50 days) of the culture period. The crude protein and lipid contents were analyzed in triplicate using standard methods (AOAC, 2016) at the Brazilian Agricultural Research Corporation (EMBRAPA), Piauí, Brazil, and the crude protein, lipid, fiber and ash contents of the feed were analyzed in triplicate at the Department of Animal Science, Federal Rural University of Pernambuco (UFRPE), Recife, Brazil. For moisture content, samples were dried in an oven at 105°C to constant weight (model 315 SE, Fanem). The difference in weight before and after drying of the sample 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 hexane solvent (98%) (model Ma 044/8/50, Marconi, São Paulo, Brazil). Ash was determined by combustion in an oven at 550°C (model Q318 D24; Quimis, São Paulo, Brazil).

The shrimp samples for mineral composition were dried in an oven until they reached a constant weight and then kept in a desiccator until they were sent to the EMBRAPA, Piauí, Brazil. The samples were then subjected to wet digestion with nitroperchloric mineralization and the corresponding values were recorded. Potassium (K), calcium (Ca), magnesium (Mg), copper (Cu), iron (Fe) and zinc (Zn) were analyzed by atomic absorption spectrophotometry (Agilent Technologies, US - SpectrAA 55B), while phosphorus (P) was analyzed by UV-VIS spectrophotometer (Thermo Scientific, FI - Genesys 10S UV-Vis), according to the methodology described by Nogueira et al. (1998).

2.7 Shrimp performance

The weight of the shrimps (n=15) was monitored every 10 days to determine growth and to adjust the amount of feed. After 25 days and at the end of the experimental period, all shrimp were counted and weight, feed conversion ratio (FCR), survival and yield were determined using the following equations 1 to 6:

1. Biomass gain= (final biomass (g) - initial biomass (g))
2. Average final weight= (final biomass (g) / number of individuals at the end of culture)
3. Feed conversion ratio (FCR)= (amount of feed offered / biomass gain)
4. Survival = (number of individuals at end of culture / number of individuals at start of culture) x 100)
5. Yield kg m^{-2} = (final biomass (kg) / experimental unit area (m^2))
6. Yield kg ha^{-1} = (final biomass (kg) / experimental unit area (m^2)) x 10,000

2.8. Economic benefits

The costs of inputs and feed processing, as well as shrimp performance, were extrapolated on a per hectare basis, after which the return on investment (ROI) was determined (Equation 7) according to Philips and Philips (2019). For the economic estimation, the cost of the control diet was USD 0.94 and the cost of the commercial *Lithothamnium* was USD 0.5 kg^{-1} (Lothar, PRIMASEA, Brazil) and the selling price of the shrimp was USD 3.70 kg^{-1}, considering the exchange rate in Reais of USD = R$ 4.95 (06.03.2024).

7. ROI = (Monetary gain from applying the input - Cost of applying the input) / Cost of applying the input)

2.11. Statistical Analysis

The sample data were previously analyzed for homogeneity of variance using the Cochran test and for normality using the Lilliefors test. Analysis of variance (ANOVA) was used for normal data (zootechnical performance and proximate and mineral composition) ($p < 0.05$). Repeated measures ANOVA ($p<0.05$) was used for water quality (calcium, potassium, total alkalinity, calcium hardness, Na/K, DT/AT, NAT). For non-normal data, the Kruskal-Wallis test ($p<0.05$) was used (lipids in proximate composition), and for significant differences, the Dunn test ($p<0.05$) was used to determine differences between treatments, and the Friedman test ($p<0.05$) with Holm-Bonferroni correction was used for magnesium, chloride, sodium, sulfate, total hardness, magnesian hardness, Mg/Ca, Ca/K, dissolved oxygen, temperature, salinity, pH, $N\text{-}NO_2$, $N\text{-}NO_3$, and SS. For digestive enzyme and antioxidant analyses, Bartlett's and Kolmogorov-Smirnov tests for homogeneity and normality, respectively, were performed, followed by one-way ANOVA and Tukey's test ($p < 0.05$) for homogeneous data (25 days - leucine aminopeptidase, trypsin, chymotrypsin and MDA; 50 days - trypsin, lipase and chymotrypsin). 05) for homogeneous data (25 days - leucine aminopeptidase, trypsin, chymotrypsin and MDA; 50 days - trypsin, lipase and chymotrypsin) and Games-Howell ($p < 0.05$) for non-homogeneous data (25 days - amylase and lipase; 50 days - amylase and leucine aminopeptidase).

3. Results

3.1 Water quality

The physicochemical and ionic composition of the water from the different treatments are summarized in Tables 3 and 4, respectively. No significant differences between treatments were observed throughout the experimental period.

Table 3. Physico-chemical water variables of rearing *P. vannamei* feeding with different *Lithothamnium* addition strategies in the diet.

