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Effects of dietary nucleotides supplementation on growth, total haemocyte count, lysozyme activity and survival upon challenge with *Vibrio harveyi* in pacific white shrimp, *Litopenaeus vannamei*

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ABSTRACT

The use of soybean meal (SBM) as replacement for fish meal (FM) in diets for Pacific white shrimp (PWS), *Litopenaeus vannamei*, involves a negative impact on the health of PWS. Dietary nucleotides modulate the immune response; therefore, they might be able to counteract this effect by enhancing PWS immunity. Based on this hypothesis, this study was aimed at evaluating the effects of nucleotide supplementation in PWS receiving diets in which FM had been partially replaced by SBM. A 70-day feeding trial was conducted to evaluate the effects of dietary nucleotides on PWS growth performance, protein levels and retention rate, total hemocyte count (THC) and lysozyme activity. Ten experimental diets were formulated. The control diet included 10% FM and 43% SBM. Another diet was formulated by reducing FM to 5% and by increasing SBM up to 50%. The rest of diets included 0.05 or 0.1% nucleotide supplementation with varying degrees of FM replacement by SBM. A total of 900 PWS post larvae with an average initial body weight of 4.24 ± 0.03 g were randomly assigned to ten study groups, with six replicates per group and 15 PWS per aquaria tank. After the performance trial, disease resistance was evaluated in a 7-day challenge test with *Vibrio harveyi* at the consistent concentration of 10^5 CFU mL^{-1}. Nucleotide supplementation led to significantly higher THC and lysozyme activity ($P < 0.05$) and a significantly increased PWS survival in the *V. harveyi* challenge test ($P < 0.05$). No significant differences were found between groups regarding growth parameters or protein analyses ($P > 0.05$). In conclusion, the present study shows a positive impact of nucleotide supplementation on immune response and disease resistance against *V. harveyi*. Nucleotides could therefore be used as functional dietary ingredients, especially in PWS which receive diets with FM replacement by plant-protein sources.

1. Introduction

The production of Pacific white shrimp (PWS), *Litopenaues vannamei,* as the most popular cultured shrimp species worldwide, has experienced an increasing trend and has provided significant economic value in the global market over the past few years (FAO, 2020a; Anderson et al., 2019; Roy et al., 2009; Tacon and Metian, 2008). This trend involves an increase in demand for high-quality feed to fulfill the specific nutrient requirement and hence optimize the growth and health status of PWS (Sookying, 2010). In order to produce good quality feed, fish meal (FM) has traditionally been considered as the preferred protein source because of its nutritional properties, content in essential amino acids, fatty acids, vitamins, minerals and growth factors, and because of its high palatability and absence of anti-nutritional factors. Recently, the development of cost-effective practical diets to support the optimum growth of PWS has been established by using 12% FM inclusion level. Lower amounts might negatively affect feed intake and, consequently, limit weight gain (Suárez et al., 2009). However, as the margin decreases, feed manufacturers and fish producers prefer to use alternative protein sources to produce more cost-effective diets (Sookying et al., 2013). Several alternative protein sources have been evaluated to replace FM and other animal proteins. This has resulted in increased usage of plant-based proteins, such as soybean meal (SBM), cotton seed meal, and corn gluten meal (Cook et al., 2016; Molina-Poveda et al., 2015; Riche, 2015; Zhou et al., 2015; Lech and Reigh, 2012; Riche and Williams, 2011; Yang et al., 2009; Lim, 1996).

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Among alternative protein sources, processed soybean (*Glycine max*) is a valid, inexpensive candidate to replace FM thanks to its nutritional qualities (Lech and Reigh, 2012). Crude soybean is widely available, highly digestible, easily shipped and stored, and competitively priced, compared to other vegetable protein sources (Amaya et al., 2007; Gatlin et al., 2007; Davis and Arnold, 2000). In addition, SBM has a favorable amino acid profile, with exception of lysine, and total sulfur-based amino acids (methionine + cystine), and it is also reported to provide adequate amounts of gross energy, crude lipid and crude protein digestibility in PWS (Yang et al., 2009). However, SBM contains anti-nutritional factors, such as lecithin, phytic acid, saponins, phytosterols and allergens (NRC, 2011), which limit a wider use of such protein source. Therefore, given the current situation, there is a need for novel functional ingredients with the ability to improve PWS health and counteract the negative effects of SBM if used as replacement for FM.

