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RESEARCH ARTICLE

Immunostimulant effect of nucleotide supplementation on shrimp *Penaeus vannamei* submitted to bacterial challenge with *Vibrio parahaemolyticus*

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Abstract

Aim of the study: Vibriosis and other bacterial infections significantly challenge Pacific white shrimp (*Penaeus vannamei*) production. This study evaluated the immunostimulant effect of nucleotide supplementation on hemato-immunological parameters, digestive gland morphology, presumptive *Vibrio* count, and survival of *Penaeus vannamei* exposed to *Vibrio parahaemolyticus*.

Area of study: Aquaculture nutrition and health management, specifically focusing on the mitigation of bacterial disease (Vibriosis) in cultured Pacific white shrimp.

Material and methods: Three nucleotide supplementation levels were evaluated: NT75 (75 mg/kg), NT150 (150 mg/kg), and NT300 (300 mg/kg), alongside a control group (C). After 60 days of feeding the experimental diets, the shrimp were challenged by immersion in a bacterial suspension of *Vibrio parahaemolyticus* (10^6 CFU mL^{-1}) for 30 days. Mortality and clinical signs were monitored, and hemato-immunological, histological, and bacteriological parameters were analyzed.

Main results: Nucleotide-supplemented treatments exhibited improved growth performance and reduced pre-challenge mortality. Following the bacterial challenge, all treatments showed an acute, albeit non-significant, change in total and differential hemocyte counts. However, histological analysis of the digestive gland revealed severe lesions, including tubule deformities and necrosis, across all treatment groups. On days 5 and 15, significant differences in *Vibrio* sp. counts within the digestive gland were observed. Specifically, differences were noted between Control (C) and NT75, and between NT75 and NT300, indicating varying bacterial colonization over time.

Research highlights: Higher nucleotide supplementation levels improved shrimp performance, reduced pre-challenge mortality, and enhanced the acute immune response following a bacterial challenge, suggesting their utility as an effective immunostimulant against Vibriosis.

Keywords: immune response; immunostimulant; nucleotides; Pacific white shrimp; vibriosis.

Efecto inmunoestimulante de la suplementación con nucleótidos en el camarón *Penaeus vannamei* expuesto a la bacteria *Vibrio parahaemolyticus*

Resumen

Objetivo del estudio: La vibriosis y otras infecciones bacterianas representan un desafío significativo para la producción de camarón blanco del Pacífico (*Penaeus vannamei*). Este estudio evaluó el efecto inmunoestimulante de la suplementación con nucleótidos sobre parámetros hematoinmunológicos, la morfología de las glándulas digestivas, el recuento presuntivo de *Vibrio* y la supervivencia de *Penaeus vannamei* expuesto a *Vibrio parahaemolyticus*.

Área de estudio: Nutrición y gestión sanitaria en acuicultura, con especial atención a la mitigación de la enfermedad bacteriana (vibriosis) en camarón blanco del Pacífico de cultivo.

Material y métodos: Se evaluaron tres niveles de suplementación con nucleótidos: NT75 (75 mg/kg), NT150 (150 mg/kg) y NT300 (300 mg/kg), junto con un grupo control (C). Después de 60 días de alimentación con las dietas experimentales, los camarones fueron sometidos a un desafío mediante inmersión en una suspensión bacteriana de *Vibrio parahaemolyticus* (10^6 UFC mL^{-1}) durante 30 días. Se monitorizaron la mortalidad y los signos clínicos, y se analizaron parámetros hematoinmunológicos, histológicos y bacteriológicos.

Resultados principales: Los tratamientos suplementados con nucleótidos mostraron un mejor rendimiento del crecimiento y una reducción de la mortalidad previa al desafío. Tras el desafío bacteriano, todos los tratamientos mostraron un cambio agudo, aunque no significativo, en los recuentos totales y diferenciales de hemocitos. Sin embargo, el análisis histológico de la glándula digestiva reveló lesiones graves, incluyendo deformidades tubulares y necrosis, en todos los grupos de tratamiento. En los días 5 y 15, se observaron diferencias significativas en los recuentos de *Vibrio* sp. dentro de la glándula digestiva. Específicamente, se observaron diferencias entre el Control (C) y el NT75, y entre el NT75 y el NT300, lo que indica una colonización bacteriana variable a lo largo del tiempo.

Aspectos destacados de la investigación: Niveles más altos de suplementación con nucleótidos mejoraron el rendimiento del camarón, redujeron la mortalidad previa al desafío y potenciaron la respuesta inmunitaria aguda tras un desafío bacteriano, lo que sugiere su utilidad como inmunoestimulante eficaz contra la vibriosis.

Palabras clave: camarón blanco del Pacífico; inmunoestimulante; nucleótidos; respuesta inmune; vibriosis.

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Introduction

In 2022, global Pacific white shrimp (*Penaeus vannamei* Boone, 1931) production reached 6.8 million tons, representing 53.3% of the world's crustacean aquaculture (FAO, 2024). Brazil's contribution is significant, with the Northeast region cultivating 99.6% of the nation's shrimp, totaling 127.5 thousand tons in 2023 (IBGE, 2024).

However, this productive potential is constantly limited by the emergence of diseases. Infectious agents, such as viruses and bacteria, have resulted in high mortality in crustacean production, which can cause irreparable economic losses. Among bacteria, species of the *Vibrio* genus, are responsible for many diseases

in shrimp, either as the main pathogen or as opportunistic agents, and can affect animals from the post-larval phase to the adult phase (De Souza Valente & Wan, 2021). Faced with these challenges, improved management strategies are crucial to mitigate losses and boost sector productivity and growth.

In addition to improvements in production systems, the use of functional feed additives has greatly increased in aquaculture. These additives are products designed to enhance animal health and improve nutrient utilization. Furthermore, they exhibit fewer toxic characteristics compared to antibiotics, representing an economically viable option aligned with ecological principles (Vijayaram et al., 2022).

