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Dietary Folic Acid Modulates Growth Performance, Expression of Antioxidant- and Immune-Related Genes, and Response to Acute Hepatopancreatic Necrosis Disease in Pacific White Shrimp (*Penaeus vannamei*)

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Abstract

This study evaluated the effects of dietary folic acid supplementation on growth performance, antioxidant- and immune-related gene expression, and the response to acute hepatopancreatic necrosis disease (AHPND) in Pacific white shrimp (*Penaeus vannamei*). Juveniles (12 shrimp per tank; four replicate tanks per treatment) were fed diets containing nominal supplemental folic acid levels of 0, 2, 5, or 10 mg kg^{-1} for 35 days, and 30 shrimp per dietary treatment (three replicate aquaria; 10 shrimp per aquarium) were subsequently challenged with an AHPND-causing strain of *Vibrio parahaemolyticus*. Among the supplementation levels evaluated, the 5 mg kg^{-1} diet produced the highest final weight (14.8 ± 0.5 g), weight gain (7.9 ± 0.3 g), a specific growth rate (2.14 ± 0.1% day^{-1}), and the lowest apparent feed conversion ratio (1.3 ± 0.1), whereas the Control showed values of 12.3 ± 0.7 g, 5.4 ± 0.3 g, 1.6 ± 0.1% day^{-1}, and 1.6 ± 0.1, respectively ($p \leq 0.05$). Dietary folic acid modulated the relative expression of antioxidant-related genes (*SOD*, *CAT*, and *GPX*) in the gills and hepatopancreas and altered basal and post-challenge expression of the immune-related genes *ALFP*, *CTL3*, *GILT*, *MNK*, and *SR* in the gills. At 72 h post-challenge, final cumulative mortality differed among treatments ($F_{3,8} = 5.405$, $p = 0.025$), with lower mortality in T5 (36.7 ± 7.6%) than in the Control (53.3 ± 2.9%; Tukey, $p = 0.022$). These findings indicate that dietary folic acid supplementation influenced growth performance, challenge outcomes, and transcriptional responses in juvenile *Penaeus vannamei*. Among the supplementation levels evaluated, the 5 mg kg^{-1} diet consistently produced the most favorable combination of growth performance, lower final challenge mortality, and modulation of antioxidant- and immune-related gene expression.

Keywords: AHPND; functional feed additive; innate immunity; oxidative stress; bacterial challenge; transcriptional response

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1. Introduction

Aquaculture has gained significant importance over the past few decades due to the ever-increasing global demand for food [1]; in fact, most crustacean production comes from aquaculture [2]. Among all farmed species, the Pacific white shrimp, *Penaeus vannamei*, stands out for its growth, environmental adaptability, and nutritional profile [3,4].

However, an increase in the number of cultured organisms is generally accompanied by an increase in the incidence and prevalence of diseases [5]. For example, acute hepatopancreatic necrosis disease (AHPND) is one of the most common bacterial diseases, responsible for a large number of problems at aquaculture farms worldwide [6]. It is important to emphasize that this disease is caused by various strains of the genus *Vibrio*, and even today it has a significant socioeconomic impact on shrimp farming, as it causes lethargy, slow growth, hepatopancreas atrophy, and high mortality rates in the early days of cultivation (70–100%), making it one of the main health constraints in shrimp farming [7,8].

To prevent or mitigate the adverse effects caused by this disease, producers have used antibiotics indiscriminately; however, the excessive use of these drugs has led to the emergence of resistant bacterial strains, which has even affected the microbial communities present in aquaculture systems [9,10]. In response to this challenge, the use of feed additives has been proposed to enhance the immune response while improving aquaculture production; these additives can regulate the physiological functions of white shrimp [11,12].

Vitamins play an important role in promoting growth and maintaining overall health in crustaceans; however, the optimal requirements for many of these vitamins remain unclear today. Within this group of substances, folic acid plays a fundamental role in cellular metabolism, actively participating in the synthesis of proteins and nucleic acids [13,14].

Folate is also a central component of one-carbon metabolism, which integrates the folate and methionine cycles and provides one-carbon units for nucleotide synthesis and methyl-group transfer. Through the methionine cycle, folate availability contributes to the generation of S-adenosylmethionine (SAM), a major methyl donor involved in DNA and histone methylation and, consequently, in epigenetic regulation of gene expression [13,15]. One-carbon metabolism is also linked to cellular redox homeostasis and immune regulation, providing a plausible mechanistic connection between dietary folate availability and transcriptional responses [15]. However, these mechanisms remain poorly characterized in crustaceans.

Dietary requirements for folic acid have been established for various species of interest to aquaculture; research has covered a wide range of aquatic organisms, including freshwater species such as tilapia (*Oreochromis niloticus* × *O. aureus*) [16], the green catfish *(Mystus nemurus*) [17], as well as marine species such as grouper (*Epinephelus malabaricus*) [18], tiger shrimp (*Penaeus monodon*) [19], giant freshwater prawn (*Macrobrachium rosenbergii*) [20], and red swamp crayfish (*Procambarus clarkii*) [21].

In addition to its role in the metabolic processes of various crustacean species, evidence suggests that its inclusion in the diet is associated with the expression of genes related to the antioxidant and immune systems of the Chinese mitten crab, *Eriocheir sinensis* [22], the giant freshwater prawn *M. rosenbergii* [20], and the Oriental river prawn, *Macrobrachium nipponense* [23]. However, little is known about the optimal folic acid requirements for enhancing the immune and antioxidant systems in crustaceans compared to mammals and fish.

Crustaceans rely on an innate immune system, characterized by structural barriers that prevent the entry and spread of pathogens. This defense mechanism comprises two main interconnected components: the humoral and cellular systems, which are synergistically activated in response to the presence of a pathogen [24].

At the molecular level, the crustacean response to AHPND is mediated by the coordinated activation of various gene groups within the humoral system. Among the most relevant are interferon-gamma-inducible lysosomal thiol reductase (*GILT*), anti-lipopolysaccharide factor (*ALF*), MAP serine kinase (*MNK*), scavenger receptors (*SR*), and C-type lectin 3 (*CTL3*), selected based on transcriptomic data, which showed that they are significantly upregulated by *V. parahaemolyticus* infection; the characterization of these genes has provided insight into how organisms recognize pathogen-associated molecular patterns (PAMPs) and how they activate immune signaling pathways to limit the spread of infectious agents [25].

Given the limited information available on the effects of dietary folic acid in *P. vannamei*, this study evaluated the effects of folic acid supplementation on growth performance, survival, and the transcriptional responses of antioxidant- and immune-related genes. However, whether dietary folic acid modulates these transcriptional pathways in *P. vannamei* and whether such changes are associated with the response to AHPND remain largely unknown. We hypothesized that dietary folic acid supplementation would improve growth performance and modulate the expression of antioxidant-related genes (*SOD*, *CAT*, and *GPX*) and *V. parahaemolyticus*-responsive immune-related genes (*ALFP*, *CTL3*, *GILT*, *MNK*, and *SR*), potentially influencing the response of juvenile shrimp to a controlled challenge with *V. parahaemolyticus*.

