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Aurantiochytrium sp. Meal as Feed Additive for Pacific White Shrimp Reared under Low Temperature and Challenged by WSSV in Association with Thermal Stress

Flávia Banderó Hoffling [1], Alex Silva Marquezi [2], Isabela Pinheiro [1], Cedric Simon [3], Artur Nishioka Rombenso [3], Walter Quadros Seiffert [1], Felipe do Nascimento Vieira [1] and Delano Dias Schleder [2,*]

[1] Laboratório de Camarões Marinhos, Federal University of Santa Catarina, Florianópolis 88061-600, Brazil; flaviabandh@gmail.com (F.B.H.); isabelapinheiro@outlook.com (I.P.); walter.seiffert@ufsc.br (W.Q.S.); felipe.vieira@ufsc.br (F.d.N.V.)

[2] Instituto Federal Catarinense, Campus Araquari, Araquari 89245-000, Brazil; lex_vet@hotmail.com

[3] Commonwealth Scientific and Industrial Research Organisation, CSIRO, 306 Carmody Road, St Lucia 4067, Australia; cedric.simon@csiro.au (C.S.); artur.rombenso@csiro.au (A.N.R.)

* Correspondence: delano.schleder@ifc.edu.br

Abstract: A study was conducted to test the inclusion of *Aurantiochytrium* sp. meal in the rearing of *Penaeus vannamei* grown in a clear water system and at a suboptimal temperature of 22 °C. The doses tested were 0 (control), 1, 2, 3, and 4% at a stocking density of 100 shrimp/m^3. Rearing was carried out with aeration and individual heaters, and seawater temperature was controlled with a chiller. After nine weeks, shrimp were weighed and hemolymph was collected for hemato-immunological tests, and growth performance were calculated. Shrimp raised at 22 °C and fed a 1% of dietary supplementation of *Aurantiochytrium* sp. meal were superior in immunological parameters. After the dietary assay, a total of 42 shrimps (10.9 ± 0.06 g) per treatment were infected with White Spot Syndrome Virus (WSSV). They were orally infected with 2.6 ± 10^6 virus particles per g of the animal, maintained at a suboptimal temperature of 22 °C for 108 h, and acclimated to an optimal temperature of 28 °C for an additional 48 h. At 7 days post infection, surviving shrimp were collected for hemato-immunological analysis. Cumulative mortality results showed that shrimp fed diets containing 3% and 4% *Aurantiochytrium* sp. meal had higher survival than other treatments when challenged with WSSV.

Keywords: DHA; disease resistance; fatty acids; hemato-immunological response; nutrition; *P. vannamei*

Key Contribution: The inclusion of *Aurantiochytrium* sp. meal as a feed additive increased the resistance of challenged *P. vannamei* to the White Spot Syndrome Virus associated with thermal stress.

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1. Introduction

Temperature is one of the main environmental factors in Pacific white shrimp (*P. vannamei*) farming [1]. Low or suboptimal temperatures due to cold fronts, during autumn and winter seasons, are challenging for shrimp, as they are ectothermic organisms that thermoregulate inefficiently and at a high energy cost. The ideal temperature range for the species is between 27–30 °C [2,3] and values outside this optimal range can increase susceptibility to diseases. Tropical and subtropical regions go through periods of autumn and winter with occurrence of low temperatures for the species *P. vannamei*, causing damages in the performance of the animals and productive cycles of the farms, such as the appearance of viral and bacterial diseases. The disease that causes the most damage in the shrimp industry is known as White Spot Syndrome Virus (WSSV); a viral disease reported in shrimp farming worldwide [4,5] which has a negative impact on the

industry, capable of causing widespread mortalities in crops in a few days [6]. Historical data suggest that sudden fluctuations in water temperature and low temperatures are associated with disease outbreaks [7]. A sudden drop of 4 degrees in optimal temperature (29–32 °C) in *P. vannamei* ponds was observed to trigger the effects of WSSV after tropical storms and showed a significant increase in viral load after typhoon passage [8]. Vidal and collaborators [9] observed that low temperatures (25.8 ± 0.7 °C) were critical for the overall mortality of shrimp infected with WSSV compared to infected animals kept at temperatures around 30–32 °C.

The general definition of optimal temperature range is that in which shrimp demonstrate fast and efficient growth. Wyban et al. [2] reported that 23 °C is a sub-optimal temperature for all three size classes of *P. vannamei*, as it impacts the growth rates and feed conversion ratio (FCR). In addition, shrimp grow less at temperatures of 20–24 °C when compared to rearing at temperatures of 28–32 °C [1,3,10,11]. *P. vannamei* is the most widespread shrimp species in aquaculture around the world due to its plasticity to different rearing conditions, being farmed in several subtropical regions and countries. Particularly in ectotherms, cold can change the dynamics of the shrimp cell membrane, reducing its fluidity and impacting its functioning, impairing the transport of nutrients and ions [12].

One of the known biochemical strategies to increase cell membrane fluidity under cold stress is to increase the proportion of long-chain polyunsaturated fatty acids (LC-PUFA) in the animal's body composition [12,13]. The process for the synthesis of fatty acids through chain elongation from 18:0 to 24:0 requires specific substrates and mitochondrial and microsomal enzymatic synthesis. However, the synthesis of certain fatty acids such as linoleic acid (18:2 n-6, LOA) and α-linolenic acid (18:3 n-3, ALA) by the desaturase enzyme system is not possible, and it is this limitation that leads to the frequent consideration of fatty acids as essential nutrients in diets for aquatic organisms [14]. For marine shrimp (*penaeidae*), requirement levels of about 1% linoleic acid (18:2 n-6) and about 1.5% α-linolenic acid (18:3 n-3) are suggested [15].

Regarding LC-PUFA, crustaceans are incapable of synthesizing them from monounsaturated fatty acids, so there is a dietary requirement for some n-3 and n-6 -LC-PUFA fatty acids [15]. Generally, the most essential LC-PUFA for various physiological processes are 20:4 n-6 (ARA—Arachidonic acid), 20:5 n-3 (EPA—Eicosapentaenoic acid), and 22:6 n-3 (DHA—Docosahexaenoic acid) [16]. For *P. vannamei*, the requirement is 0.50% of EPA or DHA while the requirement for *P. monodon* is 1.2% of each [15]. The inclusion of LC-PUFA in their diet may have beneficial physiological effects, particularly under low temperatures. These LC-PUFA perform important functions directly linked to the immune system and inflammatory responses [17–19].