Variables	Treatments				
	CT	LT2	LT4	CTL2	CTL4
Temperature (°C)	25.80±0.61[a]	25.83±0.73[a]	25.75±0.62[a]	25.87±0.66[a]	25.87±0.64[a]
DO (mg L^{-1})	6.88±0.52[a]	6.95±0.52[a]	6.97±0.49[a]	6.90±0.50[a]	6.98±0.52[a]
Salinity (g L^{-1})	4.03±0.61[a]	4.20±0.55[a]	3.99±0.57[a]	4.15±0.69[a]	4.19±0.77[a]
pH	8.08±0.41[a]	8.10±0.44[a]	8.07±0.45[a]	8.05±0.44[a]	8.05±0.45[a]
TAN (mg L^{-1})	0.56±0.38[a]	0.51±0.42[a]	0.57±0.35[a]	0.61±0.38[a]	0.63±0.43[a]
N-NO_2 (mg L^{-1})	0.35±0,.8[a]	0.33±0.47[a]	0.38±0.36[a]	0.27±0.19[a]	0.37±0.40[a]
N-NO_3 (mg L^{-1})	14.88±26.47[a]	14.69±26.57	14.80±26.51[a]	14.80±26.5[a]0	14.75±26.53[a]
SS (mL L^{-1})	0.63±0.45[a]	0.63±0.48[a]	0.35±0.23[a]	0.46±0.37[a]	0.53±0.49[a]

Data correspond to the mean ± standard deviation. Results were analyzed by repeated measures ANOVA ($p < 0.05$) for parametric data and Friedman 's test ($p \leq 0.05$) for non-parametric data. Mean values on the same line with different superscripts differ significantly. Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals. DO – dissolved oxygen; TAN – total ammonia nitrogen; N-NO_2 - nitrogen-nitrite; N-NO_3 - nitrogen-nitrate; SS- settleable solids.

Table 4. Ion composition of the water used to grow *P.* at low salinity with different strategies for adding *Lithothamnium* to the diet.

Íons	Treatments				
	CT	LT2	LT4	CTL2	CTL4
Calcium (mg L^{-1})	84.97±29.00[a]	86.91±30.18[a]	85.20±29.26[a]	85.54±29.83[a]	89.09±32.44[a]
Magnesium (mg L^{-1})	114.10±37.04[a]	126.12±40.54[a]	116.29±37.07[a]	117.85±33.43[a]	117.06±36.99[a]
Potassium (mg L^{-1})	27.26±5.67[a]	27.42±6.40[a]	27.25±6.47[a]	26.68±7.39[a]	25.79±6.26[a]
Sodium (mg L^{-1})	1,172.12±179.80[a]	1,293.50±279.60[a]	1,214.74±200.58[a]	1,178.66±213.06[a]	1,224.56±203.74[a]
Sulphate (mg L^{-1})	294.20±54.36[a]	308.61±63.24[a]	291.06±67.51[a]	310.24±63.17[a]	284.44±74.87[a]
Chloride (mg L^{-1})	1,808.83±431.18[a]	1,9916.14±431.48[a]	1,874.60±309.54[a]	1,818.92±328.84[a]	1,889.75±314.42[a]
TA (mg $CaCO_3$ L^{-1})	114.29±22.41[a]	118.21±22.41[a]	113.21±22.58[a]	130.57±30.35[a]	110.00±25.19[a]
TH (mg $CaCO_3$ L^{-1})	682.00±145.53[a]	736.29±167.28[a]	691.57±151.42[a]	698.86±147.47[a]	704.43±149.11[a]

Mg:Ca	1.68±1.15[a]	1.78±1.11[a]	1.69±1.14[a]	1.69±1.12[a]	1.66±1.15[a]
Ca:K	3.32±1.47[a]	3.39±1.49[a]	3.36±1.52[a]	3.25±1.57[a]	4.02±2.88[a]
Na:K	45.04±12.54[a]	50.07±16.66[a]	47.33±14.75[a]	43.57±13.55[a]	52.77±25.17[a]
DT:AT	6.36±2.37[a]	6.58±2.37[a]	6.44±2.30[a]	7.64±2.32[a]	6.89±2.68[a]

Data correspond to the mean ± standard deviation. Results were analyzed by repeated measures ANOVA ($p < 0.05$) followed by the Tukey test for parametric data and Friedman's test ($p \leq 0.05$) followed by Conover's multiple comparison test with Holm-Bonferroni correction for non-parametric data. Mean values on the same line with different superscripts differ significantly. Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals. TA – total alkalinity; TH – total hardness; DCa – dureza cálcica; DMg – Dureza magnesiana.