2. Materials and Methods

2.1. Ethics Statement and Handling of Organisms

Throughout the experiment, animal welfare was ensured, and scientific procedures involving organisms were conducted under appropriate and ethical conditions in accordance with the protocols of the Official Mexican Standard (NOM-062-ZOO-1999).

2.2. Experimental Organisms

A total of 200 juvenile *P. vannamei* (6.89 ± 0.2 g) were obtained from a commercial aquaculture farm located in Guasave, Sinaloa, Mexico (25°27′47.2″ N, 108°42′00.8″ W). After an inspection to rule out malformations or signs of disease, the specimens were transported in containers filled with seawater and under constant aeration to the IPN-CIIDIR Sinaloa Unit facilities. In the laboratory, the shrimp were kept in two 500-L tanks to acclimate them to a salinity of 30 g L^{-1} and a temperature of 28 ± 1 °C, under continuous aeration. Prior to the experiment, a subsample of 10 organisms was analyzed using real-time PCR to confirm the absence of white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV), following standard protocols [26,27].

Shrimp were acclimated for 7 days and fed a commercial diet at 5% of their biomass during the acclimation period.

2.3. Experimental Design and Growth Performance

Following acclimation, 192 juvenile *P. vannamei* with an average initial body weight of 6.89 ± 0.20 g were randomly distributed among 16 experimental tanks with a nominal capacity of 80 L and a working volume of 70 L. Four experimental diets containing nominal folic acid supplementation levels of 0 (control), 2.0, 5.0, and 10.0 mg kg^{-1} were evaluated using four replicate tanks per treatment, with 12 shrimp per tank. The dietary treatments were randomly assigned to the experimental tanks, and each tank was considered an independent experimental unit.

The tanks were connected to a closed recirculating aquaculture system consisting of a 500 L reservoir, mechanical filtration with 20 µm synthetic filter wool, and a biological

filtration unit containing sintered ceramic media to promote nitrification. After filtration, the water was returned to the experimental tanks.

Shrimp were fed the experimental diets twice daily at 8:30 a.m. and 6:30 p.m. for 35 days. The initial daily feeding rate was 5% of the tank biomass and was adjusted weekly according to the biomass recorded during biometric measurements. Water temperature and dissolved oxygen were measured twice daily using a portable dissolved oxygen meter (Pro20, YSI Inc., Yellow Springs, OH, USA). The pH and salinity were measured using a pH meter (Pro1020, YSI Incorporated, Yellow Springs, OH, USA) and a refractometer (B08TJ28XC1, Hallocool®, Shenzhen, China), respectively. Water-quality variables were measured at the individual tank level, and the values reported for each dietary treatment represent the mean of the corresponding replicate tanks. Water lost through evaporation was replaced daily to maintain the working volume and salinity of the system. Biometric measurements were performed weekly, and the total biomass and mean body weight of the shrimp in each replicate tank were recorded. Weight gain (WG), specific growth rate (SGR), apparent feed conversion ratio (aFCR), and survival rate (SR) were calculated for each experimental unit using the following equations:

$$WG\ (g) = Wf - Wi \tag{1}$$

$$SGR\left(\%\ d^{-1}\right) = \frac{(\ln Wf - \ln Wi)}{t} \times 100 \tag{2}$$

$$aFCR = \frac{dry\ feed\ supplied\ (g)}{biomass\ gain\ (g)} \tag{3}$$

$$SR(\%) = \frac{N_f}{N_i} \times 100 \tag{4}$$

where W_i and Wf are the initial and final mean body weights, respectively; t is the duration of the feeding trial in days; and N_i and N_f are the initial and final numbers of shrimp, respectively.

2.4. Formulation of Experimental Diets

Four experimental diets were prepared with nominal supplemental folic acid levels of 0, 2.0, 5.0, and 10.0 mg kg^{-1} diet, designated as Control, T2, T5, and T10, respectively. All experimental diets were prepared from the same basal formulation and differed only in the amount of supplemental folic acid added. The ingredient composition, nominal folic acid supplementation levels, and proximate composition of the basal diet are presented in Table 1.

Table 1. Ingredient composition, nominal supplemental folic acid levels, and analyzed proximate composition of the basal experimental diet.

Ingredient Composition (g 100 g^{-1} Diet).	Control	T2	T5	T10
Fish meal	22.5	22.5	22.5	22.5
Soybean meal	29.9	29.9	29.9	29.9
Unfortified wheat flour	37.5	37.5	37.5	37.5
Gelatin	2.1	2.1	2.1	2.1
Sodium alginate	2.0	2.0	2.0	2.0
Fish oil	3.0	3.0	3.0	3.0
Soy lecithin	3.0	3.0	3.0	3.0
Total	100.0	100.0	100.0	100.0
Nominal supplemental folic acid (mg kg^{-1} diet)	0	2	5	10

Table 1. *Cont.*

Ingredient Composition (g 100 g^{-1} Diet).	Control	T2	T5	T10
Analyzed proximate composition of the basal diet (% as-fed basis)				
Crude protein		34.1 ± 0.4		
Ether extract		8.5 ± 0.7		
Ash		7.5 ± 0.02		
Moisture		8.3 ± 0.1		
Crude fiber		2.8 ± 0.5		
Nitrogen-free extract [1]		38.8		

Control, T2, T5, and T10 correspond to nominal supplemental folic acid levels of 0, 2, 5, and 10 mg kg^{-1} diet, respectively. Proximate-composition values are expressed as mean ± SD on an as-fed basis. [1] Nitrogen-free extract was calculated by difference: NFE (%) = 100 − [crude protein + ether extract + ash + moisture + crude fiber].

Folic acid (purity ≥ 97%; Sigma-Aldrich, St. Louis, MO, USA) was prepared as a 10 mg mL^{-1} solution in distilled water, with the pH adjusted to 9 to facilitate folic acid solubilization. To prepare each kilogram of diet, the dry ingredients listed in Table 1 were thoroughly mixed. Sodium alginate was included at 20 g kg^{-1} diet as a binder. The corresponding folic acid solution was diluted in 600 mL of distilled water and gradually incorporated into the ingredient mixture. The Control diet received the same volume of pH-adjusted distilled water without supplemental folic acid.

The mixture was homogenized until a uniform consistency was obtained and subsequently extruded through a meat grinder to produce pellets. The pellets were dried at room temperature in the dark for 48 h and stored at −20 °C until use.