Nucleotides are low molecular weight intracellular compounds that play key roles in biochemical processes. Sources include *de novo* synthesis, recovery via salvage mechanisms, and dietary intake. Exogenous nucleotide supply can improve health and might become essential in certain situations where there is physiological stress, immunosuppression, or infection (Hess et al., 2012). Nucleotides have not traditionally been used in aquaculture precisely because they are normally not considered essential from a nutritional standpoint (Xiong et al., 2018; Andrino et al., 2012; NRC, 2011). However, since several published articles mention that nucleotide deficiency may impair liver, heart, intestine, and immune functions (Xiong et al., 2017; Li et al., 2007; Grimble et al., 2000a), nucleotide administration through diet might be adequate to guarantee their supply and availability to the aquatic organisms, especially during the high demand for various physiological and metabolic processes (Hossain et al., 2020; Whitehead et al., 2006). A prior study in which nucleotides were added to PWS diets with high FM inclusion level (Guo, 2016; Cheng et al., 2011a) showed that, although there was no significant effect to the survival, growth and whole-body composition of shrimp, a positive effect was achieved on gut health, immune response and disease resistance to *Vibrio parahaemolyticus*. On the other hand, a recent study revealed a beneficial effect of nucleotide supplementation on performance of gilthead seabream receiving a diet in which FM had been replaced by a mixture of plant and animal protein sources, including SBM. More specifically, adding nucleotides resulted in increased final body weight, weight gain, and specific growth rate and exerted a positive impact on liver enzymes and on the expression of several beneficial genes (El-Nokrashy et al., 2021). Moreover, in another study with 46% inclusion level of FM, the supplementation with 0.2–0.6% nucleotides enhanced the specific growth rate, feed conversion efficiency, total haemocytes count, respiratory burst activity, phenoloxidase activity and survival against White Spot Syndrome Virus (WSSV) infection in shrimp fed diets containing nucleotides as compared to those fed without nucleotide supplementation (Andrino et al., 2012).

The aim of the present study was to investigate the effects of dietary nucleotide supplementation on performance, protein levels and retention rate, immune response, and disease resistance in PWS receiving diets with low FM levels and high inclusion levels of SBM.

2. Material and methods

2.1. Experimental diets

The composition and proximate analysis of the experimental diets are presented in Table 1. All diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid). In this growth trial, control diet was designed with 10% FM, 43% SBM and 10% corn gluten meal as the primary ingredients without nucleotide supplementation. Diet 1 was formulated by replacing 5% FM with SBM, without nucleotide supplementation. Diet 2 and 3 were formulated by incorporating two inclusion levels (0.05% and 0.1%) of nucleotides (Nucleoforce™, Bioiberica, S.A. U., Esplugues de Llobregat, Spain) of yeast origin. Diets 4–9 were formulated by using 0.05 or 0.1% nucleotides and varying degrees of FM replacement by SBM. All experimental diets were produced at the Main Center of Mariculture Development of Lampung (Lampung, Indonesia)

Table 1
Composition (% *as is*) of diets containing two inclusion levels of nucleotide and fed to L. *vannamei* for 70 days.

Ingredients (% *as is*)	Diet code									
	Control	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9
Menhaden Fishmeal[a]	10.00	5.00	10.00	10.00	8.00	8.00	6.00	6.00	3.00	3.00
Soybean meal[b]	43.00	50.00	45.85	45.85	44.80	44.80	47.50	47.50	51.85	51.85
Corn Gluten Meal[c]	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Menhaden fish oil[c]	5.64	5.64	5.64	5.64	5.64	5.64	5.64	5.64	5.64	5.64
Soy-Lecithin[c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Nucleotides[d]	0.00	0.00	0.05	0.10	0.05	0.10	0.05	0.10	0.05	0.10
Corn starch[c]	8.06	6.06	5.16	5.16	8.21	8.21	7.51	7.51	6.16	6.16
Wheat products[c]	17.00	17.00	17.00	17.05	17.00	17.05	17.00	17.05	17.00	17.05
Mineral premix[e]	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
Vitamin premix[f]	1.90	1.90	1.90	1.90	1.90	1.90	1.90	1.90	1.90	1.90
KP-dibasic[c]	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Choline chloride[c]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Proximate analysis (% *as is*)[g]										
Crude protein	35.72	34.91	35.84	35.81	35.42	35.77	35.48	35.77	35.39	35.24
Lysine	1.89	1.74	1.83	1.92	1.79	1.84	1.72	1.78	1.74	1.74
Methionine	0.84	0.82	0.88	0.87	0.83	0.87	0.76	0.84	0.71	0.75
Moisture	8.12	8.78	8.14	8.24	8.35	8.49	8.51	8.48	8.11	8.74
Crude Fat	7.88	7.02	7.72	7.79	7.64	7.71	7.59	7.52	7.39	7.44
Crude Fiber	2.89	3.55	3.14	3.22	3.37	3.41	4.42	4.39	4.83	4.66
Ash	6.11	7.35	6.56	6.78	6.43	6.29	6.33	6.53	6.47	7.01