172

173 *3.2 Activity of Digestive Enzymes*

174 The enzyme activities in the shrimp hepatopancreas are summarized in figure 1.
175 After 25 days of culture, trypsin activity in the control and CTLT2 treatments was
176 significantly different from CTLT4 and LT2. After 50 days, the control showed the
177 highest activity and the other treatments were significantly similar. For chymotrypsin, the
178 highest values were observed in the LT4 treatment at 25 days of culture and in CTLT2
179 and CTLT4 at 50 days of culture, although CTLT4 was the same as the control and LT4.
180 With regard to leucine aminopeptidase, the LT4 treatment stood out at 25 days of culture,
181 but at 50 days the highest values were observed in the control treatment. For amylolytic
182 activity, the LT2 treatment showed the highest results at 25 days, and at 50 days the
183 CTLT4 and LT2 treatments showed the highest values, although they were significantly
184 equal to the control and CTLT2. Lipase activity was significantly higher in the LT4
185 treatment at 25 days. At 50 days, the highest values were observed in the control, CTLT2
186 and CTLT4, but the latter two were statistically equal to LT2 and LT4.

187

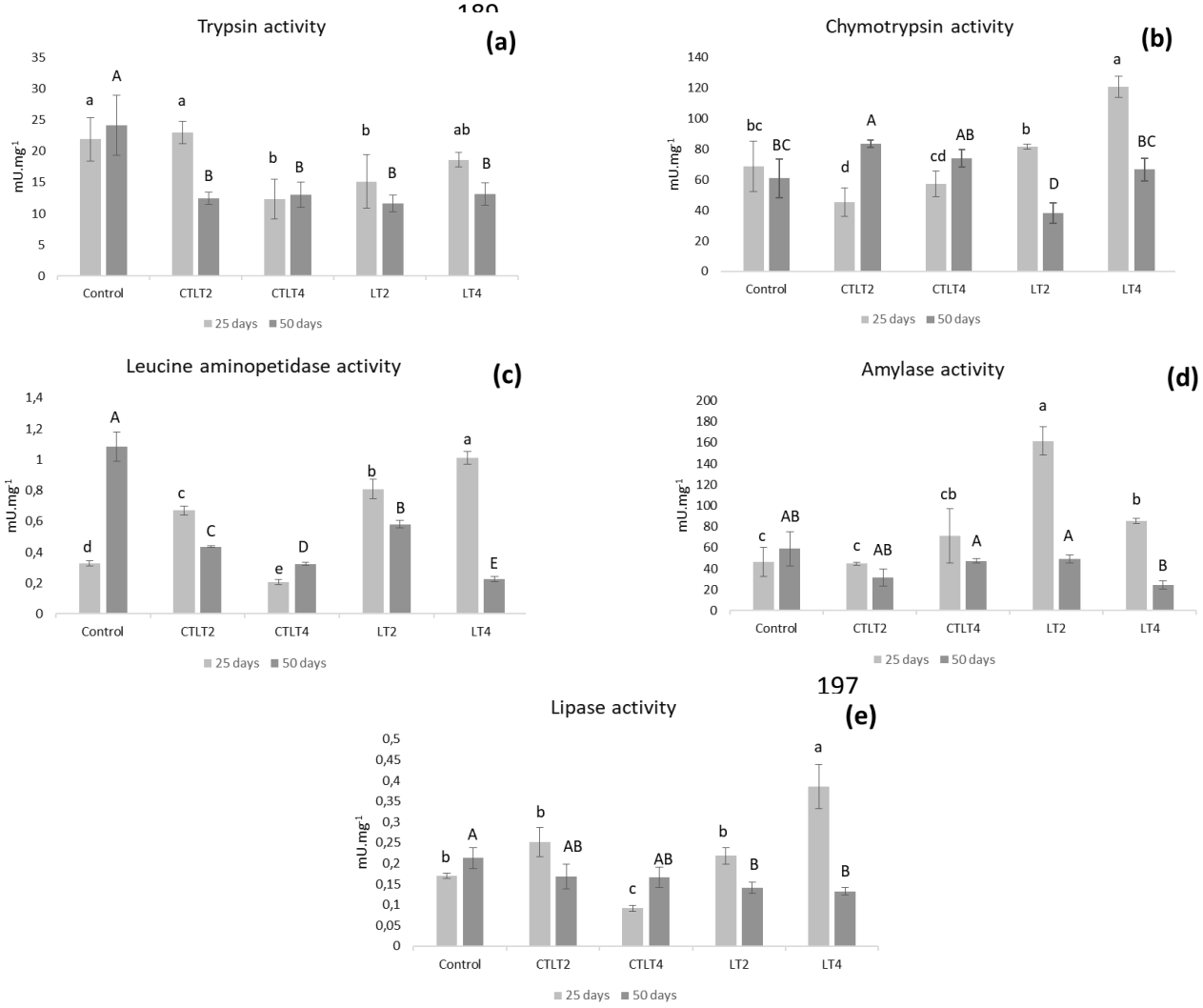

Figure 1. Activity of digestive enzymes in the crude extract of the hepatopancreas of Penaeus vannamei, cultivated in oligohaline waters in a symbiotic system, fed diets with different strategies for the addition of Lithothamnium. Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals. Data are expressed as mean ± standard deviation, by ANOVA ($p < 0.05$) followed by Tukey's test for homogeneous data and Games-Howell's test for non-homogeneous data. Different lowercase letters indicate a statistical difference between treatments at 25 days of culture. Different capital letters indicate a statistical difference between treatments at 50 days of culture. (a) Trypsin activity using BApNA 8.0 mM (Nα-benzoyl-DL-arginine-p-nitroanilide) as substrate. (b) Chymotrypsin activity using SApNA 8.0 mM (Suc-Ala-Ala-Pro-Phe p-nitroanilide) as substrate. (c) Leucine aminopeptidase activity using 8.0 mM SApNA (Suc-Ala-Ala-Pro-Phe p-nitroanilide) as substrate. (d) Amylase activity using 2% starch as substrate. (e) Lipase activity using 8.0 mM p-NPP (p-nitrophenyl palmitate) as substrate.

220 *3.3 Antioxidant Activity*