2.5. Preparation of the Bacterial Inoculum and Preliminary Dose–Response Assay

A previously characterized strain of *V. parahaemolyticus* causing AHPND (VpAHPND-E9) was cultured in Tryptic Soy Broth (TSB) containing 3% NaCl and incubated at 30 °C for approximately 9 h until an optical density of 0.8 at 580 nm was reached. Once this growth was achieved, the bacteria were transferred to 50-mL Falcon tubes and centrifuged (Thermo Scientific Micro 21R) at 2000× *g* for 20 min at room temperature (18 °C). The supernatant was then decanted, and the bacteria remaining at the bottom of the tube were resuspended in saline solution (3% NaCl) and adjusted to an optical density of 1.0 at 580 nm, corresponding to 3×10^7 CFU mL^{-1}. A preliminary dose–response assay was conducted using four bacterial concentrations: 1×10^4, 5×10^4, 1×10^5, and 2.6×10^5 CFU mL^{-1}. These concentrations were selected to span the infective range previously reported for immersion challenges with AHPND-causing *V. parahaemolyticus* in *P. vannamei*, in which mortality was shown to be strongly dependent on bacterial density [7]. Because virulence can vary among bacterial strains and experimental conditions, the present assay was used as a preliminary dose–response test to select an operational challenge concentration rather than to estimate a formal LD_{50}. Each concentration was evaluated in triplicate using three independent aquaria, each containing 10 shrimp. Mortality was monitored every 6 h for 72 h. The concentration of 2.6×10^5 CFU mL^{-1} produced approximately 50% cumulative mortality and was therefore selected as the challenge concentration for the subsequent infection assay.

2.6. Challenge Bioassay and Tissue Sampling

After the 35-day feeding trial, 30 shrimp from each dietary treatment were randomly allocated to three independent challenge aquaria, with 10 shrimp per aquarium. Each challenge aquarium was considered an independent experimental unit. The aquaria contained 40 L of seawater maintained at 27 ± 1 °C and 30 g L^{-1} salinity, with continuous aeration.

Shrimp were challenged by immersion with the AHPND-causing *Vibrio parahaemolyticus* strain E9 (VpAHPND-E9). The bacterial suspension was added to each aquarium to obtain a final concentration of 2.6×10^5 CFU mL^{-1}. This concentration was selected based on the preliminary dose–response assay described in Section 2.5, in which it produced approximately 50% cumulative mortality within 72 h. Clinical signs and mortality were recorded every 6 h after inoculation.

For gene-expression analyses, four shrimp per dietary treatment were sampled at 0, 6, and 12 h post-infection (hpi). Samples collected at 0 hpi were obtained immediately before bacterial inoculation. Different shrimp were sampled at each time point because tissue collection required destructive sampling. Gill tissue was collected for the analysis of immune-related genes, whereas gill and hepatopancreas tissues were collected for the analysis of antioxidant-related genes. Tissue samples were immediately placed in TRIzol™ reagent and stored at −80 °C until RNA extraction. For molecular analyses, tissue samples were identified using numerical codes during sample processing, and treatment identities were linked to the corresponding samples only after molecular data acquisition had been completed.

2.7. Total RNA Extraction

Total RNA was extracted from gill and hepatopancreas tissue samples using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, with the modifications described by Álvarez-Ruiz et al. [28]. Briefly, each tissue sample was placed in a 1.5 mL microcentrifuge tube containing 600 µL of TRIzol and homogenized using a sterile pestle. The homogenates were incubated for 5 min at room temperature and centrifuged at 12,000× *g* for 10 min at 4 °C. Subsequently, 500 µL of the supernatant was transferred to a new tube, mixed with 100 µL of chloroform, and vigorously shaken for 15 s. Samples were incubated at room temperature for 2–3 min and centrifuged at 12,000× *g* for 15 min at 4 °C.

After phase separation, 220 µL of the aqueous phase was transferred to a new tube and mixed with 250 µL of cold isopropanol to precipitate the RNA. Samples were incubated for 10 min, with gentle mixing every 2–3 min, and centrifuged at 12,000× *g* for 10 min at 4 °C. The supernatant was discarded, and the RNA pellet was washed twice with 250 µL of cold 75% ethanol. After each wash, samples were centrifuged at 14,000× *g* for 2 min at 4 °C, and the ethanol was carefully removed. Finally, the RNA pellet was air-dried for 5–15 min, resuspended in 40 µL of ultrapure water, quantified, and stored at −80 °C until further analysis.

2.8. RNA Quality Assessment, DNase Treatment, and cDNA Synthesis

The concentration and purity of total RNA were determined by spectrophotometry using a NanoDrop 2000c (Thermo Scientific, Vantaa, Finland), selecting only those samples with a 260/280 absorbance ratio between 1.8 and 2.0. The samples were diluted with ultrapure water to a final concentration of 200 ng μL^{-1}.

To eliminate potential contamination with genomic DNA, 25 µL of the dilution was treated with 1 U of DNase (Sigma-Aldrich) following the manufacturer's protocol. Subsequently, cDNA synthesis was performed from 1.0 µg of total RNA using the commercial ImProm-II™ kit (Promega, Madison, WI, USA) and oligo dT20 primers, in accordance with the manufacturer's instructions.

2.9. Quantitative Real-Time PCR and Relative Gene Expression Analysis

The cDNA obtained from each biological sample was diluted 1:10 with ultrapure water. Each sample was analyzed in technical duplicate using 5 µL of diluted cDNA as the template. Quantitative real-time PCR (qPCR) was performed in a CFX96 C1000 Touch Real-

Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), and fluorescence data were analyzed using CFX Manager™ software version 3.0.

A common 2× reaction mix was prepared in bulk to minimize pipetting variation. For each reaction, a 7.5 µL aliquot of the mix contained 3.0 µL of 5× PCR buffer, 1.0 µL of 25 mM $MgCl_2$, 0.30 µL of 10 mM dNTP mix, 0.75 µL of 20× EvaGreen, 0.09 µL of GoTaq DNA polymerase (5 U μL^{-1}; Promega, Madison, WI, USA), and 2.36 µL of ultrapure water. Each 15 µL qPCR reaction contained 7.5 µL of the common reaction mix, forward and reverse primers at a final concentration of 0.23 µM each, 5 µL of diluted cDNA, and ultrapure water to reach the final reaction volume.

The amplification program consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. At the end of each run, a melting-curve analysis was performed from 65 to 95 °C, with temperature increments of 0.5 °C every 5 s and continuous fluorescence acquisition, to verify the specificity of the amplification products.

The amplification efficiency of each target and reference primer pair was determined from standard curves generated using serial dilutions of cDNA. All primer pairs exhibited amplification efficiencies greater than 90%, indicating adequate performance for relative gene-expression analysis. *β-actin* and elongation factor 1-alpha (*EF1-α*) were initially evaluated as candidate reference genes. Their expression stability was assessed using the geNorm algorithm [29] implemented in the RefFinder web application. *β-actin* showed the highest expression stability under the experimental conditions and was therefore selected as the reference gene.

Relative gene-expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [30], with *β-actin* as the reference gene and the control group sampled at 0 hpi as the calibrator.