Polyunsaturated fatty acids come from trophic organisms in marine environments, such as heterotrophic microorganisms, macroalgae, and microalgae [17,20]. Protists of the Order Thraustochytrids, Family Thraustochytriidae, like the *Aurantiochytrium* sp., are capable of synthesizing PUFA and LC-PUFA and display high production capability of LC-PUFA, especially DHA [21]. The genus *Aurantiochytrium* is classified as marine heterotrophic protists, non-photosynthetic, without plastid, with a wide geographic distribution, native to mangroves and marine environments [22–24], and can be a promising biotechnological proposal for aquafeeds [25].

Species classified as major producers of fatty acids such as *Aurantiochytrium* sp., *A. limacinum* (*Schyzochytrium limacinum*), and *A. mangrovei* (*Schyzochytrium mangrovei*) have been widely used in diets for aquatic organisms in recent years [22,26–30]. The classical pathway occurs with elongation and desaturation and is present in bacteria, plants, fungi, and microalgae, and polyketide synthase (PKS) has been identified in bacteria and algae [31]. The production of fatty acids in *Aurantiochytrium* sp. occurs through the alternative pathway, catalysed by the PUFA synthase, homologous to PKS [32]. The two forms can complement each other.

Thus, the inclusion of *Aurantiochytrium* sp. as a feed additive in practical diets for Pacific white shrimp could be the key to better maintenance of the animals in periods

of cold stress, helping to deal with suboptimal temperatures, reducing their deleterious effects and causing a protective effect against diseases. The present study aimed to evaluate the performance of *P. vannamei* juveniles receiving five dietary treatments with 0, 1, 2, 3, and 4% of *Aurantiochytrium* sp. meal in a clean water system at a suboptimal temperature (22 °C) regarding zootechnical, immunological, microbiological parameters, and the WSSV challenge.

2. Materials and Methods

2.1. Shrimps, Feeding and Maintance

The shrimp used for the study were obtained from the company Aquatec (Post-larvae aged 10 days, Rio Grande do Norte state, Brazil), stored and kept in a greenhouse, in a BFT system, and fed commercial feed 40% CP (Guabi) until reaching the desired weight. When reaching an average weight of 3.8 ± 0.02 g, the temperature of the matrix tank was adjusted from 24 °C to 22 °C, a week before transferring the shrimp. The diets (Table 1) were formulated according to the nutritional requirements of *P. vannamei* [15] to be isonitrogenous and isoenergetic (Table 2), undergoing a pelleting process. The control diet contained soy lecithin and fish oil to meet the nutritional requirements of *P. vannamei* for phospholipids. The amount of soy lecithin decreased as the amount of *Aurantiochytrium* meal increased, and the amount of soy and fish oils together was 3% for all diets, with minor fluctuations aiming to increase the n-3:n-6 ratio, as the level of *Aurantiochytrium* meal increase. The feeding rate was calculated according to the methodology of Van Wyk and Scarpa [33] and the feed was provided four times a day (08:00, 11:00, 14:00, and 17:00). The animals (total or partial) were weighted weekly for adjustments in feed supply.

Table 1. Experimental diets with inclusion of *Aurantiochytrium* sp. meal for *P. vannamei* rearing in a clear water system under suboptimal temperature (22 °C).

Ingredient (g/kg as Fed)	% Inclusion of *Aurantiochytrium* sp. Meal				
	Control	1	2	3	4
Soybean meal	300	290	290	290	290
Fishmeal	160	160	160	160	160
Wheat meal	159	166	165	165	165
Poultry meal	140	140	140	140	140
Kaolin	100	100	91.5	83	75.5
Vitamin and mineral premix [a]	25	25	25	25	25
Soy lecithin	22	15	15	13.5	11
Fish oil	20	25	27.5	30	30
Soybean oil	10	5	2.5	0	0
Magnesium sulphate	15	15	15	15	15
Monocalcium phosphate	10	10	10	10	10
Sodium chloride	15	15	15	15	15
Potassium chloride	15	15	15	15	15
Carboxymethylcellulose	5	5	5	5	5
DL-Methionine	2.5	2.5	2.5	2.5	2.5
Vitamin C	1	1	1	1	1
Choline hydrochloride	0.1	0.1	0.1	0.1	0.1
Aurantiochytrium sp meal. [b]	0	10	20	30	40

[a] Vitamin and Mineral Premix (In Vivo Mix, Paulínea, Brazil) compound by 900 mg kg^{-1} vit. A, 25 mg kg^{-1} vit. D, 46,900 mg kg^{-1} vit. E, 14,000 mg kg^{-1} vit. K, 20,000 mg kg^{-1} vit. B2, 40,000 mg kg^{-1} pantothenic acid, 70,000 mg kg^{-1} niacin, 50 mg kg^{-1} vit. B12, 750 mg kg^{-1} biotin, 3000 mg kg^{-1} folic acid, 30,000 mg kg^{-1} vit. B1, 33,000 mg kg^{-1} vit B6, 20 mg kg^{-1} magnesium, 6.1 mg kg^{-1} potassium, 23,330 mg kg^{-1} copper, 1000 mg kg^{-1} iodine, 6500 mg kg^{-1} manganese, 100 g kg^{-1} zinc, 125 mg kg^{-1} selenium (In Vivo Nutrição e Saúde Animal Ltd.a—São Paulo, SP, Brazil). [b] *Aurantiochytrium* sp. meal—ALL-G-RICH® produced and supplied by Alltech Inc. (Nicholasville, KY, USA) and imported by Alltech do Brasil Agroindustrial Ltd.a (Araucaria, Paraná, Brazil).

Table 2. Proximal composition and fatty acid composition of experimental diets with inclusion of *Aurantiochytrium* sp. meal for *P. vannamei*.

Chemical Composition (%)	% Inclusion of *Aurantiochytrium* sp. Meal				
	Control	1	2	3	4
Moisture content	9.5	9.5	9.5	8.5	9.6
Crude protein	40.8	40.0	40.4	40.1	40.3
Crude lipid	8.1	8.9	9.7	10.5	10.8
Carbohydrate	25.3	25.5	25.7	24.7	25.8
Ash	16.3	16.1	14.7	16.2	13.5
Dry matter (%)	90.5	90.5	90.5	91.5	90.4
Gross energy (MJ/kg) [a]	16.6	16.3	16.6	16.6	16.7
Fatty acids (% DM)					
14:0	0.12	0.17	0.22	0.27	0.30
16:0	1.89	2.42	2.99	3.5	3.85
18:0	0.60	0.62	0.64	0.69	0.66
18:1 n-9	2.14	2.23	2.24	2.31	2.19
18:2 n-6	2.22	1.95	1.79	1.66	1.63
18:3 n-3	0.20	0.17	0.15	0.15	0.14
ARA [b]	0.06	0.06	0.07	0.07	0.07
EPA [c]	0.08	0.10	0.11	0.12	0.12
DHA [d]	0.25	0.48	0.73	0.91	1.11
SFA	2.77	3.4	4.05	4.68	5.04
MUFA	2.47	2.62	2.65	2.78	2.63
PUFA	2.88	2.86	2.95	3.03	3.17
LC-PUFA	0.33	0.58	0.84	1.03	1.23
n-3	0.53	0.76	1.0	1.19	1.37
n-6	2.31	2.05	1.9	1.78	1.74
n-3:n-6	0.23	0.37	0.53	0.67	0.79
DHA:EPA	3.13	4.80	6.64	7.58	9.25
PUFA:SFA	1.04	0.84	0.73	0.65	0.63

[a] Gross Energy are express in MJ kg^{-1}; [b] Arachidonic acid (20:4 n-6); [c] Eicosapentaenoic acid (20:5 n-3); [d] Docosahexaenoic acid (22:6 n-3). All data of chemical composition are in terms of dry matter. Fatty acid group: SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, LC-PUFA = long chain PUFA (with 20 to 22 carbons).