221 The antioxidant capacities of the shrimp are presented in Table 5. The CAT
222 (catalase activity) and GSH (reduced glutathione) of the shrimp fed with diets containing
223 *Lithothamnium* showed no significant differences between the treatments after 25 days of
224 culture, but when evaluating the MDA/μM (malondialdehyde) concentrations, the LT2,
225 LT4, baseline and control treatments were statistically equal compared to CTLT4. After
226 50 days, the antioxidant activities showed no significant difference between the
227 treatments.

228

Table 5. Antioxidant activities in the shrimp hepatopancreas with different *Lithothamnium* addition strategies in shrimp diets.

Activities	Initial	CT	LT2	LT4	CTL2	CTL4
				25 days		
CAT	0.104±0.099^{a}	0.055±0.037^{a}	0.115±0.094^{a}	0.061±0.051^{a}	0.174±0.086^{a}	0.039±0.021^{a}
GSH	0.025±0.004^{a}	0.022±0.004^{a}	0.030±0.006^{a}	0.022±0.004^{a}	0.025±0.005^{a}	0.028±0.008^{a}
MDA/µM	7.65±1.80^{bc}	889±2.06^{bc}	6.06±1.28^{ab}	5.90±0.79^{ab}	8.40±3.32^{bc}	12.61±3.68^{cd}
				50 dias		
CAT	0.104±0.099^{a}	0.573±0.391^{a}	0.287±0.196^{a}	0.331±0.084^{a}	1.103±0.878^{a}	0.419±0.232^{a}
GSH	0.025±0.004^{a}	0.022±0.004^{a}	0.027±0.005^{a}	0.027±0.005^{a}	0.028±0.008^{a}	0.032±0.008^{a}
MDA µM^{-1}	7.65±1.80^{a}	6.14±1.04^{a}	7.93±3.28^{a}	5.47±0.32^{a}	8.32±2.12^{a}	6.54±1.11^{a}

Data correspond to the mean ± standard deviation. Results were analyzed by ANOVA ($p < 0.05$) followed by the Tukey test for homogeneous data and Games-Howell test ($p \leq 0.05$) for no homogeneous data. Mean values on the same line with different superscripts differ significantly. CAT catalase activity - U mg. Protein^{-1}; GSH = reduced glutathione -µM mg. Protein^{-1}; MDA/µM = malondialdehyde - µM mg. Protein^{-1}. Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals.

238

239 *3.4 Proximate and mineral composition*

240 The levels of crude protein and the minerals magnesium, potassium, copper, phosphorus,
241 iron and zinc were not significantly different between the treatments. However,
242 significant differences were observed for calcium, which was higher in the CTL4
243 treatment, and for lipids, which were lower in the CTLT2 and CTLT4 treatments (Table
244 6).

245

Table 6. Proximate and mineral composition of shrimp fed different strategies for adding *Lithothamnium* in the shrimp diet.