2.10. Primers and Target Genes

Specific primers (Table 2) were used for five *V. parahaemolyticus*-responsive genes. Gene designations were retained according to the source study from which the primer sequences were obtained. The immune-related targets were reported as MNK1/2 MAP kinase-interacting serine (*MNK*), C-type lectin 3 (*CTL3*), scavenger receptor B1 (*SR*), gamma-interferon-inducible lysosomal thiol reductase (*GILT*), and anti-lipopolysaccharide factor-like protein (*ALFP*) [25]. Because Qin et al. [25] did not assign the *ALFP* primer pair to a specific ALF isoform, no isoform designation was inferred in the present study.

Table 2. Primer sequences used for RT-qPCR analysis of immune-related, antioxidant-related, and reference genes in *P. vannamei*.

Gene	Abbreviation	Sequence 5′–3′	Amplicon Size (bp)	Reference
Anti-lipopolysaccharide factor-like protein	*ALFP*	CGAATCTGCGAACTCCAT GAATAAGAACAGTAGTGACCC	187	[25]
C-type lectin 3	*CTL3*	AAACCCTGGATTCGTCAA AAACCTTAGCTTAGAGTGGC	172	[25]
Gamma-interferon-inducible lysosomal thiol reductase	*GILT*	GAGTGCAAGGGCAACATG GGAACGAAGTAGAGGGAAGG	248	[25]
MNK1/2 MAP kinase-interacting serine	*MNK*	AGCATGAACCAGGATGAGG AGCCTGTGGCAGTAACGAG	142	[25]
Scavenger receptor B1	*SR*	GCTGGTGGAGATGTGGTTC TGGTGTTGTTCTTCGGGTA	176	[25]

Table 2. *Cont.*

Gene	Abbreviation	Sequence 5′–3′	Amplicon Size (bp)	Reference
Catalase	*CAT*	ACTCCCATTGCTGTTCGT ATCCCAATTTCCTTCTTCTG	114	[31]
Glutathione peroxidase	*GPX*	AGGGACTTCCACCAGATG CAACAACTCCCCTTCGGTA	117	[32]
Superoxide dismutase	*SOD*	ATCCACCACACAAAGCATCA AGCTCTCGTCAATGGCTTGT	229	[33]
Reference gene	Abbreviation	Sequence 5′–3′		Reference
β-actin	β-actin	CCACGAGACCACCTACAAC AGCGAGGGCAGTGATTTC	338	[29]

2.11. Statistical Analysis

Statistical analyses were performed using STATISTICA software (Version 7.0; StatSoft, Inc., Tulsa, OK, USA). The replicate tank was considered the experimental unit for growth-performance variables. Normality of model residuals and homogeneity of variances were evaluated using the Shapiro–Wilk and Levene tests, respectively. Weight gain, specific growth rate, apparent feed conversion ratio, and final survival from the feeding trial were analyzed using one-way ANOVA, with dietary folic acid level as the fixed factor. When significant differences were detected, Tukey's multiple-comparison test was applied.

Final cumulative mortality at 72 h post-challenge was calculated for each replicate aquarium and analyzed using one-way ANOVA, with dietary treatment as the fixed factor and the replicate aquarium as the experimental unit (n = 3 aquaria per treatment). When a significant treatment effect was detected, Tukey's multiple-comparison test was applied. Differences were considered significant at $p \leq 0.05$.

Relative gene-expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed using ΔCt values rather than fold-change values. The effects of dietary folic acid level, sampling time, and their interaction were evaluated using a two-way ANOVA. When significant effects were detected, Tukey's multiple-comparison test was applied. Differences were considered significant at $p \leq 0.05$. The data supporting the statistical analyses are provided in Supplementary Data S1.

3. Results

3.1. Growth Performance and Feed Utilization

Water-quality parameters remained stable throughout the 35-day feeding trial and were similar among dietary treatments (Table 3). Dissolved oxygen, temperature, pH, and salinity remained within suitable ranges for the culture of juvenile *P. vannamei*.

Table 3. Water-quality parameters recorded during the 35-day feeding trial.

Water-Quality Parameter.	Control	T2	T5	T10
Dissolved oxygen (mg L^{-1})	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1
Temperature (°C)	27.4 ± 0.5	27.5 ± 0.5	27.7 ± 0.3	27.5 ± 0.5
pH	7.9 ± 0.01	7.9 ± 0.01	7.9 ± 0.01	7.9 ± 0.01
Salinity (g L^{-1})	30 ± 1	30 ± 1	30 ± 1	30 ± 1

Values are presented as mean ± SD. Control, T2, T5, and T10 correspond to diets containing nominal supplemental folic acid levels of 0, 2, 5, and 10 mg kg^{-1} diet, respectively.

Initial body weight did not differ among dietary treatments. After 35 days, significant differences were observed in final body weight, weight gain, specific growth rate, apparent feed conversion ratio, and survival ($p \leq 0.05$) (Table 4). Shrimp fed the T5 diet exhibited the highest final body weight, weight gain, and specific growth rate. The T2 and T10 groups showed intermediate growth values, whereas the Control group exhibited the lowest growth performance.

Table 4. Growth performance, feed utilization, and survival of juvenile *P. vannamei.*

Response Variable	Control	T2	T5	T10
Initial body weight (g)	6.9 ± 0.2	6.9 ± 0.2	6.9 ± 0.2	6.9 ± 0.2
Final body weight (g)	12.3 ± 0.7 ^{c}	13.7 ± 0.3 ^{b}	14.8 ± 0.5 ^{a}	13.6 ± 0.3 ^{b}
WG (g)	5.4 ± 0.3 ^{c}	6.8 ± 0.3 ^{b}	7.9 ± 0.3 ^{a}	6.7 ± 0.4 ^{b}
SGR (% day^{-1})	1.6 ± 0.1 ^{c}	1.9 ± 0.2 ^{b}	2.14 ± 0.1 ^{a}	1.94 ± 0.1 ^{b}
aFCR	1.6 ± 0.1 ^{b}	1.4 ± 0.1 ^{a}	1.3 ± 0.1 ^{a}	1.4 ± 0.1 ^{a}
Survival (%)	83.3 ± 0.9 ^{b}	86.6 ± 1.0 ^{a}	86.6 ± 1.0 ^{a}	83.3 ± 0.9 ^{b}

Values are presented as mean ± SD (n = 4 replicate tanks per treatment). Control, T2, T5, and T10 correspond to diets containing nominal supplemental folic acid levels of 0, 2, 5, and 10 mg kg^{-1} diet, respectively. WG, weight gain; SGR, specific growth rate; a FCR, apparent feed conversion ratio. Different superscript letters within the same row indicate significant differences among dietary treatments ($p \leq 0.05$).

The apparent feed conversion ratio was significantly lower in the supplemented groups than in the Control group, with the lowest numerical value observed in T5. Survival was significantly higher in T2 and T5 than in the Control and T10 groups. Overall, among the supplemental folic acid levels evaluated, the 5 mg kg^{-1} diet produced the highest growth performance.