An example of additives that proved to be effective in aquaculture were products derived from yeast, which when used in feed supplementation led to improvements in zootechnical performance and immunity (Licona-Jain et al., 2022; Mahdy et al., 2022). Yeast products contain several immunostimulant compounds, such as mannan oligosaccharides (MOS), β-glucans and nucleotides, which can improve immune responses (Agboola et al., 2021).

Nucleotides are low molecular weight intracellular compounds that perform essential roles in physiological and biochemical processes, including genetic information management and metabolic and cell signaling mediation (Novriadi et al., 2021). While most tissues synthesize nucleotides, certain immune and intestinal cells have a high demand that requires supplementation through exogenous sources (Yong et al., 2020; Rairat et al., 2022).

Nucleotide supplementation ensures organismal homeostasis and greater nucleotide availability, particularly during periods of high physiological demand (Ding et al., 2021). In *P. vannamei*, this has been specifically observed through improved growth performance, enhanced feed efficiency (Abdel-Rahim et al., 2021), healthy development of intestinal villi (Mok et al., 2021), and increased resistance to *Vibrio harveyi* (Baumann et al. 1981; Novriadi et al., 2021) and *Vibrio parahaemolyticus* (Fujino et al., 1965; Rairat et al., 2022). Furthermore, it has led to improved survival rates and strengthened immunological and humoral responses.

Despite all these benefits provided by nucleotides, the mechanisms underlying exogenous supplementation are not yet well documented.

Nucleotides are supplemented in feed for their immunostimulant properties; however, the precise inclusion levels required to produce such benefits are currently unknown. Consequently, this study was designed to assess the immunostimulant effects of nucleotide supplementation on the growth, hemato-immunological parameters, the digestive gland morphology, presumptive *Vibrio* counts, and survival of *P. vannamei* following a bacterial challenge with *V. parahaemolyticus*.

Material and methods

Experimental conditions

For the experiment, we used 10-day-old *P. vannamei* post-larvae. These post-larvae, acquired from a commercial larviculture (Aquatec, Rio Grande do Norte, Brazil), were confirmed negative for all World Organisation for Animal Health (WOAH) notifiable diseases through confirmatory diagnosis (World Organisation for Animal Health, 2022). We maintained the larvae in an intensive system at a stocking density of 5,000 post-larvae per cubic meter (800L) until they reached an average weight of 1.78 grams.

During the nursery phase, the post-larvae were fed a commercial shrimp feed (Wean, ADM Animal Nutrition Company, Brazil) containing 45% crude protein, with particle sizes of 0.6- and 0.8-mm. Feeding occurred four times a day (8:00 am, 11:00 am, 2:00 pm, and 5:00 pm). Initially, the daily feeding rate was 35% of the shrimp's body weight, which was gradually reduced to 8.0%. Feeding rates were adjusted daily based on estimated shrimp feed consumption and mortality, as described by Van Wyk et al. (1999). Following the nursery phase, the shrimp were counted, weighed, and transferred to experimental tanks at a density of 100 shrimp per square meter.

The experiment used 12 fiberglass tanks (800 L, 1.0 m^2). Each tank was filled with 600L of seawater (32 g/L) that had been filtered to 250 µm and treated with 30 ppm of active chlorine using sodium hypochlorite. The water was dechlorinated via 48 hours of aeration. Subsequently, 200 L of water from the synbiotic used on the shrimp nursery were added to each tank. This nursery water had the following characteristics: total ammonia nitrogen (TAN) = 0.0 mg/L, nitrite nitrogen (NO_2^--N) = 1.20 mg/L, nitrate nitrogen (NO_3^--N) = 56.3 mg/L, total alkalinity = 125 mg $CaCO_3$/L, pH 7.6, settleable solids (SS) = 3 ml/L, and salinity = 29.

The synbiotic was prepared in 20 L buckets using sequential anaerobic and aerobic processes, each lasting 24 hours. The mixture consisted of 5 g/m^3 of rice bran (< 200 micrometers), 0.5 g/m^3 of raw sugar, 0.04 g/m^3

of a commercial bacterial mixture (containing *Bacillus subtilis* [2.1 × 10^7 CFU/g], *B. licheniformis* [3.7 × 10^7 CFU/g], and *Bacillus* sp. [2.8 × 10^7 CFU/g], provided by Kayros Agrícola e Ambiental, SP, Brazil), mixed with 50 ml/m^3 of sterilized seawater. Throughout the experiment, the synbiotic was added to the experimental units every three days (15 times in total), unless the settleable solids reached 10 ml/L.

Throughout the experiment, the pH was kept between 7.5 and 8.0, and alkalinity was maintained above 100 mg/L $CaCO_3$. This correction was achieved using calcium and magnesium hydroxide (hydrated lime – PRNT 80%), which constituted 10% of the total weekly feed. Water changes were not performed; however, chlorinated and dechlorinated freshwater was added as needed to offset evaporation.

Ingredients and experimental diets

Experimental diets were formulated to meet the nutritional requirements of juvenile *P. vannamei*. These diets were isoproteic, containing 360 g/kg protein, and isoenergetic, providing 4,400 kcal/kg of energy (Table 1). Four diets were created: a control diet and three test diets. The test diets were formulated by incorporating a commercial nucleotide source into the control diet, specifically replacing wheat flour. The control diet was modified by replacing wheat flour with a commercial nucleotide source at three different concentrations: 75 mg/kg (NT75), 150 mg/kg (NT150), and 300 mg/kg (NT300) of feed (Table 1). The test diets differed from the control diet only in the inclusion of nucleotides.

Table 1. Formulation and proximate composition of the control and experimental diets, supplemented with nucleotides, used to assess the growth performance of *Penaeus vannamei* cultured in a synbiotic system over a 60-day period.

Ingredients (g kg^{-1})	Diet			
	Control	NT75	NT150	NT300
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

Ingredients (g kg^{-1})	Diet			
	Control	NT75	NT150	NT300
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 Extra/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.