Composition	Treatments				
	CT	LT2	LT4	CTL2	CTL4
Dry matter	94.24±0.34^{a}	94.14±0.96^{a}	94.72±0.77^{a}	93.73±0.61^{a}	94.99±0.38^{a}
Crude Protein (%)	58.85±0.43^{a}	59.62±1.48^{a}	58.42±1.04^{a}	59.94±0.38^{a}	59.94±0.38^{a}
Lipids (%)	4.65±0.06^{a}	5.26±0.05^{a}	4.42±0.01^{a}	2.73±0.09^{b}	2.92±0.66^{b}
Calcium (g kg^{-1})	30.02±1.72^{b}	22.51±0.88^{c}	26.23±1.16^{bc}	30.1±4.87^{b}	37.87±0.94^{a}
Magnesium (g kg^{-1})	2.41±0.36^{a}	2.33±0.18^{a}	2.64±0.12^{a}	2.75±0.13^{a}	2.71±0.28^{a}
Potassium (g kg^{-1})	12.70±1.18^{a}	13.58±1.10^{a}	13.44±1.06^{a}	13.69±1.40^{a}	12.75±0.33^{a}
Phosphorus (g kg^{-1})	10.60±0.75^{a}	10.95±0.49^{a}	10.87±0.25^{a}	11.92±0.34	11.57±0.08^{a}
Copper (mg kg^{-1})	11.07±0.85^{a}	11.71±1.02^{a}	12.37±2.63^{a}	10.66±0.30^{a}	11.43±0.49^{a}
Iron (mg kg^{-1})	27.49±3.29^{a}	23.61±2.76^{a}	23.33±2.40^{a}	28.07±2.33^{a}	29.72±5.54^{a}
Zinc (mg kg^{-1})	47.82±2.52^{a}	47.30±0.38^{a}	47.84±1.41^{a}	47.60±1.86	46.68±0.01

Data correspond to the mean ± standard deviation. Results were analyzed by ANOVA ($p < 0.05$) followed by the Tukey test for parametric data. Mean values on the same line with different superscripts differ significantly. Control (CT) - diet with inorganic minerals; CTLT2 - control diet

with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals.

3.5. Shrimp performance

The shrimp performance values for the different treatments are summarized in Table 7. Significant differences were observed between the treatments for final weight. In addition, at the end of the experiment (50 days), shrimp reared in the LT2 treatment showed significantly higher weekly growth and yield, as well as a FCR decreases compared to the other treatments and the control. Yield (kg ha^{-1}) was higher in the LT2 treatment compared to the control and the CTL2 treatment.

Table 7. Shrimp performance during 50 days of feeding different strategies for *Lithothamnium* addition in shrimp diet.

Variables	Treatments				
	CT	LT2	LT4	CTL2	CTL4
Final weight (g)	7.81 ± 0.35^{b}	8.71 ± 0.32^{a}	7.93 ± 0.37^{b}	7.87 ± 0.04^{b}	8.13 ± 0.13^{b}
Survival (%)	76.67±1.15	76.67±1.15	76.67±2.31	76.67±2.31	76.00±2.00
Growht (g $week^{-1}$)	0.66 ± 0.01^{b}	0.79 ± 0.04^{a}	0.68 ± 0.05^{b}	0.67 ± 0.01^{b}	0.69 ± 0.02^{ab}
Yield (kg m^{-2})	0.37 ± 0.02^{b}	0.42 ± 0.01^{a}	0.38 ± 0.01^{ab}	0.38 ± 0.01^{b}	0.39±0.01ab
Yield (kg ha^{-1})	$2,993\pm177^{b}$	$3,337\pm83^{a}$	$3,035\pm79^{ab}$	$3,014\pm76^{b}$	$3,088\pm132^{ab}$
FCR	1.94 ± 0.21^{a}	1.55 ± 0.09^{b}	1.88 ± 0.11^{a}	1.91 ± 0.09^{a}	1.83 ± 0.15^{a}

Data correspond to the mean ± standard deviation. Results were analyzed by ANOVA ($p < 0.05$) followed by the Tukey test for parametric data. Mean values on the same line with different superscripts differ significantly. Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals.

286 *3.6 Economic benefits*

287 In the *Lithothamnium* treatments, the revenue from shrimp sales minus feed costs ranged
288 from USD 7,438 ha^{-1} to USD 5,631 ha^{-1} (Table 8). The return on investment was positive
289 in the LT2, LT4 and CTLT4 treatments compared to the control.

290

Table 8. Economic benefits of adding *Lithothamnium* to *Penaeus vannamei* diets in low salinity water.

		CT	LT2	LT4	CTL2	CTL4
Shrimp stocking	camarões ha^{-1}	500000	500000	500000	500000	500000
Survival	%	77	77	76	76	76
Final weight	g	7.8	8.7	7.9	7.9	8.1
Biomass	kg ha^{-1}	2,994	3,339	3,040	3,017	3,089
Income	USD ha^{-1}	11,078	12,354	11,248	11,163	11,431
FCR		1.94	1.55	1.88	1.91	1.83
Feed Intake	kg ha^{-1}	5,808	5,175	5,715	5,762	5,654
Feed costs (U$$/kg)	USD	0.94	0.95	0.96	0.96	0.97
Total Feed Costs	USD ha^{-1}	5,460	4,917	5,487	5,532	5,484
Net Income	USD ha^{-1}	5,618	7,438	5,761	5,631	5,947
ROI			34.16	0.26	-0.89	0.94
Comparison of net income						
LT2 x Control	USD ha^{-1}		1,820			
LT4 x Control	USD ha^{-1}			143		
CTLT2 x Control	USD ha^{-1}				13	