2.2. Proximal Analysis of Aurantiochytrium sp.

The additive used in the experimental feeds was analysed for dry matter (DM), ash, crude protein (N), gross energy, total lipids, and minerals following methodologies described in more detail by Simon et al. [34]. The DM content was determined after drying at 105 °C for 6 h using the gravimetric method. Gross energy was determined on a Parr 6200 oxygen bomb calorimeter (Par Instrument Company®, Moline, IL, USA). The ash content was determined by muffle combustion at 550 °C for 6 h. The crude lipid was extracted in chloroform–methanol–water (2:1:0.4 ratio) and measured gravimetrically. For crude protein contents, the total nitrogen content was measured and multiplied by 6.25. (CHNS autoanalyzer®—Leco Corp., St. Joseph, MI, USA).

The analysis of the composition of fatty acids followed the method of Coutteau et al. [35]. Briefly, after crude lipid analysis, the lipids were esterified via an acid-catalysed methylation and 0.3 mg of an internal standard was added to each sample (21:0 Supelco Analytical Products®, Bellefonte, PA, USA). Fatty acids were identified against the internal standard after separation by gas chromatography (GC). An Agilent Technologies 6890 N GC system (Agilent Technologies®, Santa Clara, CA, USA) equipped with a DB-23 capillary column and flame ionization detection was used. The temperature program was 50–175 °C at 25 °C min^{-1}, then 175–230 °C at 2.5 °C min^{-1}. The injector and detector temperatures were set at 250 °C and 320 °C, respectively. The carrier gas was hydrogen at a flow rate of 40 mL min^{-1}. The peaks were identified by comparing the retention times with the internal standard and later referenced with known standards (37 Comp. FAME

mix, Supelco Analytical Products®, Bellefonte, PA, USA). The chemical composition of *Aurantiochytrium* sp. meal used in the present study is presented in Table 3. The total lipid values make up most of the chemical composition of the meal and are comprised mainly of palmitic acid (16:0) and DHA (22:6 n-3).

Table 3. Chemical composition of *Aurantiochytrium* sp.

***Aurantiochytrium* sp. ALL-G-RICH**					
Chemical Composition g kg^{-1}		**Minerals mg kg^{-1}**			
Moisture content	24.65	P	1798.83	Na	1015.46
Crude protein	108.68	Ca	2898.55	Fe	13.89
Lipid	688.73	Mg	2496.27	Al	7.87
Ash	30.64	K	1519.93	Zn	12.43
Carbohydrate	147.30	S	4964.37	Si	208.24
Dry matter	975.35			-	-
Gross energy [a]	31.90			-	-
		Fatty acids composition %			
14:00	5.34	18:4 n-3	0.26	MUFA	0.85
16:00	62.98	ARA [b]	0.82	PUFA	28.39
16:1 n-7	0.11	EPA [c]	0.12	LC-PUFA	27.13
18:1 n-9	0.33	DHA [d]	26.62	n-3	27.39
18:2 n-6	0.29	SFA	68.61	n-6	1.26

[a] Gross Energy are express in MJ kg^{-1}. [b] Arachidonic acid (20:4 n-6). [c] Eicosapentaenoic acid (20:5 n-3) [d] Docosahexaenoic acid (22:6 n-3). All data of chemical composition are in terms of dry matter. Fatty acid group: SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, LC-PUFA = long chain PUFA (with 20 to 22 carbons).

2.3. Experimental System

A 9-week growth trial was conducted in an indoor clean water system with 80–100% daily water change. Six hundred shrimps with average initial weight of 3.8 ± 0.02 g were transferred to the clean water system at 22 °C, at high storage density (100 shrimp/m^3) in polyethylene boxes with 400 L of usable volume (experimental unit). Forty animals were randomly divided into 15 experimental units equipped with microporous tubes for aeration. The experiment was made with five dietary treatments with 0, 1, 2, 3, and 4% of *Aurantiochytrium* sp. meal, and treatments were run in triplicate. Room temperature was controlled using air conditioning and water was cooled using a central chiller (22 °C) to replace static water. Monitoring of temperature and dissolved oxygen was performed twice a day with the aid of a YSI multiparameter probe (Pro20). Alkalinity, pH, salinity, ammonia, and nitrite were measured once a week using methodologies described by the APHA [36].

2.4. Growth Performance

At the end of the experimental period, according to methodologies described by Van-Wyk and Scarpa [33] and Bagenal and Tesch [37], the following variables were measured: final mean weight, feed conversion ratio (FCR), weekly weight gain, yield, and survival. These variables were calculated to evaluate feeding parameters in the two phases of the study, in terms of wet weight, as follows:

$$\text{Final mean weight } (g^{-1}) = \text{final biomass/number of shrimp.} \quad (1)$$

$$\text{Feed conversion ratio } (g^{-1}) = \text{feed intake (g)/(final biomass} - \text{initial biomass).} \quad (2)$$

$$\text{Weekly weight gain (g week}^{-1}) = \text{final weight} - \text{initial weight/weeks of rearing.} \quad (3)$$

$$\text{Total weight gain } (g^{-1}) = \text{average of final biomass} - \text{average of initial biomass.} \quad (4)$$

$$\text{Productivity (kg m}^{-3}\text{)} = \text{final biomass/tank volume.} \quad (5)$$

$$\text{Survival (\%)} = (\text{number final of shrimp/number of shrimp stored}) \times 100. \quad (6)$$

2.5. Microbiological Parameters

For microbiological analysis, at the end of the experiment, the intestines of five shrimp per experimental unit (15 per treatment) were collected. The intestines were homogenized and serially diluted in concentrations of 1:10 in sterile 3% saline (NaCl). After dilution, they were seeded in petri dishes with Soy Triptone Agar (TSA) culture for total heterotrophic bacteria count and in Thiosulfate Citrate Bile Sucrose Agar (TCBS) for total vibrionaceous bacteria count. The counts were performed using the total colony-forming units (CFU mL^{-1} g^{-1} of intestine) after 24 h of incubation in an oven at 30 °C.