The commercial nucleotide source consisted of 15% RNA (nucleotides) from the yeast *Saccharomyces cerevisiae*, 50% crude protein, 15% ash and 8% water (Biotide Extra, Biorigin, São Paulo, Brazil). The experimental diets were made 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 using a hammer mill (model M300, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil) to achieve a particle size smaller than 600 µm. They were then mixed thoroughly for 15 minutes (model M2200, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil), extruded at temperatures between 80 ºC and 90 ºC (model E62, Ferraz Máquinas e Engenharia Ltda, Ribeirão Preto, São Paulo, Brazil) into 2.0 mm diameter pellets, and dried at 90 ºC-100 ºC to reduce moisture to 120 g/kg. The pellets were subsequently crumbled, sealed in bags, and stored frozen until needed.

The shrimp received the experimental diets for 60 days prior to the bacterial challenge. During this period, the feeding rate was adjusted daily based on Van Wyk's (1999) methodology, and feed was administered four times a day at 8:00 am, 11:00 am, 1:00 pm, and 4:00 pm.

Water quality

Water quality parameters were checked twice a day at 8:00 a.m. and 4:00 p.m. for dissolved oxygen (DO) and temperature using a water quality meter (AT-160, AlfaKit, Brazil). Salinity (AZ86031), pH (Asko AK90, Brazil), and settleable solids (Imhoff cone) were measured three times per week (Avnimelech, 2012). Additionally, we used an Alfakit AT 10P II Photocolorimeter to measure 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) every 10 days.

Bacterial challenge

For the bacterial challenge, a lyophilized strain of *V. parahaemolyticus* (ATCC 17802) was obtained from the Fundação Oswaldo Cruz (FIOCRUZ), and activated and cultivated in Tryptone Soy Broth (TSB) supplemented with 2% sodium chloride for 24 hours at 30 ºC to obtain the infectious material. Then, the culture was centrifuged at 2,200 x*g* for 5 minutes, with the supernatant discarded and the pellets washed twice and finally resuspended in sterile 2% saline solution. The concentration of 10^8 CFU mL^{-1} was determined

using the 0.5 McFarland standard as reference. From this material, the culture was diluted to 10^6 CFU mL^{-1}, the concentration that was used in the bacterial challenge.

The bacterial challenge was conducted after 60 days of feeding experimental diets. Shrimp averaging 9 g were immersed in a *V. parahaemolyticus* suspension (10^8 CFU mL^{-1}) for 15 minutes. Subsequently, they were transferred to 40 L tanks of clean, chlorinated and dechlorinated water in a recirculating system at a density of 100 shrimp m^{-2}. Additional infectious material was added to reach a final concentration of 10^6 CFU mL^{-1} of *V. parahaemolyticus* (Tran et al., 2013).

The 30-day challenge maintained identical water quality management practices as the preceding phase, except for the measurement of settleable solids, as the water was visibly clear. Shrimp were fed the experimental diets three times daily (8 am, 12 pm, and 4 pm), with quantities adjusted based on Van Wyk's (1999) methodology. The challenge employed a completely randomized design, with four replicates per treatment. Daily monitoring of the shrimp occurred to record mortality and clinical signs, and to assess hemato-immunological, histological, and bacteriological parameters.

Shrimp performance

To evaluate shrimp performance, biometrics were conducted every 10 days during the period of feeding with experimental diets. to determine: 1- relative weight gain (%WG = 100 × [final weight (g) − initial weight (g) / initial weight (g)]), 2- biomass increase (%BI = 100 × [(final biomass (kg) − initial biomass (kg)] / initial biomass (kg)]), 3- mortality rate (Mortality % = (initial number of shrimp - final number of shrimp / initial number of shrimp) × 100), and 4- average growth (AG), and feed conversion rate (FCR) = (Consumed feed (g))/ (Biomass gain (g)). All animals were collected and weighted. These variables were not evaluated during the bacterial challenge period for biosafety reasons.

Hemato-immunological parameters

Hemato-immunological analysis was performed both before and during the experimental challenge, at day 1, day 3, and every 5 days after inoculation of the infectious material into the water of the experimental units. One shrimp from each experimental unit was sampled at each time interval to collect hemolymph for determining the total and differential hemocyte count, following the protocol outlined by Guertler et al. (2013). Hemolymph was extracted from the hemocoel of the first abdominal segment using a 1 mL syringe pre-filled with Alsever MAS (Modified Alsever Solution) anticoagulant (336 mmol/L NaCl, 115 mmol/L glucose, 27 mmol/L sodium citrate, 9 mmol/L EDTA, pH 7.2), in a 1:2 (v:v) ratio. A portion of the hemolymph was separated and preserved in MAS with 4% formaldehyde (1:3).

The total hemocyte count (THC) was conducted using 100 µL of the anticoagulant-treated hemolymph, and 0.1 µL of this mixture was placed in a Neubauer chamber for cell counting (Beçak & Paulete, 1976). Hemocyte counts were determined in duplicate using an optical microscope.

The differential hemocyte count (DHC) was determined using a modified method based on Celi et al. (2013). This involved spreading 5 µL of hemocyte cell suspension, fixed in 4% formaldehyde and air-dried, onto a slide. The slide was then sequentially immersed in absolute methanol (6 minutes), Giemsa solution (1:10 dilution, 10 minutes), 70% ethanol (1 minute), and absolute xylene (6 minutes). The relative percentages of different hemocyte types were then determined through triplicate observations of the slide using optical microscopy.

Histopathological analysis

For histopathological analysis of the digestive gland morphology and lesions suggestive of *V. parahaemolyticus* infection, 15 shrimps from each treatment were collected after the bacterial challenge. The shrimp were preserved by injecting Davidson's solution (AFA's), where they remained for 70 hours. After fixation, the samples were transferred to a 70% ethanol solution for further processing into histological slides. The slides were stained with hematoxylin and eosin (HE), following the method described by Lightner (1996). All slides were examined under an optical microscope, and the lesions were assessed according to the criteria outlined by Caro et al. (2020).

Histological changes in the digestive gland were assessed through semiquantitative analysis by calculating the Histological Alteration Index (HAI). The damage was categorized into three progressive stages:

stage I (no impact on organ function), stage II (more severe damage with impaired organ function), and stage III (very severe and irreversible structural and functional changes in the organ) (Fregoso-López et al., 2017).