CTLT4x Control	USD ha^{-1}		329

Control (CT) - diet with inorganic minerals; CTLT2 - control diet with the addition of 2% *Lithothamnium* with commercial Binder; CTLT4 - control diet with the addition of 4% *Lithothamnium* with commercial Binder; LT2 - addition of 2% *Lithothamnium* in the diet to replace inorganic minerals and LT4 - addition of 4% *Lithothamnium* in the diet to replace inorganic minerals

4. Discussion

The water quality variables were within the recommended standards for shrimp culture, except for temperature, which, although stable, was below the values considered ideal (28-32°C) (Samocha and Prangnell, 2019). However, the survival and growth results were not affected by this factor, unlike Abdelrahman et al. (2018), who obtained lower results in *P. vannamei* cultures grown at low salinity and temperature. Nitrogen compounds, due to their toxicity to shrimp, especially at low salinities with minimal water exchange, are one of the major challenges for crustacean production in these environments (Pimentel et al., 2023a,b). The use of nursery water (inoculum) combined with organic fertilization (synbiotic) and alkalinity concentrations above 100 mg $CaCO_3L^{-1}$ helped to keep these variables within safe concentrations for the species (Pimentel et al., 20223a).

Research on the digestive processes of penaeid shrimp has been conducted with the aim of evaluating the ability of these organisms to hydrolyze, absorb and assimilate the main nutrients in the diet. Proteases, lipases and amylases are the main digestive enzymes in the hepatopancreas of shrimp that affect the digestion and absorption of food and are directly related to the growth of the animal and its adaptability to the environment (Muhlia-Almazán et al., 2003; Xia et al., 2018). In the present study, we observed significant differences in the activities of trypsin, chymotrypsin, leucine aminopeptidase, lipase and amylase in *P. vannamei* fed the experimental diets. Tan et al. (2014) evaluated the addition of 2.0 and 4.0 g kg^{-1} of volcanic sediment composed of natural minerals in the diet of *P. vannamei* and observed an increase in the activities of pepsin and hepatopancreatic lipase, and the concentration of 2.0 g kg^{-1} significantly increased proteolytic activity, demonstrating the influence of minerals on enzymatic activities.

During the experimental period, trypsin activities in the control, CTLT2 and LT4 treatments were significantly higher than the other treatments after 25 days of culture. Laining et al. (2015) emphasized the need to clarify the role of organic minerals in the growth, utilization, and immune response of aquatic animals. This observation is important because the bioavailability of organic minerals may have favored their use in molting processes related to growth and osmoregulation (Ahearn & Zhuang, 1996; Wheatly, 1999; Zanotto & Wheatly, 2002), with calcium, for example, being essential in these processes, in addition to its role in optimizing enzyme function and animal health. In the case of enzymes, calcium increased the activity of trypsin isoforms A, B, and C in *P. vannamei*, but isotrypsin C requires higher concentrations of calcium to achieve the same activity as isoforms A and B, i.e., respecting physiological limits, we can guarantee the activity of the isoforms found in the species (Sainz et al., 2004). Simon et al. (2022) emphasized that although trypsin does not depend on cofactors, it essentially requires the binding of calcium ions to its binding site to achieve full enzymatic activity and stability.

Li et al. (2008), when evaluating trypsin activity at extreme salinities, reported a significant increase in activity in *P. vannamei* exposed to 3.0‰ salinity, showing the possibility that the species may derive additional food energy to compensate for the loss due to osmoregulation. The authors emphasize that there is still no definitive evidence that increased trypsin activity could be a compensatory factor in low salinity conditions to obtain energy from protein. However, it is worth noting that even though the shrimp in this study were kept at the same salinities, the control (inorganic mineral diet) showed higher trypsin activity at the end of the experiment.

Mineral deficiencies can reduce tissue mineralization and potentially affect digestive enzyme functions (Davis et al., 1993). However, specific studies focusing on direct effects on chymotrypsin in this species are limited. Gao et al. (2012 and 2016)

demonstrated that chymotrypsin expression in *P. vannamei* was negatively regulated with decreasing salinity, but there are no specific data directly linking mineral compounds to changes in enzyme activity, which was observed in this study.

Leucine aminopeptidase from marine sources generally depends on metal ions for its activity; in this context, zinc and manganese are common cofactors essential for maintaining enzyme function. Many, but not all, aminopeptidases are metalloenzymes that contain a central zinc that is essential for enzyme activity (Wu et al., 2008). Although the diet with the highest zinc concentration showed greater enzyme activity at 25 days, the other results did not show this direct relationship.

The presence and concentration of ions in the environment play a critical role in modulating amylase activity in shrimp, either enhancing its function or leading to inhibition, depending on the specific ion and its concentration (Castro et al., 2012). The treatment that stood out in terms of growth (LT2) also stood out in terms of amylolytic activity at 25 days of culture, and at 50 days the activity was significantly similar to the control, CTLT2 and CTLT4. It is likely that the inclusion forms and their proportions did not negatively affect the digestive processes of the species. In addition, the amylase activity may indicate that the animals were making good use of the energy present in the diet, thereby increasing the overall efficiency of nutrient utilization.