3.2. Survival and Cumulative Mortality Following Challenge with VpAHPND-E9

At 72 h post-challenge, final cumulative mortality differed significantly among dietary treatments (one-way ANOVA, $F_{3,8} = 5.405$, $p = 0.025$; Figure 1). The Control exhibited the highest cumulative mortality (53.3 ± 2.9%), followed by T10 (45.0 ± 5.0%), T2 (40.0 ± 5.0%), and T5 (36.7 ± 7.6%). Tukey's multiple-comparison test showed significantly lower final cumulative mortality in T5 than in the Control ($p = 0.022$), whereas no significant differences were detected among the remaining pairwise comparisons ($p > 0.05$). Corresponding final survival values were 46.7 ± 2.9%, 55.0 ± 5.0%, 60.0 ± 5.0%, and 63.3 ± 7.6% for the Control, T10, T2, and T5 groups, respectively.

3.3. Transcriptional Response of VpAHPND-Responsive Genes

The values recorded at 0 hpi represent basal transcript abundance after 35 days of dietary treatment and immediately before exposure to VpAHPND-E9. Gene-specific treatment- and time-dependent transcriptional patterns were observed among the immune-related genes evaluated (Figure 2). At 0 hpi, the highest relative expression of *ALFP* was observed in T2, whereas *CTL3* and *GILT* showed their highest basal expression in T10. Following bacterial challenge, *ALFP* and *CTL3* generally declined from their elevated basal levels in the supplemented groups, whereas *GILT* and *MNK* showed increased relative expression at 12 hpi in selected treatments. *SR* exhibited a distinct temporal response, with higher relative expression in T5 at 6 hpi and in T10 at 12 hpi. Overall, transcriptional responses varied among genes, dietary treatments, and sampling times.

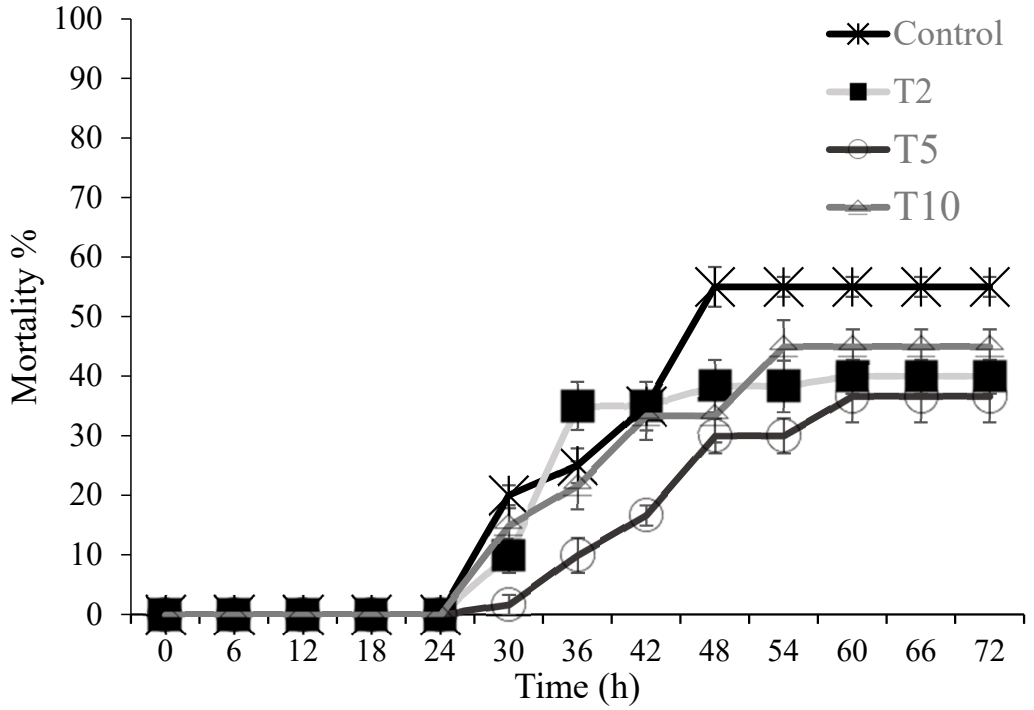

Figure 1. Cumulative mortality of juvenile Pacific white shrimp (*P. vannamei*) challenged with *Vibrio parahaemolyticus* strain E9 (AHPND) after 35 days on experimental diets. Control, T2, T5, and T10 correspond to nominal supplemental folic acid levels of 0, 2, 5, and 10 mg kg^{-1} diet, respectively. Mortality was monitored for 72 h post-challenge. Values are presented as mean ± SD of three replicate aquaria per dietary treatment. Statistical comparison was performed on final cumulative mortality at 72 h using one-way ANOVA followed by Tukey's multiple-comparison test. Final cumulative mortality was significantly lower in T5 than in the Control ($p = 0.022$), whereas the remaining pairwise comparisons were not significant ($p > 0.05$).

Two-way ANOVA revealed gene-specific effects of dietary treatment, sampling time, and their interaction. Significant treatment × time interactions were detected for all immune-related genes evaluated: *ALFP* ($F_{6,24} = 6.663$, $p < 0.001$), *CTL3* ($F_{6,24} = 6.238$, $p < 0.001$), *GILT* ($F_{6,27} = 20.017$, $p < 0.001$), *MNK* ($F_{6,25} = 16.071$, $p < 0.001$), and *SR* ($F_{6,25} = 5.998$, $p < 0.001$). Significant main effects of treatment and time were also detected for *CTL3*, *GILT*, and *MNK* ($p < 0.001$). For *ALFP*, only the time effect was significant ($F_{2,24} = 11.599$, $p < 0.001$), whereas for *SR*, only the treatment effect was significant ($F_{3,25} = 7.884$, $p < 0.001$). The remaining main effects were not significant (*ALFP*, treatment: $p = 0.076$; *SR*, time: $p = 0.078$).

3.4. Transcriptional Response of Antioxidant-Related Genes

Significant differences in antioxidant-related gene expression were observed among dietary treatments, with tissue-specific response patterns (Figure 3). In gill tissue, relative *CAT* and *GPX* transcript abundance was highest in T5, whereas *SOD* increased at the higher supplementation levels and reached its highest relative expression in T10 ($p \leq 0.05$).

In the hepatopancreas, T5 showed the highest relative expression of *CAT* and *SOD*, whereas *GPX* expression was higher in T2 and T5 than in the Control ($p \leq 0.05$). At the highest supplementation level (T10), the relative expression of the three antioxidant-related genes decreased compared with T5. Overall, the transcriptional patterns differed between tissues and among dietary folic acid levels.