The HAI value was determined using the formula: HAI = [1 × Σ (stage I)] + [10 × Σ (stage II)] + [100 × Σ (stage III)]. The mean HAI was classified into five categories: 0–10 (normal tissue function), 11–20 (mild to moderate changes), 21–50 (moderate to severe changes), 51–100 (severe changes), and >100 (irreparable changes) (Poleksic & Mitrovic-Tutundzic, 1994).

Microbiological analysis

To estimate the total number of *Vibrio* sp. in the digestive gland of challenged shrimps, samples were taken before inoculation and every 5 days thereafter. At each 5-day interval, one shrimp per experimental unit was euthanized by severing the nerve cord. The external surface of each shrimp was then disinfected by sequential immersion in 70% ethanol (15 seconds), 1.5% sodium hypochlorite solution with 0.1% tween-80 (15 minutes), and finally rinsed three times in sterile distilled water.

Fifty milligrams of the digestive gland tissue were macerated and diluted in 4.5 mL of buffered peptone water. The homogenized product was subjected to successive serial dilutions from 10^{-2} to 10^{-4} and seeded (100 µL) on Thiosulfate Citrate Bile Sucrose Agar (TCBS) (Kobayashi et al., 1963), in triplicate. The plates were incubated at 30 °C for 24 hours to subsequently count the number of colony-forming units (CFU g^{-1}), using the colony counter.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics version 27.0.0.0. The data were tested for homogeneity of variance using the Levene test ($p \leq 0.05$) and for normality using the Shapiro-Wilk test ($p \leq 0.05$). Data with normal and homogeneous distributions were analyzed using one-way ANOVA (for shrimp performance and HAI) and repeated measures ANOVA (for temperature, salinity, dissolved oxygen, pH, alkalinity, and TAN (N-NH_3), followed by the Tukey test (HSD, honest significant difference) to compare mean differences ($p \leq 0.05$). For the total and differential hemocyte count and bacterial count in the TCBS, non-parametric data, the Kruskal-Wallis test ($p \leq 0.05$) was used with the Bonferroni correction (Pairwise Comparison) (Zar, 2013).

Results

Water quality

The variables were maintained within the physiological limits for *P. vannamei* according to Van Wyk & Scarpa (1999) (data not shown). The average values of water quality parameters recorded during the bacterial challenge period are shown in Table 2, with no significant differences ($p \leq 0.05$). To measure these parameters, a water sample was taken from the system to be measured, with no cross-contamination due to the use of the probe.

Table 2. Water quality parameters monitored in *Penaeus vannamei* culture during the 30-day challenge with *Vibrio parahaemolyticus* following dietary nucleotide supplementation.

Variables	Treatments			
	C	NT75	NT150	NT300
Temperature (°C)	28.65 ± 0.64[a]	28.69 ± 0.66[a]	28.68 ± 0.58[a]	28.53 ± 0.64[a]
Salinity (ppt)	30.91 ± 0.56[a]	30.61 ± 0.37[a]	30.66 ± 0.43[a]	30.82 ± 0.47[a]
Dissolved oxygen (mg L^{-1})	8.50 ± 2.87[a]	8.41 ± 2.97[a]	8.06 ± 2.95[a]	7.84 ± 2.92[a]
pH	7.24 ± 0.26[a]	7.37 ± 0.23[a]	7.34 ± 0.30[a]	7.38 ± 0.27[a]
Alkalinity (mg $CaCO_3$ L^{-1})	102.50 ± 86.30[a]	97.50 ± 70.80[a]	102.50 ± 72.40[a]	85.00 ± 55.80[a]
N-NH_3 (mg L^{-1})	0.12 ± 0.16[a]	0.07 ± 0.08[a]	0.07 ± 0.06[a]	0.10 ± 0.07[a]

Note: Mean ± standard deviation values, C (control), NT75 (75 mg/kg), NT150 (150 mg/kg), and NT300 (300 mg/kg).

Shrimp performance

The average values for weight gain (WG), biomass increase (BI), mortality rate, average growth (AG), and feed conversion ratio (FCR) of shrimp fed the experimental diet for 60 days are presented in Table 3. Significant differences between treatments were observed for WG, BI, and AG, with higher values recorded in the nucleotide-supplemented groups. Conversely, FCR was lower in all nucleotide treatments compared to the control. No significant differences in mortality rate were found among the treatments (Table 3).

Table 3. Shrimp performance evaluated during the period of feeding with experimental diets.

Variables	Treatments			
	C	NT75	NT150	NT300
WG (%)	362.41 ± 16.08^{b}	391.91 ± 9.03^{ab}	403.14 ± 6.91^{a}	419.62 ± 12.58^{a}
BI (%)	312.97 ± 10.96^{c}	360.79 ± 11.17^{b}	367.88 ± 4.50^{b}	398.71 ± 6.48^{a}
Mortality rate (%)	10.67 ± 1.53^{a}	6.33 ± 0.58^{ab}	7.00 ± 1.00^{ab}	4.00 ± 1.73^{b}
AG (g)	1.07 ± 0.05^{b}	1.18 ± 0.03^{a}	1.21 ± 0.02^{a}	1.22 ± 0.04^{a}
FCR	1.95±0.07^{a}	1.79±0.06^{b}	1.77±0.01^{b}	1.74±0.02^{b}

Note: WG= weight gain; BI = biomass increase; AG = average growth every 10 days. N=100 animals per unit with three replicas per group; Mean ± standard deviation values; Different letters on the same line express a significant difference ($p < 0.05$); C (control), NT75 (75 mg/kg), NT150 (150 mg/kg), and NT300 (300 mg/kg).