Lipolytic activity is essential for the mobilization of energy reserves, especially during periods of fasting or stress (Rivera-Perez et al., 2011). During periods of stress, intracellular lipase expression increases, which helps maintain lipid homeostasis by mobilizing stored lipids from muscle tissue (Rivera-Perez & García-Carreño, 2011). At 25 days, lipase activity in LT4 shrimp was significantly higher than in the other treatments, suggesting that shrimp in this treatment may not be in homeostasis at this time. However, at the end of the experimental period, the activities showed a certain

similarity, including the lowest activity in the LT2 treatment, the experiment that showed the best productive performance, demonstrating a balance in this treatment.

Antioxidant activities are regulated by dietary nutrients, health status, and environmental stress. Catalase, reduced glutathione and MDA interact significantly under conditions of oxidative stress in aquatic animals and play essential roles in protecting organisms against lipid peroxidation and oxidative damage. Disease-related stress (Gonçalves-Soares et al., 2012), changes in water quality (Wang et al., 2012) lead to an increase in antioxidant enzyme activity and MDA levels in *P. vannamei*. This highlights the role of enzymes in neutralizing reactive oxygen species (ROS) during oxidative stress and lipid peroxidation. In stress situations, ROS are continuously produced in response to the stressor, but their accumulation can damage cell membranes, genetic material, vital cellular components, inactivate enzymes, and cause shrimp mortality (Chien et al., 2003).

Lin and Chen (2001, 2003); Li et al. (2007, 2008), showed that *P. vannamei* are more vulnerable to environmental stress at 3.0 ‰ salinity than at 15, 25 and 35‰. Long et al. (2023) showed that salinity has a great influence on catalase concentrations, due to the energy expenditure for osmoregulation, but it does not influence glutathione peroxidase concentrations, which remained stable throughout the times and treatments analyzed. In the present study, an increase in catalase was observed when evaluating the shrimp from 25 to 50 days, however, no significant difference was observed between treatments, including GSH and MDA. However, when assessed more broadly, at 50 days, catalase activity increased compared to 25 days in all treatments, indicating that despite the longer exposure time to low salinity concentrations, the shrimp showed a response against lipid peroxidation and oxidative damage, and the similar MDA values between times and sometimes lower when reaching 50 days, corroborates this observation.

With respect to the mineral composition of *P. vannamei*, the different forms of inclusion of *Lithothamnium* resulted in significant differences in calcium concentration. Diets containing 2% or more of dietary calcium from the addition of the inorganic mineral may inhibit the absorption of phosphorus and other nutrients by shrimp (Cheng et al., 2006). Phosphorus absorption is directly involved in all energy production reactions and plays an integral role in cellular functions as it is a key component of nucleic acids, phospholipids, phosphoproteins, ATP, alkaline phosphatase and osmoregulation in crustaceans (Lovett et al., 1994; Pinoni and López Mañanes, 2004). All the diets had 3% calcium and 1% phosphorus in their formulation, but the calcium:phosphorus ratios differed in the proximate composition of the shrimp, probably due to the way the shrimp absorb the different sources (inorganic and organic). According to Davis et al. (1993) and Cheng et al. (2006), a calcium:phosphorus ratio of 2:1-2 is more suitable for *P. vannamei* diets because it increases the absorption of phosphorus and other nutrients by the shrimp. This ratio was observed in the mineral composition of LT2 shrimp, which may have contributed to the lower FCR and higher final weight in this treatment.

The inclusion of magnesium in the diet of low salinity shrimp culture is a widely used nutritional strategy to minimize survival and growth problems (Gong et al., 2004; Cheng et al., 2005; Roy et al., 2007, 2009). Magnesium plays an important role in cellular respiration and metabolic activity, as well as being one of the components of the exoskeleton and a cofactor for the enzyme Na^+/K^+-ATPase (responsible for osmoregulation in active ion transport) (Galkanda-Arachchige et al., 2021; Nesapriyam et al., 2022). However, the results are controversial regarding the concentrations required in the diet of shrimp at low salinities. Roy et al. (2009) showed that the inclusion of magnesium chelate complex with amino acids (1.5 to 3.0 g magnesium kg $diet^{-1}$) in diets for *P. vannamei* at 5 g L^{-1} salinity in nursery and grow-out did not have a positive effect