2.6. White Spot Syndrome Virus (WSSV) Challenge with Thermal Stress

After the experimental rearing, a challenge trial with WSSV in association with thermal stress (Figure 1) was conducted in the Aquaculture Laboratory of the Instituto Federal Catarinense, campus Araquari (LAq/IFCCA), using 42 shrimp per treatment (14 per tank, triplicate). The animals (10.9 ± 0.06 g) were acclimatized for 48 h to the new tanks (50 L); after that, they were infected orally, via gavage, all at under 22 °C.

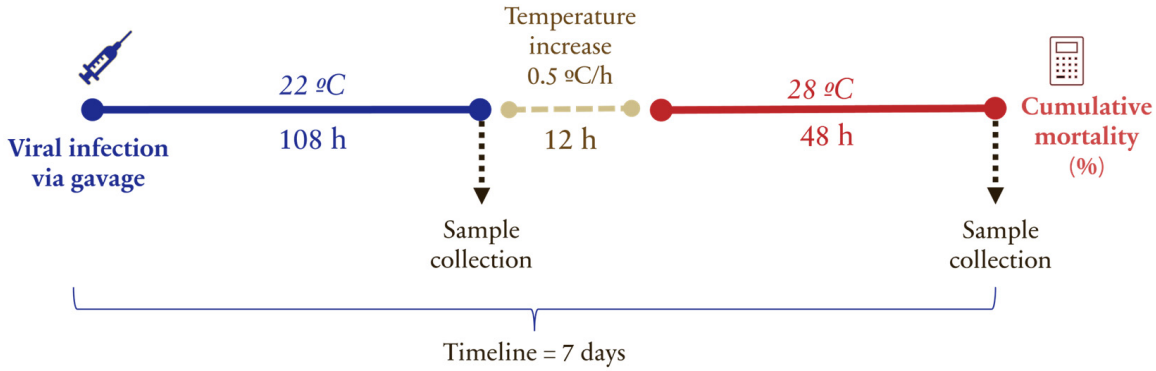

Figure 1. Timeline of the challenge with WSSV associated with thermal stress, representing the challenge sequence and sample collection.

The presence of the virus in the experimental inoculum and the animals (three shrimp per treatment) was confirmed using PCR (Hi-PCR® WSSV Detection Kit—HiMedia®). The inoculum had a concentration of 3.7×10^8 viral particles per µL. The shrimps were infected with 70 µL per g animal, totaling 2.6×10^{10} viral particles per g of animal. The viral dose was defined based on previous studies [38,39] and a pilot 50% lethal dose trial (LD 50) performed under the same temperature regime of the present study.

The WSSV inoculum was produced as described by Rezende et al. [38]. Briefly, 50 µL of infected muscle samples, homogenized (1:10 *w*/*v*) in buffer solution (330 mM NaCl, 10 mM Tris, pH 7.4), was inoculated intramuscularly into healthy SPF animals. At the first signs of disease, the animals were sacrificed, mechanically minced and homogenized (1:10 *w*/*v*) in the same buffer solution and then centrifuged at 2000× *g* for 20 min at 4 °C. The supernatant was centrifuged again at 9000× *g* for 10 min at 4 °C, filtered (0.45 µm), and stored in liquid nitrogen. The quantification of the viral dose in the WSSV inoculum was carried out in a partner laboratory with RT-qPCR, using primer pairs WS1F (5′-CGTGCTGGCGATGACATTCCAATA-3′) and WS1R (5′-TTGAACCATCAAGACTCGCCCTCT-3′), as described by Valente et al. [40].

During the infection, a concomitant thermal stress was performed aiming to replicate the temperature variations that occur in subtropical regions, particularly in late spring and early summer. The thermal stress was initiated 108 h post infection (h.p.i; 4.5 days post infection) by increasing the water temperature from 22 to 28 °C for 12 h (0.5 °C h^{-1}), then kept at 28 °C for a further 48 h, meaning a total of 168 h (seven days) of challenge trial.

Mortality was monitored every 3 h for the entire challenge. A negative control group (NC) was performed by inoculating shrimp WSSV-free inoculum and submitting them only to thermal stress.

The daily water renewal rate was 50% and the following parameters were monitored: temperature (21.8 ± 0.7 °C and 28.4 ± 0.6 °C), measured every 3 h; dissolved oxygen (7.0 ± 0.5 mg L^{-1}) measured twice a day, using an oximeter (Ecosense® D0200A, YSI, Yellow Springs, OH 45387 USA); pH (8.0 ± 0.2), measured once a day using a pHmeter (AK59 AKSO®, São Leopoldo, RS, Brazil); salinity (30 ± 2 g L^{-1}), measured once a day using a portable refractometer; ammonia (<1 mg L^{-1}), measured every 48 h, according to APHA [36].

2.7. Hemato-Immunological Assessment

At the end of the rearing period, the hemolymph of five shrimp per experimental unit (15 per treatment) was collected from the ventral sinus to form a pool, using sterile 1 mL syringes, cooled at 4 °C. For total hemocyte count (THC), 50 µL of the collected hemolymph was fixed in modified Alsever's anticoagulant solution (MAS) (27 mM sodium citrate, 9 mM EDTA, 115 mM glucose, 336 mM NaCl, pH 7.2) with 4% formaldehyde. The remaining portion was coagulated at 4 °C and centrifuged at 6000× *g* for 10 min, for two times, to obtain serum, which was aliquoted and stored at −20 °C for further analysis. The hemolymph of four shrimp per experimental unit (12 per treatment) was collected after 108 h (at the end of sub-optimal temperature period) and 168 h of challenge (after thermal increase), following the process described above, and the same hemato-immunological parameters were performed. The detailed methodologies of the hemato-immunological parameters were described by Bolívar-Ramirez et al. [41].

2.8. Statistical Analysis

All data were tested for normality (Shapiro-Wilk test) and homogeneity (Levene test) to meet the requirements of ANOVA. Gut bacteria count data were transformed to log10 (x + 1) before being submitted for analysis. Those data were subjected to one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and a post-hoc Tukey test with a 5% confidence level using the Statistica 10 program (StatSoft) [42]. Mortality data from the viral challenge associated with thermal stress was analysed using the Kaplan–Meier test with Statistica 10 software (StatSoft). To evaluate the effect of the different levels of dietary supplementation of *Aurantiochytrium* sp. on the hemato-immunological parameters, before and after the infection and thermal stress, a two-way ANOVA was performed followed by a Tukey test. All data were tested for normality and homogeneity to meet the requirements of ANOVA and MANOVA. For all tests, the significance level adopted was 5% in all the statistical analyses.