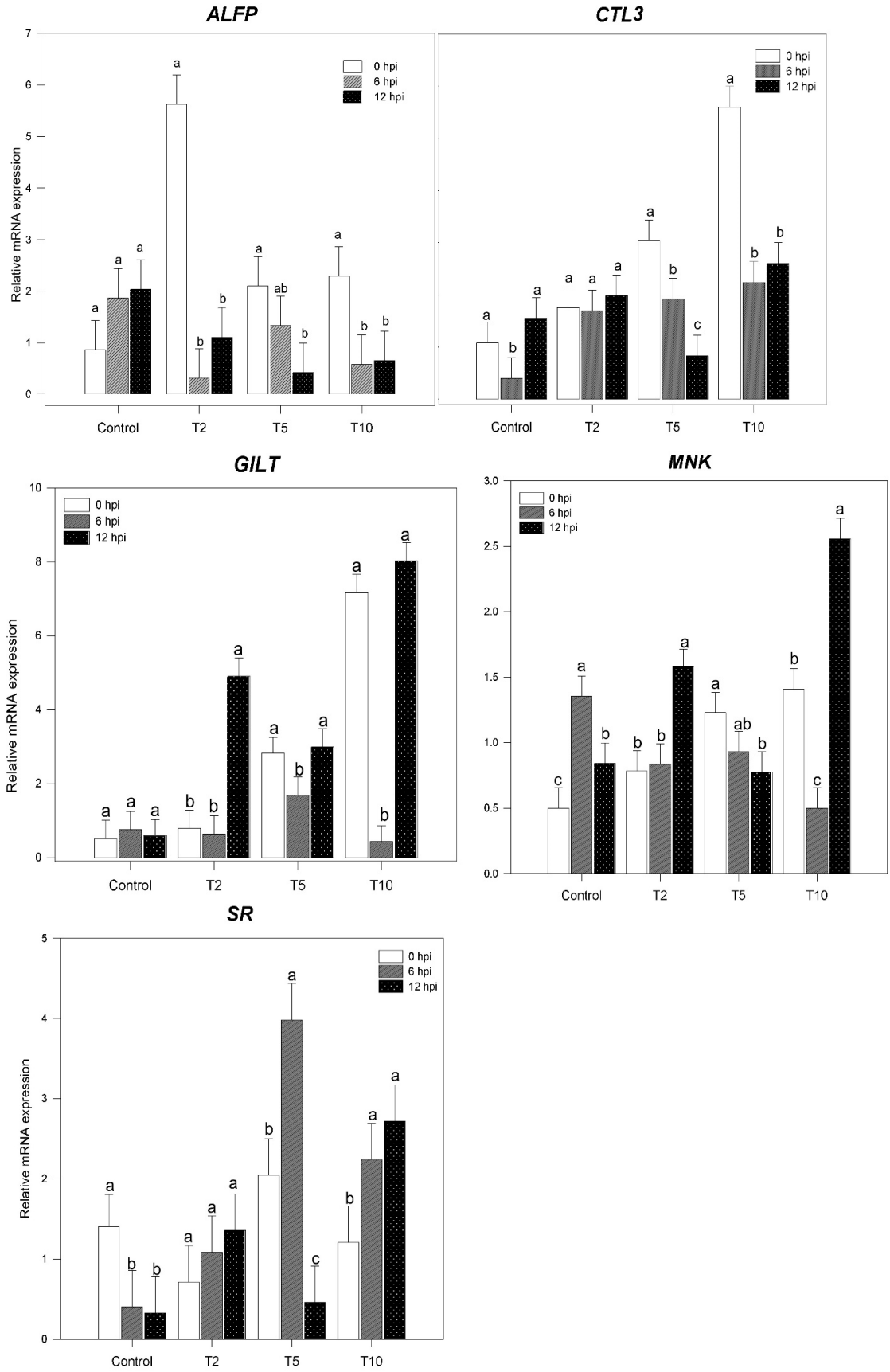

Figure 2. Relative mRNA expression of immune-related genes (*ALFP*, *CTL3*, *GILT*, *MNK*, and *SR*) in the gills of *P. vannamei* fed diets containing different levels of supplemental folic acid for 35 days (Control = 0, T2 = 2, T5 = 5, and T10 = 10 mg kg^{-1} diet). Samples were collected at 0, 6,

and 12 h post-infection (hpi) with VpAHPND-E9. Values are presented as mean ± SD. Statistical analyses were performed using ΔCt values by two-way ANOVA, with dietary treatment and sampling time as fixed factors, followed by Tukey's multiple-comparison test when significant effects were detected. Different letters indicate significant differences among sampling times within each dietary treatment ($p \leq 0.05$).

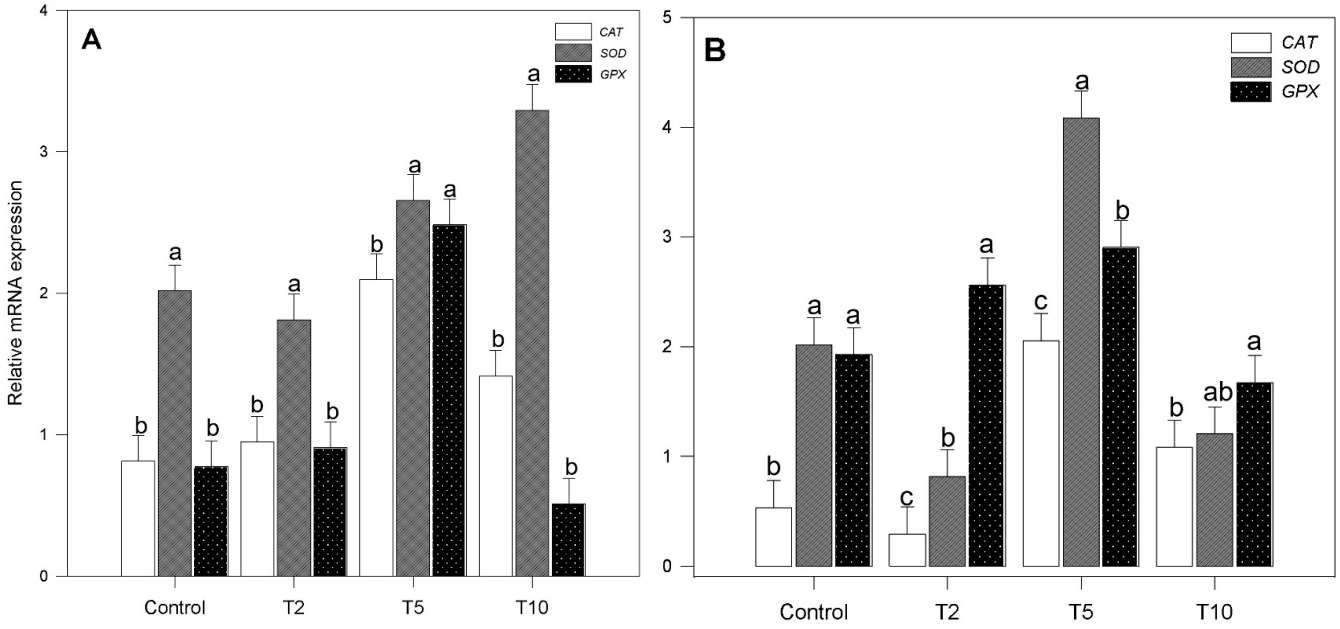

Figure 3. Relative mRNA expression of antioxidant-related genes, including catalase (*CAT*), glutathione peroxidase (*GPX*), and superoxide dismutase (*SOD*), in the gills (**A**) and hepatopancreas (**B**) of *P. vannamei* fed diets containing different levels of supplemental folic acid for 35 days (Control = 0, T2 = 2, T5 = 5, and T10 = 10 mg kg^{-1} diet). Values are presented as mean ± SD. Different letters indicate significant differences among dietary treatments for each gene ($p \leq 0.05$).