Hemato-immunological parameters

The total hemocyte counts (THC) measured before and during the bacterial challenge are detailed in Table 4. In both the control, without nucleotide supplementation, and the treatments with supplementation, there was a decrease in THC after 1 day of exposure to *V. parahaemolyticus*. Significant differences between treatments were found only on day 1 between in the control and NT75 and on day 3 between treatments NT75 and NT150 after the start of the challenge ($p \leq 0.05$). The control had the highest THC before the start of the experimental infection, but on day 1 of the infection, the average count was 0.88×10^{-6} cells mL^{-1}, the lowest among the treatments ($p \leq 0.05$).

Table 4. Total hemocyte count (THC) (10^{6} cells mL^{-1}) and differential count (DHC) (in percentage) of *Penaeus vannamei* challenged with *Vibrio parahaemolyticus*.

Day	Variables		Treatments			
			C	NT75	NT150	NT300
0	THC		6.68 ± 3.17^{a}	5.69 ± 2.97^{a}	5.72 ± 3.73^{a}	5.73 ± 3.18^{a}
	DHC	H	56.54 ± 27.26^{a}	71.27 ± 23.33^{a}	68.81 ± 21.34^{a}	68.82 ± 27.44^{a}
		SG	39.84 ± 26.69^{a}	25.48 ± 24.25^{a}	28.54 ± 21.41^{a}	28.41 ± 28.38^{a}
		G	3.61 ± 2.48^{a}	3.25 ± 2.73^{a}	2.65 ± 1.34^{a}	1.33 ± 0.92^{b}
1	THC		0.88 ± 0.44^{b}	2.30 ± 1.19^{a}	1.15 ± 0.77^{ab}	1.90 ± 1.30^{ab}
	DHC	H	59.40 ± 40.34^{a}	48.16 ± 34.68^{a}	48.27 ± 41.56^{a}	81.44 ± 29.10^{a}
		SG	39.73 ±40.53^{a}	50.36 ± 34.75^{a}	50.88 ± 41.96^{a}	17.54 ± 29.49^{a}
		G	0.87 ± 1.06^{a}	1.47 ± 1.12^{a}	0.85 ± 1.78^{a}	1.02 ± 0.63^{a}
3	THC		1.90 ± 1.28^{a}	3.09 ± 2.13^{a}	2.94 ± 2.25^{a}	1.28 ± 0.90^{a}
	DHC	H	90.57 ± 13.13^{a}	94.87 ± 4.80^{a}	94.13 ± 6.35^{a}	85.20 ± 10.59^{a}
		SG	7.83 ± 12.14^{a}	3.94 ± 5.06^{a}	3.77 ± 4.91^{a}	12.69 ± 10.63^{a}
		G	1.60 ± 2.96^{a}	1.19 ± 1.62^{a}	2.10 ± 2.32^{a}	2.10 ± 1.86^{a}

Day	Variables		Treatments			
			C	NT75	NT150	NT300
5	THC		1.23 ± 0.66^{ab}	2.21 ± 0.44^{a}	1.08 ± 0.75^{b}	1.98 ± 0.74^{ab}
	DHC	H	97.32 ± 2.86^{a}	78.51 ± 41.39^{a}	69.81 ± 44.94^{a}	89.45 ± 28.24^{a}
		SG	1.07 ± 2.45^{a}	0.06 ± 0.12^{a}	0.23 ± 0.59^{a}	0.28 ± 0.91^{a}
		G	1.61 ± 1.13^{a}	1.43 ± 1.16^{a}	2.69 ± 3.21^{a}	1.94 ± 1.73^{a}
10	THC		2.27 ± 1.14^{a}	3.44 ± 2.95^{a}	2.49 ± 2.82^{a}	1.56 ± 0.49^{a}
	DHC	H	97.69 ± 1.58^{a}	97.52 ± 1.11^{a}	95.50 ± 2.86^{a}	94.73 ± 5.75^{a}
		SG	0.62 ± 1.40^{a}	0.17 ± 0.38^{a}	1.75 ± 1.97^{a}	3.28 ± 6.09^{a}
		G	1.68 ± 1.42^{a}	2.31 ± 0.80^{a}	2.75 ± 2.14^{a}	2.00 ± 1.17^{a}
15	THC		3.95 ± 4.21^{a}	*	1.43 ± 0.94^{a}	2.99 ± 3.11^{a}
	DHC	H	98.41 ± 1.83^{a}	*	96.25 ± 4.74^{a}	98.17 ± 1.48^{a}
		SG	0.58 ± 1.12^{a}	*	0.00 ± 0.00^{a}	0.27 ± 0.63^{a}
		G	1.01 ± 0.93^{a}	*	3.75 ± 4.74^{a}	1.55 ± 1.59^{a}
20	THC		3.29 ± 2.12^{a}	2.30± 1.30^{a}	1.27± 0.69^{a}	2.34± 1.47^{a}
	DHC	H	97.54 ± 1.76^{a}	97.65 ± 1.38^{a}	89.50 ± 18.20^{a}	94.39 ± 10.32^{a}
		SG	0.27 ± 0.54^{a}	0.28 ± 0.61^{a}	9.46 ± 17.69^{a}	3.27 ± 9.02^{a}
		G	2.20 ± 1.74^{a}	2.07 ± 1.33^{a}	1.05 ± 0.97^{a}	2.34 ± 1.94^{a}
25	THC		2.80± 4.08^{a}	2.89± 2.41^{a}	1.96± 1.61^{a}	1.95± 1.36^{a}
	DHC	H	93.72 ± 10.77^{a}	92.49 ± 20.53^{a}	98.31 ± 1.97^{a}	96.63 ± 2.59^{a}
		SG	1.55 ± 5.37^{a}	6.40 ± 20.87^{a}	0.02 ± 0.08^{a}	0.83 ± 1.98^{a}
		G	4.73 ± 5.68^{a}	1.11 ± 0.98^{a}	1.66 ± 1.97^{a}	2.54 ± 1.83^{a}
30	THC		133± 0.78^{a}	2.45± 4.12^{a}	2.83± 2.30^{a}	2.39± 0.87^{a}
	DHC	H	95.07 ± 11.26^{a}	95.24 ± 8.18^{a}	97.45 ± 2.96^{a}	90.24 ± 13.77^{a}
		SG	3.14 ± 10.43^{a}	2.77 ± 7.32^{a}	0.26 ± 0.74^{a}	7.56 ± 14.79^{a}
		G	1.79 ± 1.73^{a}	1.99 ± 2.32^{a}	2.29 ± 2.37^{a}	2.21 ± 1.84^{a}

Note: *non-viable samples, H = hyaline, SG = semi-granular, G = granular; Mean ± standard deviation values; N= 3 animals per experimental unit (3 replicas) in each sampled time; Different letters on the same line express a significant difference ($p < 0.05$); C (control), NT75 (75 mg/kg), NT150 (150 mg/kg), and NT300 (300 mg/kg).