3. Results

3.1. Experimental System and Growth Performance

The temperature of the experimental period remained within the suboptimal range stipulated for the experiment (22.1 ± 0.2 °C), without statistically differing between treatments. Dissolved oxygen was within the recommended range for the species (7.1 ± 0.2 mg L^{-1}). Nitrogen compounds remained within the recommended range for the marine shrimp [43] and did not differ significantly, as did alkalinity (121.7 ± 4.0 mg $CaCO_3$ L^{-1}) and pH. The source of water used is marine water, with constant salinity (32.7 ± 0.9 mg L^{-1}) throughout the experiment. No evaluated parameter showed statistical difference (Table 4). The average weekly weight gain of shrimps was around 1.02 g per week, and the productivity was around 1.28 kg^{-1} m^3. Survival remained at values above 96.7% in all treatments. Feed conversion was above 2:1 kg in all treatments.

Table 4. Growth performance results of *P. vannamei* rearing in clear water and fed diet containing 0 (control), 1, 2, 3, and 4% of *Aurantiochytrium* sp. meal inclusion offered after nine weeks.

Treatment	Initial Weight (g^{-1})	Final Weight (g^{-1})	Weekly Weight Gain (g^{-1})	Yield (kg^{-1}/m^3)	Survival (%)	Feed Conversion Ratio (kg^{-1})
Control	3.8 ± 0.01	13.1 ± 0.67	1.04 ± 0.07	1.35 ± 0.10	100.0 ± 0.0	2.16 ± 0.15
1%	3.8 ± 0.02	12.6 ± 0.78	0.98 ± 0.08	1.24 ± 0.04	97.5 ± 2.5	2.32 ± 0.07
2%	3.8 ± 0.03	12.7 ± 0.78	0.99 ± 0.09	1.25 ± 0.08	98.3 ± 1.4	2.26 ± 0.13
3%	3.8 ± 0.04	13.9 ± 0.07	1.10 ± 0.03	1.34 ± 0.02	96.7 ± 1.4	2.16 ± 0.07
4%	3.8 ± 0.02	12.9 ± 0.41	1.01 ± 0.04	1.25 ± 0.02	97.5 ± 2.5	2.26 ± 0.03
p value	0.9931	0.2411	0.2364	0.1364	0.5251	0.3025

Data presented as mean ± standard deviation. *p*-value for one-way ANOVA.

3.2. Microbiological Analysis

Regarding the concentration of bacteria per gram of intestine, in Log 10 colony-forming units (CFU), no statistical differences were identified between treatments in bacterial counts (Figure 2).

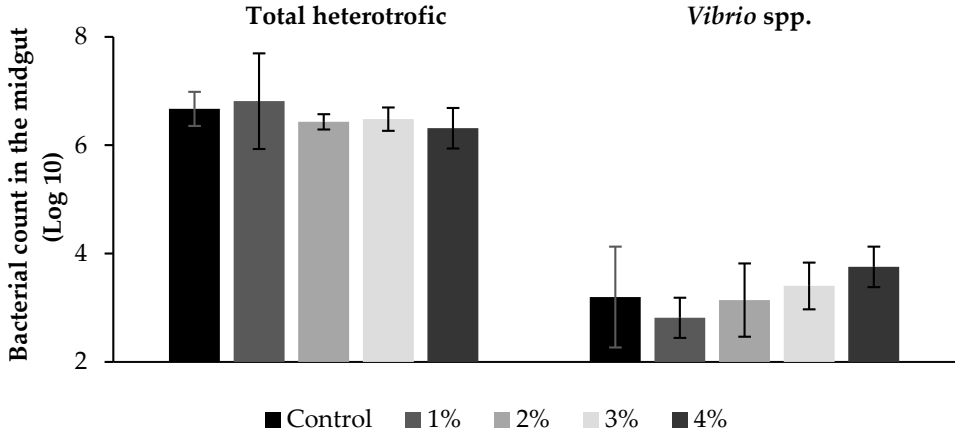

Figure 2. Concentration of bacteria (g intestine in Log 10 CFU), of *P. vannamei* rearing in clear water and fed diet containing 0 (control), 1, 2, 3, and 4% of *Aurantiochytrium* sp. meal inclusion offered after nine weeks. The concentrations of bacteria shown are total heterotrophic bacteria (*p* value = 0.7056) and total *Vibrio* spp. (*p* value = 0.4458). Data are presented as mean ± error deviation.

3.3. WSSV Challenge Associated with Thermal Stress

At the end of the challenge, animals from the 4% treatment showed the lowest mortality at 36.7% ($p < 0.01$) (Table 5). The non-supplemented treatment presented the worst resistance, with the highest mortality to the viral challenge associated with thermal stress (61.7%), and during the phase under temperature of 22 °C (40.8%). During the phase under temperature of 28 °C, these non-supplemented animals showed the third highest mortality (20.8%). The thermal variation alone was not capable of causing any mortality, as observed in the negative control (NC), since this group was not inoculated with WSSV.

In the temperature phase of 22 °C, all treatments with *Aurantiochytrium* sp. meal inclusion showed the lowest absolute mortality; however, only 3 and 4% treatments were significantly lower compared to the control ($p < 0.05$). On the other hand, animals from the 3% treatment were the ones that presented the highest mortality after the increase in temperature, so that at the end of the challenge, only 4% treatment presented lower total cumulative mortality compared to the control (0%) ($p < 0.05$) (Figure 3).

Table 5. Cumulative mortality (%) of *P. vannamei*, rearing in clear water and fed diet containing 0 (control), 1, 2, 3, and 4% of *Aurantiochytrium* sp. meal inclusion offered after nine weeks, after infection with WSSV associated with heat stress.

Treatments	Suboptimal Temperature Phase (22 °C)	Thermal Increment Phase	Optimal Temperature Phase (28 °C)	Cumulative Total Mortality
Negative control *	0	0	0	0
Control	40.8 ± 21.3 [a]	0	20.8 ± 16.6 [a]	61.7 ± 12.6 [a]
1%	26.7 ± 23 [a]	0	20.0 ± 17.3 [a]	46.7 ± 40.4 [a]
2%	21.9 ± 11.7 [a]	3.7 ± 6.4	28.5 ± 5.7 [a]	54.1 ± 13.4 [a]
3%	10.7 ± 1.0 [b]	0	46.3 ± 3.2 [b]	57.0 ± 2.6 [a]
4%	16.7 ± 15.3 [b]	3.3 ± 5.7	16.7 ± 20.8 [a]	36.7 ± 35.1 [b]
Time (h)	108	12	48	168
p value	0.0184	-	0.0170	0.0006

Mean data ± standard deviation via Kaplan–Meier analysis. * The NC (negative control) was not infected with WSSV but was subjected to the same temperature conditions. The NC data were not statistically analyzed and served as a standard to visualize the infection compared to other treatments. All treatments are statistically different from the NC ($p < 0.01$). Superscript letters show the differences between the treatments compared to the control treatment between the infection periods (22 °C, 28 °C and at the end of the challenge).