4. Discussion

Previous studies have reported favorable growth responses in crustaceans fed diets containing approximately 2 mg kg^{-1} folic acid, including tiger shrimp, *Penaeus monodon*, giant freshwater prawn, *Macrobrachium rosenbergii*, and red swamp crayfish, *Procambarus clarkii* [19–21]. However, the dietary level associated with the highest growth response may vary among species. For example, folic acid requirements of approximately 2.29–2.90 mg kg^{-1} have been reported for *Eriocheir sinensis* [22].

In the present study, dietary folic acid supplementation improved shrimp growth. These findings are consistent with previous evidence in *P. clarkii*, in which dietary folic acid supplementation improved growth performance [21]. Among the dietary treatments evaluated, the 5 mg kg^{-1} supplementation level produced the highest growth response, resulting in an approximately 2 g greater weight gain than the Control diet. Favorable growth responses have also been reported for *P. monodon* and *M. rosenbergii* fed diets supplemented with approximately 2 mg kg^{-1} folic acid [19,20].

High dietary folic acid supplementation has been associated with alterations in cellular homeostasis and oxidative status [34]. Antioxidant enzymes constitute an important component of the cellular defense against oxidative stress [35] by maintaining the balance between the generation and elimination of reactive oxygen species (ROS). Although ROS participate in normal cellular signaling and homeostasis, their excessive accumulation can damage cellular components.

A potential mechanistic link between dietary folic acid and the transcriptional responses observed in the present study may involve folate-mediated one-carbon metabolism.

Folate participates in the transfer of one-carbon units required for nucleotide synthesis and in the methionine cycle, which contributes to the generation of S-adenosylmethionine (SAM), a major methyl donor for DNA and histone methylation [13,15]. Because DNA methylation can influence transcriptional regulation, changes in folate availability could theoretically contribute to epigenetic modulation of gene expression. One-carbon metabolism is also interconnected with pathways involved in cellular redox homeostasis, providing a plausible metabolic link between folate availability and antioxidant-related transcriptional responses [15,34]. Recent evidence in crustaceans further supports a physiological role for dietary folic acid, as supplementation in *Procambarus clarkii* affected growth, glucose metabolism, antioxidant status, and immune-related parameters [21]. Nevertheless, the present study did not measure one-carbon metabolites, SAM availability, DNA methylation, redox-signaling intermediates, protein abundance, or antioxidant enzyme activities. Therefore, the observed changes in *SOD*, *CAT*, *GPX*, and immune-related transcripts should be interpreted as transcriptional modulation potentially associated with folate-dependent metabolic regulation rather than as direct evidence of enhanced antioxidant or immune function.

Antioxidant enzymes also participate in the physiological and immune responses of invertebrates [36]. Superoxide dismutase (SOD) converts superoxide anions into hydrogen peroxide and oxygen, whereas catalase (CAT) and glutathione peroxidase (GPX) contribute to the conversion of hydrogen peroxide into less reactive products, thereby supporting cellular homeostasis [37,38]. These enzymes are commonly used as indicators of physiological condition in crustaceans [39]. In the present study, antioxidant-related gene expression was evaluated in the gills and hepatopancreas. Relative *SOD* mRNA expression in the gills was higher in T5 and T10, whereas the highest hepatopancreatic *SOD* expression was observed in T5. The hepatopancreas and gills perform different physiological and metabolic functions, which may partly explain the tissue-specific transcriptional responses observed among dietary treatments. The hepatopancreas is a major site of digestion and intermediary metabolism, whereas the gills are involved in gas exchange, osmoregulation, and direct interaction with the surrounding water. A previous study [40] similarly reported increased *MnSOD* expression in response to dietary astaxanthin. Although folic acid and astaxanthin act through different biological mechanisms, both studies showed modulation of antioxidant-related transcription in gill tissue. Likewise, higher T-SOD enzymatic activity has been reported in juvenile mud crabs, *Scylla paramamosain*, fed diets containing 5.40 mg kg^{-1} vitamin A [41].

The increased relative expression of *SOD*, *CAT*, and *GPX* indicates that dietary folic acid modulated antioxidant-related transcriptional responses in shrimp. Such responses may vary among crustacean species. For example, Asaikkutti et al. [20] reported changes in SOD and CAT enzymatic activities in *M. rosenbergii* fed diets containing different levels of folic acid, although these enzymatic responses represent different endpoints from the transcriptional responses evaluated in the present study. In *E. sinensis*, Wei et al. [22] reported significantly higher SOD activity in crabs receiving 0.5 mg kg^{-1} dietary folic acid than in those fed the unsupplemented diet, with the highest activities observed at supplementation levels $\geq$ 2.0 mg kg^{-1}.

Relative *CAT* mRNA expression was higher in T5 than in the Control group in both tissues, although the magnitude of the response differed between the gills and hepatopancreas. In *M. rosenbergii*, Asaikkutti et al. [20] evaluated CAT enzymatic activity and observed higher activities at the upper dietary folic acid levels, particularly at 4 and 8 mg kg^{-1}; however, enzymatic activity and relative *CAT* mRNA abundance represent distinct biological endpoints and should not be interpreted as directly equivalent.

Consistent with these patterns, relative *GPX* mRNA expression was higher in T2 and T5 in the hepatopancreas and in T5 in the gills. Overall, the 5 mg kg^{-1} treatment produced the most consistent transcriptional response across the antioxidant-related genes evaluated. However, the physiological significance of these transcriptional changes remains to be established.

A previous study [42] reported that dietary macronutrient composition modulated antioxidant-related transcriptional responses in *Macrobrachium acanthurus*. The basal diet used in the present study contained 34.1% crude protein, suggesting that the overall nutritional composition of the diet should also be considered when interpreting the responses to folic acid supplementation. In *Macrobrachium acanthurus*, dietary protein level and lipid-to-carbohydrate ratio interacted to modify CAT and glutathione peroxidase (GP) enzymatic activities, demonstrating that macronutrient balance can influence antioxidant enzyme responses in crustaceans [43]. Together, these findings indicate that antioxidant responses in crustaceans can be influenced by both micronutrient supplementation and the broader nutritional composition of the diet.