The percentages of hyaline (H), semi-granular (SG) and granular (G) cells are described in Table 4. In the differential hemocyte count (DHC), significant differences were found between treatments and the control group only before the challenge. After the bacterial challenge, the means between treatments were similar ($p \leq 0.05$). In all groups, the highest prevalence throughout the experimental period was of hyaline cells.

The percentage of hyaline hemocytes did not differ significantly between treatments ($p \leq 0.05$). However, some notable trends emerged. Treatments NT75 and NT150 showed reductions in hyaline hemocyte percentages after 1 and 5 days, respectively. In contrast, the control (C) group initially saw an increase in hyaline hemocytes during the same period, with a decrease only observed on day 25. Interestingly, NT300 exhibited the highest initial increase in hyaline cells, and this elevated average was sustained throughout the experimental period. The percentage of semi-granular cells also did not show a significant difference between treatments ($p \leq 0.05$). The incidence of this cell type was higher up to day 3 in all treatments after infection by *V. parahaemolyticus*. The granular cells were present in low proportions in relation to other cell types throughout the experimental period, with no significant difference after the beginning of the experimental challenge ($p \leq 0.05$).

Cumulative mortality and histopathological analysis

Histopathological analysis of the digestive gland of shrimp challenged with *V. parahaemolyticus* revealed histological changes in the three stages according to the histological alteration index (HAI) (Table 5). The stages indicated histological changes, and the rate of irreparable changes was higher in the C group (60%) than the other treatments. Amo the treatments, the highest rate of moderate to severe changes was observed in the NT300 treatment (85.7%). Regarding HAI, there was no significant difference between the treatments ($p \leq 0.05$).

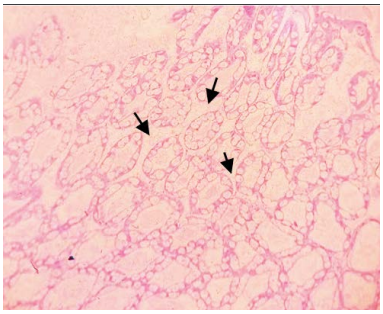
Table 5. Percentage values of the Histopathological Alteration Index (HAI) of the digestive gland of *Penaeus vannamei* subjected to challenge with *Vibrio parahaemolyticus*.

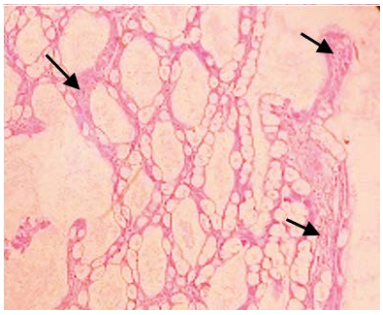
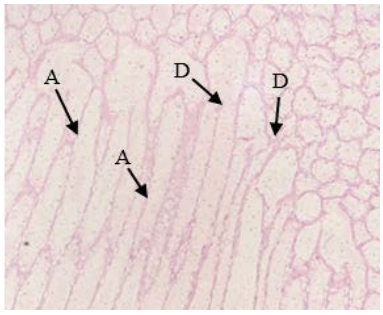
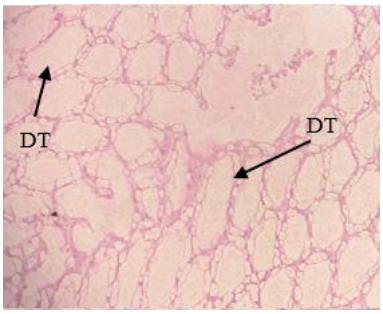
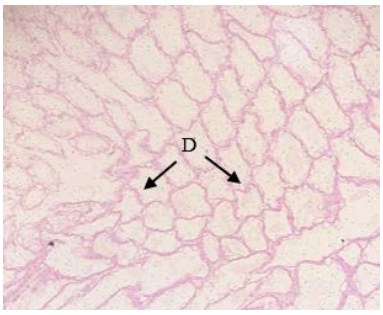
Stages	Categories	Treatment			
		C	NT75	NT150	NT300
I	Normal tissue functioning	0.0%	0.0%	0.0%	0.0%
II	Mild to moderate change	0.0%	0.0%	6.7%	0.0%
	Moderate to severe change	40.0%	60.0%	60.0%	85.7%
	Serious change	0.0%	0.0%	0.0%	0.0%
III	Irreparable change	60.0%	40.0%	33.3%	14.3%
HAI		80.00 ± 47.35[a]	64.87 ± 44.37[a]	64.20 ± 44.22[a]	47.43 ± 36.08[a]

Note: Stage I (normal tissue functioning), Stage II (mild to moderate change, moderate to severe change and severe change), Stage III (irreparable change). The average HAI was classified into five categories: 0–10 (normal tissue function), 11–20 (mild to moderate changes), 21–50 (moderate to severe changes), 51–100 (severe changes), and >100 (irreparable alterations); C (control), NT75 (75 mg/kg), NT150 (150 mg/kg), and NT300 (300 mg/kg).