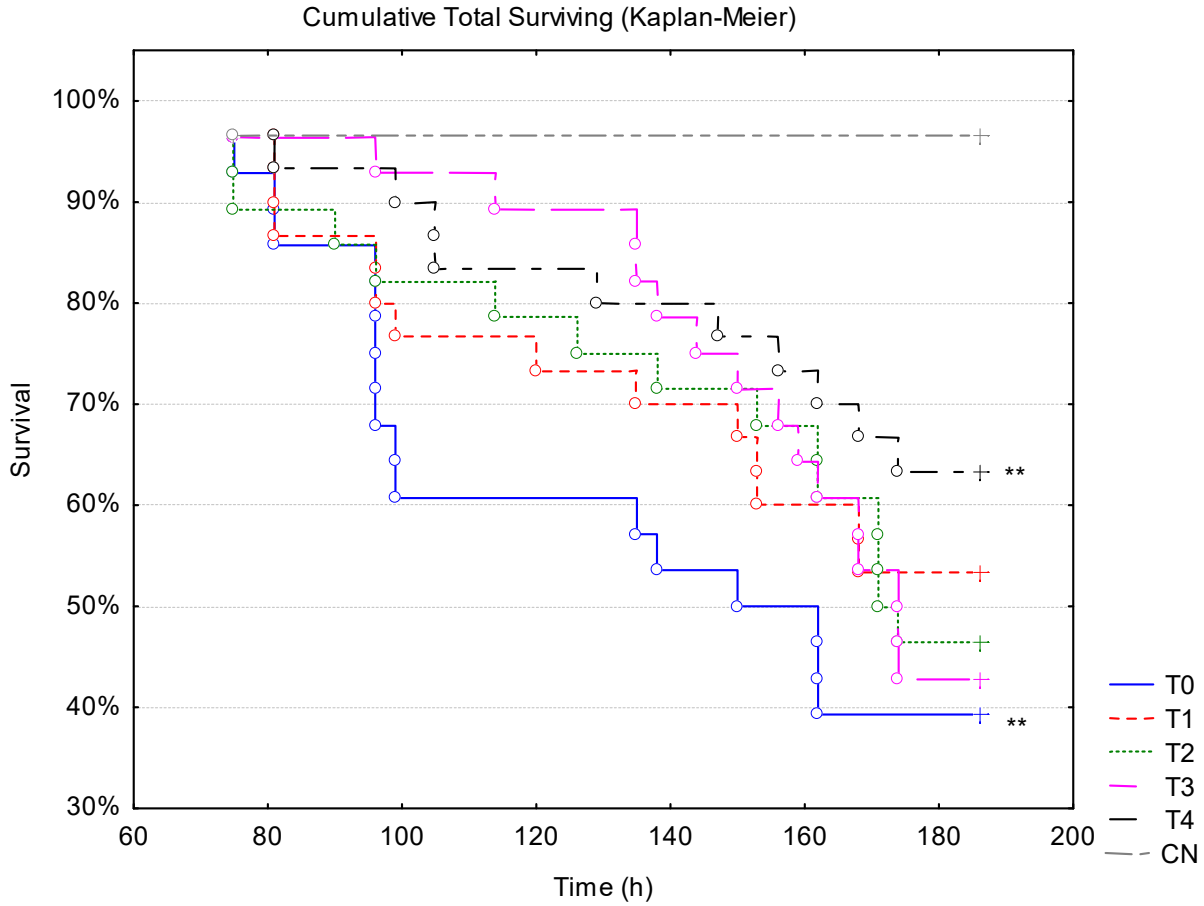

Figure 3. Cumulative mortality (%) of *P. vannamei* rearing in clear water and fed diet containing 0 (control), 1, 2, 3, and 4% of *Aurantiochytrium* sp. meal inclusion offered after nine weeks, after infection with WSSV associated with thermal stress via Kaplan–Meier analysis *p* value = 0.00069). All dietary treatments were significantly different from the negative control (*p* value < 0.01). ** Treatment T0 × T4 (*p* value = 0.0259). Subtitle: T0 (control), T1 (1% *Aurantiochytrium* sp. meal), T2 (2% *Aurantiochytrium* sp. meal), T3 (3% *Aurantiochytrium* sp. meal), and T4 (4% *Aurantiochytrium* sp. meal).

3.4. Hemato-Immunological Parameters

Before infection, there were no differences in hemato-immunological parameters among the treatments, except for THC, which was higher in the 1%-supplemented treatment (Table 6). After the challenge, hemocyte count significantly reduced after WSSV infection in all groups when at 22 °C, and even more after thermal stress ($p < 0.01$). Thermal stress alone also caused a significant reduction in THC in the NC group in comparison to first sample (Table 6). In general, the average values in the agglutination test showed a reduction throughout the challenge, differing only before and after the infection ($p < 0.05$). Protein concentration and specific PO also showed a significant reduction after WSSV infection, with no difference among treatments ($p > 0.05$), differing only before and after infection ($p < 0.05$).

Table 6. Total hemocytes count (THC), protein concentration, phenoloxidase (PO), enzyme activity and serum agglutinating activity of *P. vannamei,* rearing in clear water and fed diet containing 0 (control), 1, 2, 3, and 4% of *Aurantiochytrium* sp. inclusion offered after nine weeks.