[a] High protein fish meal (Peru) supplied by Agri Permata Asia, Jakarta, Indonesia.
[b] De-hulled solvent extract soybean meal, Bogor Ingredients, Indonesia.
[c] Supplied by Bogor Ingredients, Indonesia.
[d] Nucleoforce™, Bioiberica SAU, Barcelona, Spain.
[e] Trace mineral premix (g/100 g premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.550; ferrous sulfate, 2.000; magnesium sulfate anhydrous, 13.862; manganese sulfate monohydrate, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193; alpha-cellulose, 69.664.
[f] Vitamin premix (g/kg premix): thiamin·HCL, 4.95; riboflavin, 3.83; pyridoxine·HCL, 4.00; Ca-pantothenate, 10.00; nicotinic acid, 10.00; biotin, 0.50; folic acid, 4.00; cyanocobalamin, 0.05; inositol, 25.00; vitamin A acetate (500,000 IU/g), 0.32; vitamin D3 (1000,000 IU/g), 80.00; menadione, 0.50; alpha-cellulose, 856.81.

using standard procedures for manufacturing shrimp feed and labeled as control diet and diets 1–9. Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until use.

2.2. Growth trial

The growth trial was conducted at the PT. Batam Dae Hae Seng research station (Batam, Indonesia). A total of 900 PWS post larvae (PL) were obtained from PT. Maju Tambak Sumur (Kalianda, Lampung, Indonesia) and were acclimatized to the culture system. PL were fed with commercial feed (Evergreen Feed, Lampung, Indonesia) for three weeks until they reached the suitable size. PWS (4.24 ± 0.03 g initial mean weight) were then randomly distributed into 60 tanks with size of 70 × 35 × 40 cm (98 L per aquaria tank). Six replicate groups of PWS were administered different types of experimental diets using nutrition research standard protocol for 70 days and fed by hand four times daily, at 07:00, 11:00, 15:00 and 20:00 h. The formulae to calculate the daily feed inputs (g) were presented as follows:

$$\text{Daily feed inputs (g)} = \text{Estimated FCR} \times \text{Expexted Growth} \times \text{number of shrimp} /7$$

Feed inputs were pre-programmed assuming the normal growth of shrimp with an estimated feed conversion ratio of 1.5 across the growth trial. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Uneaten feed, feces, and molts were removed by siphoning the aquaria tank prior to the first feeding.

2.3. Water quality and growth sampling

For water quality analysis: pH, dissolved oxygen (DO), water temperature and salinity were measured four times daily using Aqua TROLL 500 Multiparameter Sonde instrument and connected to AquaEasy apps (Bosch, Singapore) for data monitoring and recording system. Total ammonia-nitrogen (TAN), nitrate and nitrite were measured once in a week by using absorption spectrophotometry (DR890, HACH, USA). At the end of feeding period, all shrimp were grouped and individually weighed to calculate the final biomass, final body weight (FBW), percentage of weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR) and thermal unit growth coefficient (TGC) as follows:

$$\text{Protein retention rate (\%)} = \frac{(\text{final weight} * \text{final protein})\text{-}(\text{initial wiight} * \text{ini.protein})}{\text{intotal protein intake (dry matter)}} \times 100$$

$$\text{PWG} = \frac{(\text{average individual final weight-average individual initial weight})}{(\text{average individual initial weight})} \times 100$$

$$\text{FCR} = \frac{feed\ \ given\ \ (g)}{alive\ \ weigh\ \ gain\ \ (g)}$$

$$\text{SR} = \frac{final\ \ number\ \ of\ \ fish}{initial\ \ number\ \ of\ \ fish} \times 100$$

$$\text{TGC} = \frac{\text{FBW}^{1/3}\text{- IBW}^{1/3}}{\sum \text{TD}} \times 100$$

where FBW is final body weight, IBW is initial body weight, T is water temperature (°C) and D is number of trial days.

2.4. Total hemocyte count

At the end of growth trial, hemolymph was sampled from two intermolt shrimp per tank or ten PWS per treatment and total hemocytes count was determined. Hemolymph (100 μL) of individual shrimp was withdrawn from the pleopod base of the second abdominal segment with a sterile 1-mL syringe (25 G × 13 mm needle). Before hemolymph extraction, the syringe was loaded with a precooled (4 °C) solution (10%-EDTA, Na2) used as an anticoagulant. The hemolymph with anticoagulant solution were diluted in 150 μL of formaldehyde (4%) and then 20 μL were placed on a hemocytometer (Neubauer) to determine the total hemocyte count (THC) using an optical microscope (Olympus, DP72).