The histological findings are described in Table 6. All groups showed hemocytic infiltrations in different proportions and in different regions of the digestive gland. Control and NT150 presented, in most of the tissues analyzed, discrete peripheral infiltrations and a higher incidence of granular hemocytes. NT75 showed, for the most part, a moderate infiltration, spread throughout the the digestive gland, and with a predominance of granular hemocytes. NT300 presented an infiltration similar to the C and NT150 treatments, differing only by presenting a lower quantity of hyaline hemocytes than the other treatments. Cumulative mortality throughout the bacterial challenge did not show significant differences between treatments, varying between 29% and 33%. The highest mortality peak occurred on the 5th day in all treatments (Table 7).

Table 6. Classification of changes found in the digestive gland of *Penaeus vannamei* examined at the end of the challenge with *Vibrio parahaemolyticus*.

Change	Classification	
Edema Stage I	Intense (> 2/3 of the field) Moderate (1/3–2/3 of the field) Discrete (< 1/2 of the field)	

Change	Classification	
Hemocytic infiltration Stage I	Intense (> 40% of the field) Moderate (10–40% of the field) Discreet (< 10% of the field)	
Deformation (D)/ Stretching (A)/ Tubule dilation (DT) Stage II	Intense (> 2/3 of the field) Moderate (1/3–2/3 of the field) Discrete (< 1/2 of the field)	  

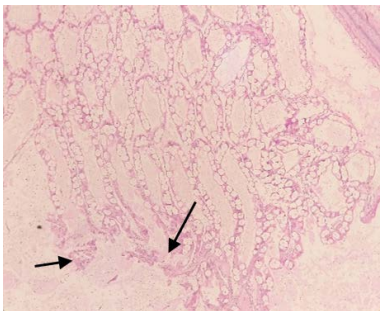
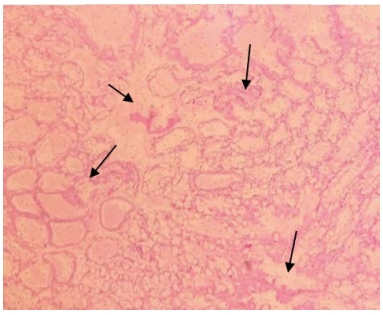
Change	Classification	
Necrosis	Intense (> 2/3 of the field)	
Stage II (Discrete)	Moderate (1/3–2/3 of the field)	
Stage III	Discreet (< 1/2 of the field)	

Table 7. Cumulative mortality (%) of *Penaeus vannamei* throughout bacterial challenge with *Vibrio parahaemolyticus*.

Variables		Treatments			
		C	NT75	NT150	NT300
Initial number of animals		88	88	88	88
		Cumulative mortality (%)			
Cumulative mortality	Day 1	0	0	0	0
	Day 5	17	20	17	16
	Day 10	19	25	22	22
	Day 15	36	33	34	31
	Day 20	42	41	47	39
	Day 25	48	45	53	43
	Day 30	57	53	59	55
Averages		31.29 ± 20.05[a]	31.29 ± 20.05[a]	33.14 ± 21.37[a]	29.43 ± 18.41[a]

Note: Mean ± standard deviation values; N=100 animals per unit with three replicas per group; Different letters on the same line express a significant difference ($p < 0.05$); C (control), NT75 (75 mg/kg), NT150 (150 mg/kg), and NT300 (300 mg/kg).

Microbiological analysis

Table 8 details the total presumptive mean counts of *Vibrio* sp. on TCBS agar in *P. vannamei* digestive gland samples during the experimental challenge. Significant differences ($p \leq 0.05$) between groups were only noted on days 5 and 15. On day 5, the average count in the Control was significantly higher than in the NT75 treatment ($p \leq 0.05$), and the count in NT75 was significantly lower than in NT300. The counts on day 5 ranged from 0 to 2.00×10^5 CFU g^{-1} in C, 0 to 5.00×10^7 CFU g^{-1} in NT300, and were 0 CFU g^{-1} in NT75.

In the third sampling, NT150 treatment showed a significantly higher average count than the Control ($p \leq 0.05$). Both Control and NT150 had counts of 0 CFU g^{-1}, with a range of 0 to 3.00×10^8 CFU g^{-1} for NT150.

No significant differences ($p \leq 0.05$) were observed between treatments in the second (day 10) and fourth (day 20) collections. By the fifth (day 25) and sixth (day 30) sampling, all treatments registered counts of 0 CFU g^{-1}.

Table 8. Total bacterial count (10^6 cells L^{-1}) and presumptive *Vibrio* per treatment (percentage of sucrose) in TCBS in *Penaeus vannamei* (the digestive gland) throughout the bacterial challenge.

Days	Treatments				
	C	NT75	NT150	NT150	NT300
0	1.01 ± 0.90[a]	1.96 ± 1.79[a]	1.93 ± 1.41[a]	1.93 ± 1.41[a]	1.05 ± 1.00[a]
5	0.07 ± 0.08[a]	0.00 ± 0.00 [b]	0.28 ± 0.60[ab]	0.28 ± 0.60[ab]	6.78 ± 14.80[a]
10	60.89 ± 15.21[a]	6.3 ± 15.21[a]	12.61 ± 28.19[a]	12.61 ± 28.19[a]	1.77 ± 3.69[a]
15	0.00 ± 0.00[b]	0.68 ± 1.40[ab]	31.16 ± 91.62[a]	31.16 ± 91.62[a]	0.57 ± 1.30[ab]
20	0.05 ± 0.17[a]	0.24 ± 0.90[a]	0.23 ± 0.68[a]	0.23 ± 0.68[a]	0.00 ± 0.02[a]
25	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
30	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	Sucrose percentage				
Positive	100[a]	100[a]		95.04[a]	100[a]
Negative	0[a]	0[a]		4.96[a]	0[a]

Note: Mean ± standard deviation values; N= 3 animals per experimental unit (3 replicas) in each sampled time; Different letters on the same line express a significant difference ($p < 0.05$); C (control), NT75 (75 mg/kg), NT150 (150 mg/kg), and NT300 (300 mg/kg).

Discussion

Our findings indicate that nucleotide supplementation significantly improved shrimp performance, particularly in the NT150 and NT300 treatments compared to the control (C) group, suggesting a positive effect on biomass increase with nucleotide inclusions of 150 and 300 mg per kg of feed. This observation aligns with previous studies that highlight the potential benefits of nucleotide supplementation for promoting growth in shrimp (Noviadri et al., 2021; He et al., 2023).