	Treatments	THC (10^6)	Protein Concentration (mg mL^{-1})	PO activity (U min^{-1} mg^{-1} Protein)	Agglutination Titer (Log2)
1 st sample: after 9 weeks of trial, before infection WSSV (22 °C)	Control	53.90 ± 5.77^{B}	167.9 ± 43.8	60.9 ± 43.6	9.33 ± 0.58^{ABC}
	1%	100.78 ± 19.44^{A}	163.4 ± 48.9	87.7 ± 37.8	10.33 ± 0.58^{A}
	2%	53.53 ± 20.43^{B}	161.6 ± 10.0	59.4 ± 21.6	9.00 ± 1.00^{ABC}
	3%	55.76 ± 12.47^{B}	164.7 ± 37.7	49.6 ± 26.5	9.91 ± 0.87^{AB}
	4%	57.13 ± 30.52^{B}	199.4 ± 63.2	47.0 ± 19.9	9.77 ± 0.88^{AB}
2nd sample: 4.5 days after infection WSSV (22 °C)	Negative control	27.58 ± 13.14	143.6 ± 9.3	30.0 ± 11.2	8.74 ± 1.08
	Control	23.21 ± 7.12^{B}	135.4 ± 17.7	38.9 ± 21.0	10.02 ± 0.58^{AB}
	1%	25.89 ± 12.90^{B}	139.1 ± 13.9	25.4 ± 13.1	8.02 ± 0.73^{BC}
	2%	21.00 ± 8.77^{B}	131.1 ± 12.5	19.8 ± 1.8	8.45 ± 0.56^{ABC}
	3%	27.73 ± 15.22^{B}	109.5 ± 24.9	43.3 ± 16.5	8.42 ± 0.38^{ABC}
	4%	16.83 ± 4.90^{BC}	99.4 ± 11.5	32.3 ± 2.9	8.06 ± 0.93^{BC}
3 rd sample: 7 days after infection WSSV (28 °C)	Negative control	14.71 ± 5.68	115.2 ± 6.0	15.4 ± 4.5	8.63 ± 0.40
	Control	3.76 ± 3.54^{C}	117.1 ± 14.5	14.8 ± 8.0	8.4 ± 0.65^{ABC}
	1%	6.03 ± 6.88^{C}	114.2 ± 44.0	18.4 ± 11.6	7.37 ± 0.48^{C}
	2%	8.28 ± 4.40^{C}	115.9 ± 5.8	21.3 ± 9.3	8.09 ± 0.36^{BC}
	3%	4.70 ± 7.08^{C}	134.1 ± 24.3	22.4 ± 11.3	8.0 ± 1.00^{BC}
	4%	9.07 ± 7.11^{C}	118.4 ± 23.9	13.3 ± 3.5	9.24 ± 0.54^{ABC}
Factor 1: Feed		0.0207	0.9538	0.7044	0.1715
Factor 2: Time infection/thermal stress		<0.01	<0.01	<0.01	<0.01
Interaction between 1 and 2		0.0309	0.8450	0.4169	<0.01

Data presented as mean ± standard deviation. *p*-value for Factorial ANOVA. Different letters in each column indicate significant differences by Tukey's test at a significance level of 5% for the interaction between factor 1 and 2. The negative control data were not statistically analyzed and served only as a standard to visualize the infection compared to other treatments.

Regarding the negative control group, although the thermal stress was not able to cause any mortality, it caused significant disturbances in the hemato-immunological parameters. The variation profile of hemato-immunological parameters evaluated highlights that supplementation can mitigate the effects of low temperatures, with or without infection, depending on the level of supplementation.

4. Discussion

4.1. Growth Performance and Diet Composition

During the entire rearing period, the animals from the different dietary treatments were kept under a suboptimal water temperature of 22 °C. However, their final survival was not affected by this temperature, remaining above 96.7% in shrimp from all dietary

treatments. The survival demonstrates the adaptability of the species to the temperature range of 22 °C and the importance of not having fluctuations in temperature, despite being at a temperature below the ideal recommended for the species. No statistical differences were identified regarding the growth performance parameters among the treatments.

Considering the low temperature and the high density (100 shrimp/m^3) used in the experiment, with a high survival rate, the weekly growth was higher than that found by other authors. Walker et al. [1] found values below those found in the present study for the weekly growth of shrimp grown at 20 and 24 °C (0.14 to 0.49 g $week^{-1}$) in clear water culture conditions with constant renewals and lower density. Prates et al. [11] subjected shrimp to treatments at low temperatures (20 and 24 °C) in a biofloc system with super-intensive density (300 shrimp/m^3) and found weekly growth rates of 0.33 to 0.75 g $week^{-1}$, values below those found in the present study, despite the use of flocs as a dietary feed supplement. These better results from the present study compared to those mentioned may be related to the better fatty acid profile of the diets.

It is known that different factors such as rearing temperature, life stage, lipid source, and so on can affect the lipid dynamic and requirements in aquatic animals [29,44]. In the present study, the control diet had 2.2% soy lecithin inclusion and a total lipid content of 8.1%; meanwhile, the soy lecithin inclusions and total lipid content of the supplemented diets varied from 1.5 to 1.1% and from 8.1 to 10.8% DM, with increasing levels of *Aurantiochytrium* sp. meal, respectively. These inclusion ranges did not significantly increase the growth performance of the animals. However, they maintained them at levels similar to those of animals raised at optimal temperatures. This fact demonstrates that the dietary inclusion of *Aurantiochytrium* sp. meal is probably safe for the cultivation of *P. vannamei* under suboptimal temperature (22 °C), since they resulted in a weekly growth of around 1.02 g.

At suboptimal temperatures, the addition of phospholipids (4.22% of soy lecithin) improved the weight gain of Nile tilapia (*Oreochromis niloticus*) reared at 22 °C [45]. For shrimps, a previous study by Zhang et al. [46] found better overall health outcomes for *P. vannamei* on diets containing 10–12% lipids and only 1% dietary lecithin at 28 ± 2 °C. Liou et al. [47] observed the best growth and immune responses of *P. vannamei* at 29.0 ± 1.0 °C with 11% lipids and 1.8% soy lecithin. In another study, diets including 5% fish oil supplemented with 3.1% soy lecithin (97.6% phospholipids) resulted in effective improvement in shrimp growth compared to other sources of plant oil, at 32.8 ± 1.0 °C [48]. The best performance of shrimp grown at optimal temperatures in comparison with the present study may be related to the use of lipid sources and fatty acids in the diet for growth, and not just for maintaining membrane fluidity and the immune system.

Shrimp can activate some lipid mediators and related metabolic pathways to regulate the balance of the organism itself when in a situation of temperature fluctuations [49]. The presence of phospholipids may have contributed to the shrimp response to cold. González-Félix et al. [48] noted that sources of LC-PUFA are as important as phospholipid intake. Shrimp that were fed diets including plant oils associated with soy lecithin did not improve performance when compared to animals fed diets including fish oil without lecithin. This may be since fish oil has high levels of n-3 fatty acids, which are less significant in plant oils. The isolated inclusion of EPA or DHA in the diet of *P. monodon* increased the growth of the animals, with the level of 4% of both EPA and DHA separately, within diets with 7.5% of total lipid, promoting maximum growth [50]. In the present study, shrimp fed diets with the inclusion of 1, 2, 3, and 4% *Aurantiochytrium* sp. meal had DHA contents of 0.48, 0.73, 0.91, and 1.11%, respectively, while shrimp fed the control diet had about 0.25% DHA corroborating the results of Gonzalez-Felix et al. [48], where the inclusion of soy lecithin in all diets may have reduced the DHA requirement of shrimp.