2.5. Lysozyme activity analysis

Lysozyme activity was measured by using a lysozyme detection kit (Sigma-Aldrich, Cat. no. LY0100) according to the manufacturer's instructions. The results of lysozyme activity were defined by the lysis of the *Micrococcus lysodeikticus* cells. The reactions were conducted at 25 °C and absorbance at 450 nm was measured on the ultraviolet/visible spectrophotometer (Perkin Elmer, Lambda XLS, USA).

$$\text{Lysozyme activity}\left(\frac{\text{Units}}{\text{mL}}\right) = \frac{(\Delta\text{A450/min}Test - \Delta\text{A450/min}Blank)(df)}{(0.001)(0.03)}$$

df=dilution factor
$0.001 = \Delta A_{450}$ as per the unit definition
0.03 = Volume (in mL) of enzyme solution

2.6. Protein level and retention analysis

Upon termination of the trial, four PWS from each tank (i.e. twenty-four per treatment) were randomly sampled and stored at −60 °C for body composition analysis. Prior to the protein analysis, dried whole shrimp were rigorously blended and chopped in a mixer according to the standard methods established by Association of Official Analytical Chemists (AOAC, 1990). Protein content of whole shrimp body were analyzed by using Kjeldahl method and conducted at PT. Angler Bio-Chem Lab (Surabaya, East Java, Indonesia). Protein retention rate (%) was calculated using formula as follows:

2.7. Challenge test

2.7.1. Bacterial strains

Pure cultures of *Vibrio harveyi* were obtained from the Batam Nara Laboratory (Batam, Indonesia). The isolates of the bacterial strains, previously stored in 30% glycerol at −80 °C, were aseptically inoculated in 30 mL marine broth by incubation overnight at 25–28 °C with constant agitation. 150 μL was subsequently transferred and grown to stationary phase in 30 mL marine broth six hours before challenge. The bacterial densities were determined spectrophotometrically at an optical density of 550 nm, and were calculated using the equation: Concentration (CFU/mL) = [1200 * 10^6 *OD] according to McFarland standard,

(BioMerieux, Marcy L'Etoile, France), assuming that an $OD_{550} = 1.000$ corresponds to 1.2×10^9 cells/mL.

2.7.2. Challenge with Vibrio harveyi

After the sampling campaign, all tanks consist with 7 shrimps received a suspension of *Vibrio harveyi* to reach the density of 1×10^5 CFU/mL. Each group of treatments had 6 replicates and were fed 4 times per day using the last amount of feed during the growth trial. The culture experiment was continued for 7 days and every day 25% of water was changed to avoid deterioration of the water quality, and water containing the corresponding strain concentration was added to maintain the concentration. Shrimp mortality determination was performed every day during the 7 days of the challenge test. Cumulative mortality rate was calculated.

2.8. Statistical analysis

All data were analyzed using one-way analysis of variance to determine the significant difference ($P < 0.05$) among the treatment means followed by the Tukey's multiple comparison test to determine difference between treatments means in each trial. The pooled standard errors were used across all the growth parameters as the variance of each treatment is the same. Statistical analyses were conducted using SAS system (V9.4, SAS Institute, Cary, NC, USA).

3. Results

3.1. Water quality

Variations in morning and afternoon pH, salinity (‰), water temperature (°C) and dissolved oxygen (mg L^{-1}), together with ammonia (mg TAN L^{-1}) and nitrate (mg NO_2-N L^{-1}) are displayed in Table 2. All these parameters remained within the acceptable ranges for L. *vannamei.*

3.2. Growth and body composition analysis

Performance parameters are presented in Table 3. Nucleotide supplementation led to better performance results, especially in the study groups in which FM had been partially replaced by SBM, but no significant differences were found between groups ($P > 0.05$). Protein level and protein retention results are shown in Fig. 1. Since all diets were produced by using highly digestible ingredients and targeting similar protein and lipid level, there were no significant differences ($P > 0.05$) in terms of protein level in the whole body of PWS nor in protein retention rate, as expected. However, the inclusion of nucleotides in general could enhance the nutrient utilization in shrimp similarly to the group of PWS fed with control diet. Interestingly, a reduction in FM and the supplementation with nucleotides did not affect the protein deposition in shrimp.

3.3. Immune parameters

The supplementation with nucleotides allowed a significant increase ($P < 0.05$) in THC and lysozyme activity, compared to the group without nucleotide supplementation in which FM had been partially replaced by SBM (Figs. 2 and 3). A dose effect was observed for nucleotides, with 0.1% achieving better results than 0.05%.

Table 3
Growth performance of pacific white shrimp L. *vannamei* (Mean initial weight 4.24 ± 0.03 g) fed experimental diets for 70 d. Values represent the mean of six replicates. Results in the same columns with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test.

Diet code	Final Biomass (g)	Final Mean Weight (g)	Survival (%)	WG (%)	FCR[2]	TGC[3]
Control diet	217.43	16.50	87.78	290.99	2.80	0.0463
Diet 1	222.97	15.74	96.67	272.78	2.95	0.0443
Diet 2	219.02	16.21	90.00	283.59	2.85	0.0455
Diet 3	215.41	16.65	86.67	294.89	2.79	0.0467
Diet 4	232.42	16.58	93.33	292.00	2.80	0.0464
Diet 5	235.40	16.76	93.33	298.48	2.78	0.0469
Diet 6	231.73	16.55	93.33	291.52	2.79	0.0464
Diet 7	215.37	16.38	87.78	286.60	2.83	0.0458
Diet 8	230.98	16.33	94.43	286.09	2.83	0.0472
Diet 9	225.55	16.86	88.89	300.38	2.75	0.0472
P-value	0.5834	0.6238	0.1194	0.5738	0.5358	0.5855
PSE[4]	6.8429	0.2924	2.1505	7.0733	0.0469	0.0007

Note: [1]WG = Weight gain; [2]FCR = Feed conversion ratio; [3]TGC = Thermal growth coefficient; [4]PSE = Pooled standard error.