Nucleotides are essential molecules for the synthesis of DNA and RNA in shrimp, influencing processes such as cell replication and tissue regeneration. They also play crucial roles in genetic coding, metabolic function and cell signaling. Also, they have an impact on immunity, resistance to infections, growth, and intestinal and liver function, as well as protection against stress and DNA damage. This suggests their potential for use in therapeutic and nutritional applications (Ding et al., 2021). Its presence on the digestive tract, favor the absorption of nutrients and increase resistance to disease (Javahery et al., 2019).

This broad spectrum of benefits, particularly their role in immunity, is further supported by our findings regarding mortality rates. Supplementation with nucleotides provided immunological support, as evidenced by the significantly lower mortality rate in the NT300 treatment (4%) compared to the control (C) treatment (10%) during the experimental diet period. This difference suggests that nucleotide supplementation is associated with greater resistance to stress factors, resulting in reduced mortality (Novriadi et al., 2021).

One of the mechanisms of *P. vannamei* immune response is associated with hemocytes (THC), which are responsible for encapsulation, coagulation, melanization, and phagocytosis, acting directly on the infectious site and combating pathogenic organisms (Lin & Söderhäll, 2011; Söderhäll et al., 2016; Lu et al., 2021). In this study, both shrimp from the control treatment (C) without nucleotide supplementation and those from nucleotide supplemented treatments showed a decrease in total hemocytes count (THC) after 24 hours of exposure to *V. parahaemolyticus*. This initial decrease may indicate an acute immune response to the bacterial challenge, characterized by the mobilization of hemocytes against the infection.

Significant differences in THC were only detected at two specific time points: after 24 hours between treatments C and NT75, and after 5 days between treatments NT75 and NT150. These variations may suggest a differential response of shrimp to supplementation with different doses of nucleotides, promoting greater synthesis and release of hemocytes by hematopoietic tissue, suggesting the effectiveness of the immune response over time (Lin & Söderhäll, 2011).

Due to the immunostimulant effect of nucleotide supplementation, an increase in THC is expected when comparing shrimp with and without supplementation, however, in this study, there were no significant differences between the supplemented groups and the control group. This differs from the study by Abdel-Rahim et al. (2021), in which 0.5 g kg^{-1} of nucleotide associated with a macroalgae was used and there was a significant increase in THC, suggesting an enhancement of the nucleotide's immunological action.

Hemocytes circulating in the hemolymph have distinct shapes and functions, and influence both the innate immune system and physiological functions. Hyaline hemocytes carry out phagocytosis, semi-granular ones promote encapsulation and cytotoxicity, as well as storage and release in the proPO (prophenoloxidase) system, and lastly granular hemocytes act on melanization, antimicrobial peptides and cytotoxicity, as the main defense against pathogens (Lin & Söderhäll, 2011; Suleman et al., 2019; Vogt, 2022).

Infectious events require many hemocytes, mainly granular and semi-granular, which initiate a complex defense action, in which, through degranulation, they stimulate phagocytosis by hyaline cells (Johansson et al., 2000). This degranulation results in cell death and a consequent temporary decrease in hemocytes provided with granules circulating in the hemolymph (Liu et al., 2021), which are quickly restored by synthesis and released by the hematopoietic tissue (Lin & Söderhäll, 2011). Therefore, a greater prevalence of hyaline cells is expected in an infectious condition, corroborating the results of this study, in which there was a predominance of hyaline cells.

Although there are no significant differences between the average DHC in the treatments, the effect of supplementation with immunostimulants on the semi-granular and granular cell counts of the supplemented shrimp can be observed. According to Javahery et al. (2019) supplementation led to better levels of granular and semi-granular cells, and in a study by Mulyadi et al. (2020), where hyaline and semi-granular cells were dominant, immunostimulant supplementation also influenced granular cells.

Vibrio infection can damage the digestive gland and other tissues and organs, limiting the functions of metabolism, transport, and catabolism. The immune system requires high amounts of energy to fight infectious agents. This energy is contained in lipid reserves present in the digestive gland (Jarc & Petan, 2019; Yin et al., 2022). Given that immunostimulants promote nutrient absorption, we would expect supplemented shrimp to exhibit higher lipid storage and greater resistance to pathogens, unlike the non-supplemented group. However, in this study, low lipid storage, tubule deformations and necrosis were observed in all treatments. Rairat et al. (2022) also observed signs of atrophy and low lipid storage in the positive control group and the group with low nucleotide inclusion (0.25 g kg^{-1}). This necessitates further research into the effect of nucleotide supplementation on the digestive gland.

Analysis of total presumptive mean counts of *Vibrio* sp. in *P. vannamei* the digestive gland samples during the experimental challenge provides important insights into the dynamics of bacterial colonization in this organ and its relationship with the different treatments applied. Supplementation with nucleotides in treatments other than C effectively limited *V. parahaemolyticus* growth after inoculation. This was evident in the significantly higher bacterial counts observed in the Control at various time points, an effect consistent with previous research (Rairat et al., 2022).

Studies on the immune response of shrimp challenged with *Vibrio* spp. evaluated the infection through injection challenge, although there is a lack of studies that evaluate the immune response of shrimp infected with *Vibrio* spp. by immersion (Segarra et al., 2023; Licona-Jain et al., 2022). Therefore, the assessment made by this study is of great importance as it resembles the form of contagion in shrimp farms, and a scenario closer to reality.

Nucleotide supplementation at 300 mg/kg of feed enhanced shrimp biomass gain and lowered pre-challenge mortality. While an acute immune response was observed post-challenge, no significant differences were found between supplemented and unsupplemented groups. Histological examination indicated moderate to severe hepatopancreatic alterations across all groups, highlighting the necessity for further research into the impact of supplementation on this tissue, the mechanisms driving the immune response, and the overall efficacy of nucleotide supplementation.

Data availability: Data will be available on reasonable request.

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