Because shrimp lack an adaptive immune system, their defense relies primarily on interconnected humoral and cellular responses [44]. Humoral defenses include melanization through the prophenoloxidase system and the production of antimicrobial peptides, whereas cellular responses involve processes such as recognition, encapsulation, nodulation, and phagocytosis following the detection of pathogen-associated molecular patterns [45].

Gene-expression analyses provide useful information on the molecular responses associated with immune and antioxidant pathways during infection. AHPND is associated with PirA and PirB toxins, which cause epithelial damage and cellular detachment in the shrimp hepatopancreas [8]. A previous transcriptomic study [25] identified several genes potentially involved in the response of *P. vannamei* to *V. parahaemolyticus*, including *ALF*, *CTL3*, *GILT*, *MNK*, and *SR*.

ALF is an antimicrobial peptide with activity against Gram-positive and Gram-negative bacteria and is particularly relevant in defense against *Vibrio* species [46]. Functional evidence for a protective role of specific ALF family members has been reported in *L. vannamei*, where RNAi-mediated knockdown of *LvALF1* increased mortality following challenge with *Vibrio penaeicida* [46]. In the present study, the supplemented treatments, particularly T2, showed higher basal *ALFP* expression than the Control group at 0 hpi, followed by a decline after challenge. A previous study [25] also reported tissue- and time-dependent *ALFP* transcription following infection with VpAHPND. The higher basal *ALFP* expression observed in the supplemented groups may indicate that dietary folic acid modified the constitutive transcriptional state of this antimicrobial peptide. However, because antimicrobial activity, bacterial load, and functional immune priming were not evaluated, these results should not be interpreted as direct evidence of enhanced pathogen clearance.

The basal expression pattern of *CTL3* suggests that dietary folic acid may influence the transcription of pathogen-recognition receptors before bacterial exposure. Nevertheless, additional functional studies are required to determine whether this response represents immune priming.

GILT also showed a treatment-dependent transcriptional response. This enzyme participates in lysosomal protein processing through the reduction of disulfide bonds, which may contribute to the processing of microbial proteins. The relatively high basal *GILT* expression observed in supplemented shrimp suggests that dietary folic acid altered the constitutive transcriptional state of this gene before bacterial exposure. Following challenge, *GILT* expression varied among treatments and sampling times, indicating a dynamic transcriptional response to VpAHPND-E9. However, because lysosomal activity, protein processing, and bacterial clearance were not directly measured, the functional consequences

of these transcriptional changes remain to be determined. In shrimp treated with the probiotic *Bacillus velezensis* S141, increased *GILT* expression in the gills was observed together with increased tolerance to VpAHPND challenge [47]. In addition, altered *GILT* transcription has been reported in *Exopalaemon carinicauda* under impaired physiological conditions and in survivors of bacterial infection, supporting its possible participation in host defense [48].

The expression dynamics of *MNK* indicate that dietary folic acid influenced the transcriptional response of this kinase during both the basal and post-challenge periods. Because MNK participates in signaling pathways associated with translational regulation, its modulation may contribute to the cellular response during infection. Qin et al. [25] reported differential *MNK* transcription following *V. parahaemolyticus* challenge, supporting the responsiveness of this transcript during bacterial infection.

The transcriptomic response of scavenger receptor-related genes during *V. parahaemolyticus* infection has previously been reported in *P. vannamei* [25]. In the present study, changes in *SR* mRNA expression at 6 and 12 hpi indicate that dietary folic acid modified the transcriptional response of this receptor following challenge.

Notably, T5 combined the highest growth performance with the lowest final cumulative mortality and distinct transcriptional responses in several antioxidant- and immune-related genes. However, the present design does not establish a causal relationship between these transcriptional patterns and the observed mortality response.

Taken together, the present findings indicate that dietary folic acid supplementation influenced both zootechnical performance and molecular responses in juvenile *P. vannamei*. Although the experimental design does not establish a causal relationship between transcriptional profiles and survival outcomes, the 5 mg kg^{-1} supplementation level consistently combined superior growth performance, significantly lower final cumulative mortality following challenge, and distinct antioxidant- and immune-related transcriptional responses. These results support further investigation of folic acid as a functional dietary component for shrimp culture under disease-challenge conditions.

Study Limitations and Future Perspectives

The present findings should be interpreted within the scope of the experimental design. Gene-expression analyses required destructive sampling at each time point, and the challenge trial included three replicate aquaria per dietary treatment, which should be considered when interpreting temporal transcriptional patterns and mortality differences. In addition, total folate concentrations in the experimental diets were not analytically quantified; therefore, the nominal supplementation levels should not be interpreted as dietary folate requirements for *P. vannamei*. Because the molecular analyses were based on relative mRNA abundance, further studies incorporating protein or enzyme activity, bacterial load, and other functional endpoints would help determine the physiological significance of the observed transcriptional responses and their relationship with one-carbon metabolism. Validation under production-relevant conditions and with additional pathogens would further clarify the practical applicability of dietary folic acid supplementation in shrimp culture, including its incorporation into commercial vitamin–mineral premixes.

Despite these limitations, the consistent response observed across growth-performance variables, challenge outcomes, and transcriptional analyses strengthens the evidence that dietary folic acid level is an important factor influencing shrimp performance under the conditions evaluated.

5. Conclusions

The present study shows that dietary folic acid supplementation was associated with improved growth performance and with modulation of antioxidant- and immune-related transcriptional responses in juvenile *Penaeus vannamei*. Among the dietary treatments evaluated, 5 mg kg^{-1} consistently produced the most favorable growth performance and was associated with significantly lower final cumulative mortality following challenge with VpAHPND-E9. These findings support further investigation of folic acid as a dietary component capable of influencing growth performance, challenge outcomes, and transcriptional responses in shrimp. However, the observed changes in relative mRNA abundance alone should not be interpreted as direct evidence of enhanced antioxidant or immune function without complementary functional measurements. Further studies linking transcriptional responses with enzyme activity, bacterial load, and other functional immune and antioxidant indicators are warranted.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/aquacj6030040/s1, Supplementary Data S1: Dataset supporting the water-quality, growth-performance, mortality, and gene-expression analyses presented in this study.

Author Contributions: G.L.-V.: Formal analysis, investigation. J.A.E.-G. and P.Á.-R.: Formal analysis, investigation, project administration, funding acquisition, writing—original draft. G.L.-C.: Investigation, visualization, project administration, writing—review and editing. M.M.-M., J.S.-A., F.A.F.-H., M.I.B.-V., C.G.-J. and N.M.P.-L.: Supervision, conceptualization, methodology, writing—review and editing. Author P.Á.-R. passed away prior to the publication of this manuscript. All other authors have read and agreed to the published version of this manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study because it involved an invertebrate species (Pacific white shrimp) that is not subject to ethical approval under the applicable institutional requirements. All experimental procedures were conducted in accordance with applicable national regulations and institutional guidelines for the care and use of experimental animals, and appropriate measures were taken to minimize stress and unnecessary suffering.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are provided in Supplementary Data S1. Additional information is available from the corresponding author upon reasonable request.

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