414 on shrimp performance, while Cheng et al. (2005) recommended the inclusion of 3.46 g
415 magnesium as an inorganic mineral kg diet^{-1} to obtain better shrimp performance in 2 g
416 L^{-1} salinity water. These results show that the type of mineral added to the diet and the
417 salinity of the water influence the amount required by the shrimp.
418 Replacing magnesium oxide (an inorganic mineral) with *Lithothamnium* (LT2 and LT4)
419 reduced the amount of magnesium kg diet^{-1} from 8 g to 3 g and probably did not cause
420 any physiological damage to the shrimp reared at low salinity, as survival rates were
421 similar to the control. Furthermore, at the end of 50 days of rearing, shrimp in treatment
422 LT2 (3.0 g magnesium kg diet^{-1}) showed better growth and FCR compared to the other
423 treatments. Roy and Davis (2010) found that supplementation in excess of dietary
424 magnesium requirements, as observed in the CT, CTLT2, and CTLT4 treatments, did not
425 have benefits for survival, growth, or osmoregulatory capacity of *P. vannamei* shrimp,
426 especially when magnesium concentrations in the water were adequate.
427 The reduction in potassium chloride inclusion from 5.66 g kg diet-1 to 1.16 g kg diet^{-1} in
428 the LT4 treatment affected shrimp performance compared to LT2. This result indicates
429 that there is a limit to the replacement of potassium chloride by *Lithothamnium* in diets
430 for *P. vannamei* at low salinity. According to Hongyu et al. (2014) and Roy et al. (2007),
431 the potassium concentration in *P. vannamei* diets at low salinity should be between 1.0
432 and 1.4%. This range of K^+ concentration can affect the growth and survival of shrimp
433 because it plays a role in osmoregulation and also helps to improve the uptake of
434 extracellular nutrients such as glucose, amino acids, phosphorus, and vitamins (Davis and
435 Lawrence 1997; Cheng et al. 2005; Roy et al. 2007; Naik, 2012).
436 In crustaceans, the supplementation of bioavailable calcium in diets comes from fishmeal
437 (Van Wyk, 1999, Truong et al., 2023). However, in recent years, the shrimp feed
438 manufacturing industry has reduced the use of fishmeal and increased the use of other

terrestrial animal and vegetable meal sources that are low in bioavailable calcium (Truong et al., 2023). This reduction in dietary bioavailability and the low availability of calcium in the water in low salinity culture can lead to reduced growth and survival, as calcium is the predominant mineral in shrimp exoskeleton formation, muscle contraction, and osmoregulation (Van Wyk, 1999; Li et al., 2017). Thus, the levels of 0.3% magnesium, 1.2% potassium and 3.6% calcium in the LT2 diet with 2% *Lithothamnium* kg feed^{-1} probably provided an adequate combination of these minerals under the conditions of the ionic composition of the water in this study.

Another alternative to improve the osmoregulatory capacity of crustaceans at low salinity is to increase the inclusion of amino acids in the diet (Saoud et al., 2007; Huai et al., 2009; Jin et al., 2017). *Lithothamnium* has 7% amino acids in its composition, including essential amino acids (e.g., methionine and arginine) and trace minerals (e.g., zinc and copper), which are associated with improved shrimp performance and health of shrimp and farmed fish (Dawood 2022; Dawood et al., 2022; Truong et al., 2023), and their inclusion is likely to contribute to better growth of shrimp fed diets because they are in their organic form. The structure of the organic mineral molecule linked to amino acids allows these minerals to be transported intact to the mucosal cell, improving utilization by the shrimp (Truong et al., 2023). The chemical form of minerals can also affect the absorption and utilization of minerals, and the supplementation of trace elements in animal diets has been achieved through the use of inorganic salts such as sulfate and carbonate (Katya et. al., 2016). It is likely that this binding of *Lithothamnium* minerals with amino acids allowed for greater nutritional absorption of these minerals, as pointed out by other authors (Katya et al., 2016).

The evaluation of the shrimp potential of diets is important for the assessment of productivity, but studies aimed at the return on investment are of great importance for the

economic sustainability of production, since feed costs represent a large part of the investment in inputs. The results of the cost analysis showed that the inclusion of *Lithothamnium* in the diet in a simulation for a 1 ha production showed that the return on investment (ROI) was higher in the LT2 treatment (34. 16) compared to the control, indicating more profit and competitive advantage with the inclusion of 2% *Lithothamnium* kg feed^{-1} as a partial replacement of magnesium and calcium in the form of inorganic minerals in the formulation of low salinity shrimp feeds, opening new avenues for the substitution of these inorganic minerals with organic ones in the formulation of feeds for marine shrimp farmed at low salinity.

5. Conclusions

The addition of *Lithothamnium* (LT2) to the diet of *P. vannamei* reared in a synbiotic system at low salinity improved growth, survival and yield compared to the control diet and adequately maintained the activities of digestive enzymes. In addition, this supplement provided the most nutritionally appropriate calcium:phosphorus ratio (shrimp proximate composition) and maintained a balance of oxidative activities in the shrimp hepatopancreas. In addition to the shrimp performance results, the treatment also showed a higher return on investment, which can have a direct impact on production costs.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have seemed to influence the work reported in this paper.

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