3.4. Response to Vibrio harveyi challenge

The effects of different study diets on the survival of shrimp *L. vannamei* after the challenge test with *V. harveyi* are depicted in Fig. 4. All nucleotide-supplemented diets provided significantly better survival rates ($P < 0.05$) compared to the control diet, but also compared to the group fed with a diet without nucleotide supplementation and with partial FM replacement by SBM (Diet 1). The highest survival rates were achieved with diets 3 and 5 with 10% and 8% inclusion levels of FM, respectively, and supplemented with 0.1% nucleotides.

4. Discussion

In the coming years, the development of sustainable practical diets will depend on the reduction of FM together with the increase in inclusion levels of plant-protein sources, such as SBM (Novriadi and Davis, 2018). However, the presence of anti-nutritional factors and reduced amount of sulfur-containing amino acids limit the wider use of SBM in the diet formulation (Novriadi et al., 2019). Therefore, the use of functional ingredients such as nucleotides to improve the health of aquatic organisms, especially in PWS, could become a useful tool to counteract the negative effects of replacing FM by SBM.

In this study, a significant effect of nucleotide supplementation as a functional ingredient was observed on the immune parameters and resistance of PWS to pathogens. Although the exogenous supply of nucleotides could not provide a statistically significant improvement in growth performance of PWS, biologically, all groups supplemented with nucleotides showed better growth compared to the group of PWS fed with low inclusion levels of FM and without nucleotide supplementation. This goes in line with the study from (Guo et al., 2016), in which the supplementation with nucleotides in low FM diets ranged from 0% to 0.12% and had no significant effect on growth of shrimp after a 10-week feeding trial. In addition, Andrino et al. (2012) also found no significant difference in terms of growth when L. *vannamei* were fed with diets

Table 2
Overall water quality measurements during the grow-out phase of the experiment. Data were presented as mean ± standard deviation (range).

Time	Parameter					
	Temperature (°C)	D.O (mg L^{-1})	pH	Salinity (‰)	Ammonia (mg TAN L^{-1})	Nitrate (mg NO_2-N L^{-1})
AM	28.89 ± 0.38	5.43 ± 0.44	7.62 ± 0.33	25.24 ± 2.02	0.08 ± 0.07	31.33 ± 4.88
PM	29.74 ± 0.58	6.02 ± 0.48	7.86 ± 0.48	25.93 ± 2.62		