In a study conducted by Nobrega et al. [29] with Nile tilapia (*Oreochromis niloticus*) at a suboptimal temperature (22 °C), it was reported that the dietary supplementation with *Aurantiochytrium* sp. meal in the range of 0.45 to 1.42%, as a source of DHA, resulted in better growth, body fat composition, and n-3:n-6 PUFA ratio in muscle than the dietary

supplementation with cod liver oil. In this range of *Aurantiochytrium* sp. intake (diets with 0.5, 1, and 2%), a total of about 0.15 to 0.58% LC-PUFA was obtained. In our diets, the total range of *Aurantiochytrium* sp. uptake resulted in 0.33 to 1.23% of LC-PUFA and showed no difference in growth performance. However, it is worth noting that there are significant differences between the nutrient requirements of *P. vannamei* and tilapia for phospholipids and LC-PUFA.

Shrimp, as marine organisms, have a greater affinity and requirement for these compounds than Nile tilapia [15], which can be cultured without an n-3 source when the growth temperature is within the optimal range [51]. In the present study, the tested suboptimal temperature with the increase of the dietary n-3:n-6 ratio and the addition of the ingredient rich in DHA and PUFA beyond the known requirement had no adverse effects on shrimp survival and growth. On the contrary, the shrimp appeared to adapt well to the low temperature in a system without fluctuations, and no differences in growth performance were observed even when the n-3:n-6 ratio was increased with the increase in feed additive dose.

Farmed shrimp tend to have their feeding behaviour affected by low temperature, which may reduce feed intake and remain immobile inside the tank [52]. However, this was not the case in our study, in which the animals showed active feeding behaviour and consumption, with low feed waste. This may be related to the restrict water temperature control, stabilizing the temperature along the experiment, and the adequate temperature acclimatization of the animals prior to the experiment.

Still, the suboptimal temperature affected the feeding rate, which was less efficient when compared to studies carried out at ideal temperatures [53,54]. Similar values of feed conversion and survival were found in a study using increasing levels of the same *Aurantiochytrium* sp. meal in replacement of fish oil for *P. vannamei* reared at an optimal temperature of 28 °C [55].

4.2. Microbiological and Hemato-Immunological and Analyses and Challenge with WSSV in Association with Thermal Stress

There were no significant differences in the quantification of total heterotrophic and total vibrionaceous bacteria. The presence of vibrio bacteria is natural in the marine environment and intestinal tract of shrimp. The experiment's water changes were performed daily at rates of 80–100% of the total volume, a factor that influenced the maintenance of low levels of nitrogenous compounds in the system, possibly controlling the proliferation of vibrionaceous bacteria. *Vibrio* spp. can grow over a wide range of temperatures (0.5 °C to 48 °C), but there is evidence that its growth rate is accelerated at higher temperatures, such as in the species of *V. parahaemolyticus* and *V. alginolyticus* at 37 to 42 ºC [56]. The total bacteria count is in line with results found in previous studies with similar methodology, as the *Vibrio* spp. represents lower values than those found in the same studies, at optimal temperatures [41,57,58]. Suboptimal temperatures may slow *Vibrio* spp. growth [56].

Regarding the challenge trial, at low temperature phase (22 °C), supplementation with *Aurantiochytrium* sp. meal at the concentrations of 3% and 4% (Table 5) increased the resistance of the animals to viral infections, being even statistically significant when compared to the control group (0%). However, when the temperature increased to 28 °C during the challenge, the group supplemented with 4% *Aurantiochytrium* sp. meal was the only one that resulted in greater survival and therefore greater resistance to WSSV.

This finding may be associated with the composition of *Aurantiochytrium* sp., which has β-1,3-glycans in the cell wall [59]. This polysaccharide has properties that promote animal health, such as antioxidant, anti-inflammatory, and immunostimulant action [60–62]. In marine shrimp, different studies have shown that dietary supplementation of β-1,3-glycans promoted greater resistance against WSSV infection [63,64]. These results are in line with what was observed in the present study.

In parallel, *Aurantiochytrium* sp. can produce LC-PUFA such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and others [65], but is particularly rich in DHA [22]. In addition, it is able to synthesize other bioactive compounds such as carotenoids, especially

squalene [66]. These compounds can be essentially useful in increasing the cold resistance of ectothermic aquatic animals and minimizing their deleterious effects, as temperature reduction can lead to loss of membrane fluidity, transmembrane protein dysfunction, protein degradation and aggregation, oxidative damage, and others [12,13,67].

Variation in the fatty acid composition of phospholipids is particularly important during the process of adaptation to temperature changes. Generally, there is an increase in the proportion of PUFA and monounsaturated fatty acids (MUFA) and a reduction of saturated fatty acids (SFA) in membrane phospholipids [68,69]. The increase in PUFA maintains the liquid-crystal state of the lipid matrix, which allows the membrane to continue functioning properly, to compensate for the cold-induced solidification of the lipid bilayers [70,71].

In this sense, this rich constitution of bioactive compounds of *Aurantiochytrium* sp. may be associated with the beneficial effects observed in the present study, particularly the increase in shrimp resistance against viral infection at low temperature, and more specifically, the supplementation levels of 3 and 4%. However, when the shrimp were submitted to thermal stress by raising the temperature, the animals supplemented with 3% of *Aurantiochytrium* sp. showed high mortality, matching the values of the control (0%). This low tolerance to thermal oscillation of animals from 3% treatment was not fully understood, requiring further investigations.

Hemato-immunological parameters showed a significant reduction throughout the bioassay, demonstrating the immunosuppressive effect of viral infection. This effect has already been demonstrated in several studies with WSSV infection [72–76]. The decrease in immune parameters was also observed in the NC group (uninfected), which highlights that heat stress can have deleterious effects with immune system disorders, as reported by Qiu et al. [77] and Zhou et al. [78]. These results reinforce the importance of developing new technologies to minimize the effects of heat stress, especially in breeding farms in subtropical regions.

Prior to the virus challenge, animals raised at suboptimal temperature and fed a 1% feed supplement with *Aurantiochytrium* sp. meal were generally superior in immune parameters. However, this was not reflected in the mortality and immune response of the animals after virus infection, which had only the second lowest mortality at the end of the challenge.

5. Conclusions

The dietary inclusion of *Aurantiochytrium* sp. meal had no effect on the growth performance of *P. vannamei* cultivated at suboptimal temperatures. However, the 4% dose resulted in lower mortality of shrimp after the viral challenge with WSSV associated with heat stress.

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Data Availability Statement: Data are contained within the article, and are available from the corresponding author upon reasonable request.

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