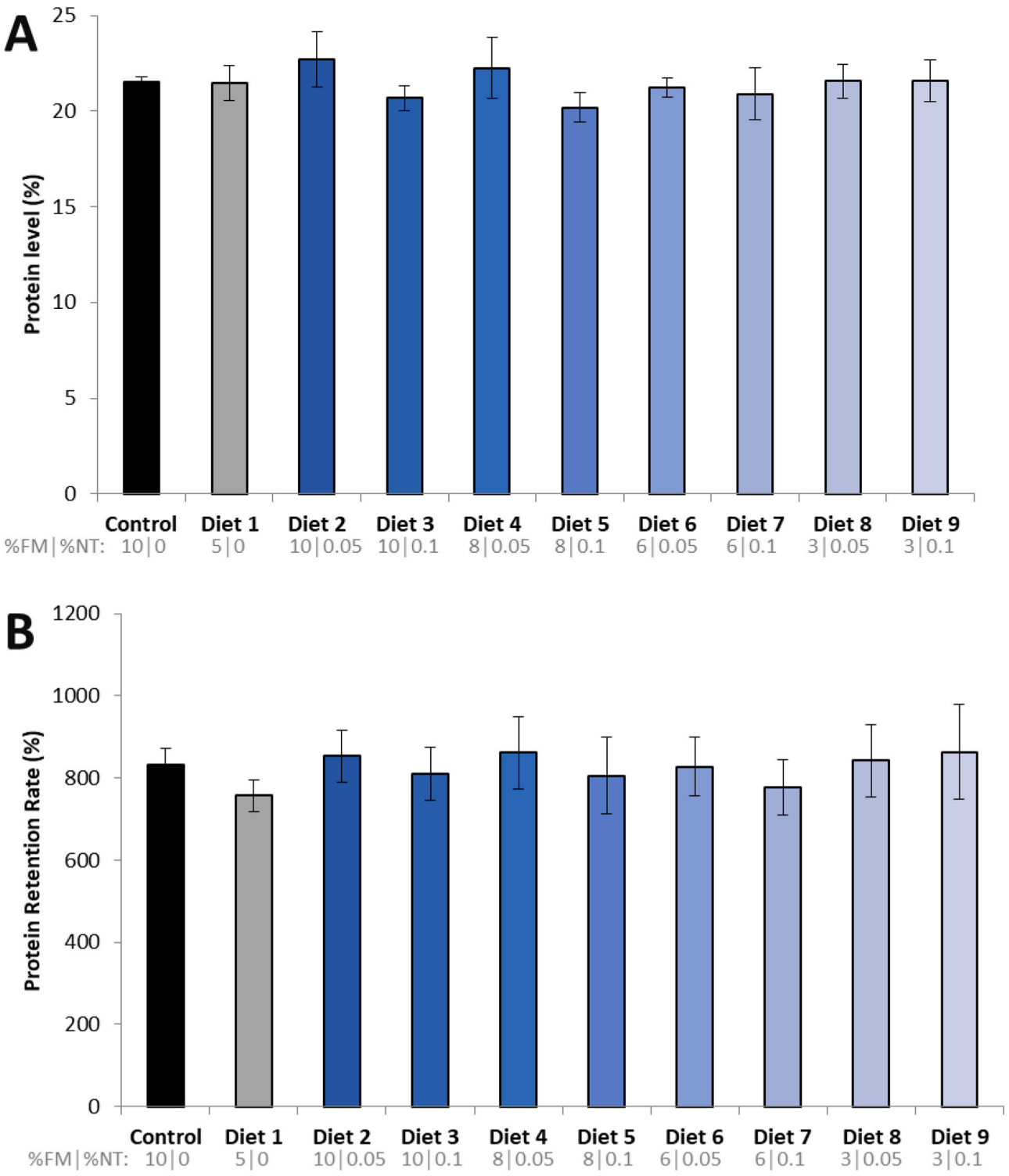

Fig. 1. Protein level (A) and protein retention rate (%) (B) of whole body of Pacific white shrimp L. *vannamei* fed experimental diets for 70 d. Values represent the mean of four replicates. Fish meal (FM) and nucleotides (NT) inclusion levels (%) are described below the name of the diet.

containing 0.2%, 0.4% and 0.6% nucleotides. According to (Guo et al., 2016), variations in size caused by the extended length of growth trial (60–70 days) could explain this lack of statistical significance. In addition, differences in the source and concentrations of nucleotides could explain the different outcomes, as also highlighted by Xiong et al. (2018). In our study, we stocked the shrimp with an average initial weight of 4.24 ± 0.03 g, while (Guo et al., 2016) started the study with average initial weight of 0.39 ± 0.00 g and Andrino et al. (2012) with average body weight between 0.2 and 0.3 g, which indicates that initial weight might affect the final results. Interestingly, the study from Murthy et al. (2009) and Xiong et al. (2017) showed that the supplementation with 0.5% nucleotide-rich yeast for 30 and 56 days provided significant improvements in growth performance.

It is generally recognized that, under normal conditions, exogenous nucleotide supply has shown positive responses on health status and disease resistance in various aquatic animals (Xiong et al., 2018; Ringø

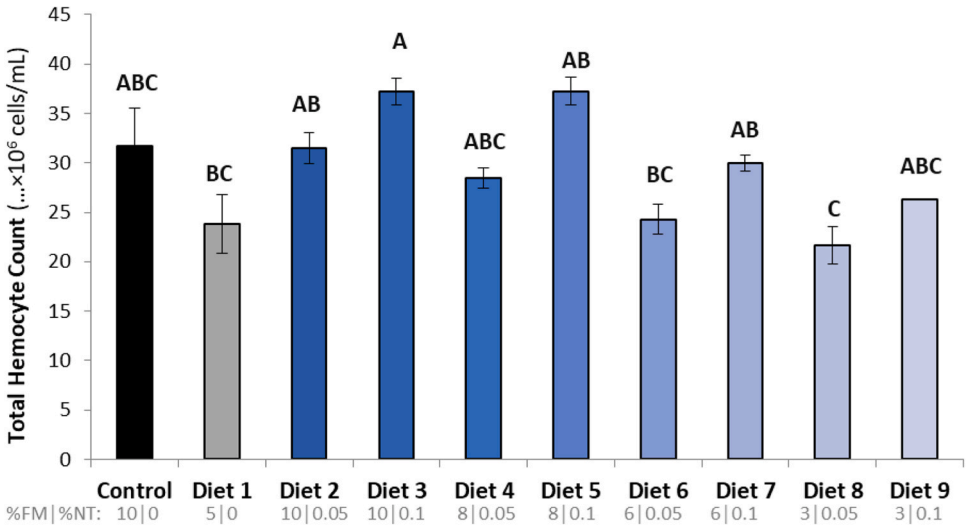

Fig. 2. Total hemocyte count of Pacific white shrimp L. *Vannamei* (10^6 cell mL^{-1}) at the end of growth trial. Values represent the mean of six replicates. Results with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test. Fish meal (FM) and nucleotides (NT) inclusion levels (%) are described below the name of the diet.

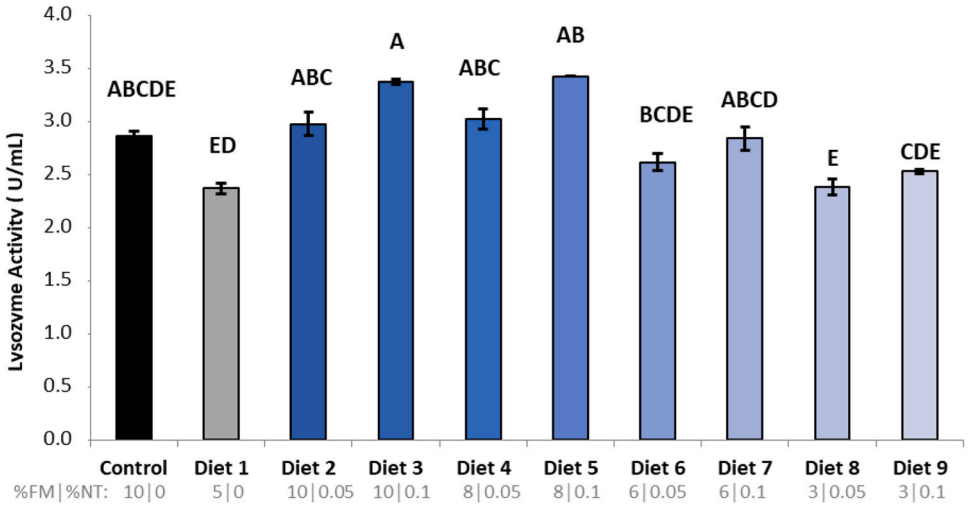

Fig. 3. Lysozyme activity of Pacific white shrimp L. *Vannamei* (U mL^{-1}) at the end of growth trial. Values represent the mean of six replicates. Results with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test. Fish meal (FM) and nucleotides (NT) inclusion levels (%) are described below the name of the diet.

et al., 2012; Murthy et al., 2009; Li and Gatlin, 2006). In the present study, enhanced THC and lysozyme activity were observed in shrimp fed the nucleotide-supplemented diets. We also observed a remarkable decrease in mortality upon challenge with *V. harveyi*. Murthy et al. (2009) also reported that 0.2% and 0.5% nucleotide supplementation significantly increased THC compared to the control group. Similar results were reported by Manoppo et al. (2011), describing that THC in nucleotide-supplemented shrimp significantly increased up to 87% higher than shrimp fed a regular diet. Since crustacean immune responses are based on both cellular and humoral components, hemocytes play a role in the recognition of microorganisms (Söderhäll and Cerenius, 1998). Under the conditions of physiological stresses which increase susceptibility to diseases, the exogenous nucleotide supply through the diet may exert a positive impact to the THC that could enhance the cellular immune response to directly attack the pathogens in shrimp L. *vannamei* (Kaizu et al., 2011).

The lysozyme activity of shrimp L. *vannamei* in this study was increased in the study group supplemented with nucleotides compared to the group fed with low inclusion levels of FM without nucleotide supplementation. Lysozyme is a glycolytic enzyme which is well known for the antimicrobial activity against gram positive and negative bacteria (De La Re Vega et al., 2006; Goarant et al., 1999), including *Vibrio* species that are pathogenic to shrimp (Supungul et al., 2010; Tyagi et al., 2007). Enhanced lysozyme activity in L. *vannamei* following nucleotide supplementation was also noticed by (Guo et al., 2016) with an increased activity from 60 to 120 mg/Kg. Furthermore, Xiong et al. (2018) also reported an increased lysozyme activity after nucleotide-rich yeast supplementation. Our results of elevated THC and lysozyme activity support the incorporation of nucleotides as functional ingredients in PWS diets to improve their immune health status.

In shrimp production systems, vibrios are among the bacterial diseases that cause multibillion dollar losses and severe mortality during the culture system (Defoirdt et al., 2007; Lightner, 1996). In many cases, vibrios may be classified as an opportunist, only causing disease when the host is immunosuppressed or stressed due to the (super) intensive culture and adverse environmental conditions (Aldeman and Hastings, 1998). Among vibrios, *V. harveyi* is one of the most important pathogens that can cause devastation to diverse range of marine invertebrates,

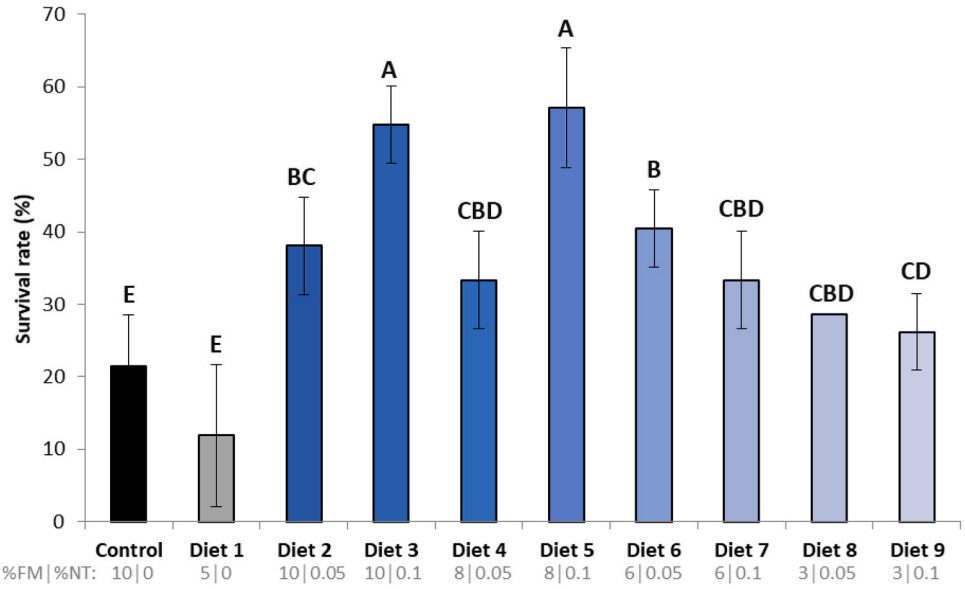

Fig. 4. Survival rates (%) of L. *vannamei* over 7 days period after the challenge test with *Vibrio harveyi* (10^5 CFU/mL). Values represent the mean of six replicates. Results with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test. Fish meal (FM) and nucleotides (NT) inclusion levels (%) are described below the name of the diet.

including L. *vannamei* (Soto-Rodriguez et al., 2010; Robertson et al., 1998;). Data from the present challenge study showed that L. *vannamei* fed with nucleotide-supplemented diets featured significantly higher cumulative survival rate than those fed with diet without exogenous supply of nucleotides. Among the dietary treatments, the inclusion of 0.1% nucleotides in diets with 10% and 8% FM provided the better resistance to *V. harveyi* infection. This agrees with the report from Manoppo et al. (2011) in which a nucleotide-supplemented diet significantly enhanced protection and survival of L. *vannamei* after being challenged with *V. harveyi* at the concentration levels of 0.1×10^6 CFU per shrimp over 14 days using intramuscularly injection method. Not only for *V. harveyi*, the immunomodulatory effects of nucleotides could also enhance the resistance of L. *vannamei* against *Vibrio parahaemolyticus* (Guo et al., 2016) and even against one of the most devastating viral pathogens like white spot syndrome virus (Andrino et al., 2012).

As it had already been reported previously in other aquatic species (El-Nokrashy et al., 2021), in the herein study in PWS, nucleotides were proved to be a useful and convenient tool for counteracting the negative effects of using high inclusion levels of SBM to replace the use of FM in the diet formulation. The protective effects of dietary nucleotides could be likely due to the enhancing effect on the immune system, as seen on improved THC and lysozyme activity. These results would suggest a promising justification for the use of nucleotides for health management in shrimp aquaculture systems.

Although this work reports health benefits of nucleotide supplementation in PWS, this study has some limitations. One of them is that a proper evaluation of the effects of nucleotides would be more adequate in a study design in which each fishmeal replacement level would be tested with and without such supplementation. In further studies, this issue should be addressed, and the same FM percentage should be maintained. Another limitation is the duration of the study. Considering the normal PWS production systems and the PWS commercial size in Indonesia and other countries, further longer-term studies are warranted.

5. Conclusion

Under the conditions of the present study, nucleotide supplementation showed a positive impact on the immune system and disease resistance against *Vibrio harveyi* in PWS. Nucleotides could therefore be used as functional dietary ingredients to improve the health of PWS. This research also provides an evidence of health-related benefits of incorporating dietary nucleotides when the amount of FM is reduced. Nevertheless, further research is needed in PWS in order to optimize the inclusion levels of nucleotides as well as cost benefits of nucleotides in a wider context throughout the commercial production cycle until shrimp reach the commercial size.

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CRediT authorship contribution statement

Conception and design of study: **Romi Novriadi**, **Sergi Segarra**, **Oriol Roigé**; Acquisition of data: **Romi Novriadi**; Analysis of data: **Ilham Ilham**; Interpretation of data: **Romi Novriadi**, **Sergi Segarra**; Drafting the manuscript: **Romi Novriadi**; Revising the manuscript: **Sergi Segarra**, **Oriol Roigé**, **Ilham Ilham**; Approval of the version of the manuscript to be published: **Sergi Segarra**, **Oriol Roigé**, **Ilham Ilham**.

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Conflicts of Interest

S.S. and O.R. are employed by Bioiberica S.A.U. The rest of the authors state no conflict of interest.

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