Effect of dietary phytase and protease supplementation on the growth performance and apparent nutrient digestibility in juvenile Pacific white shrimp (Litopenaeus vannamei) fed fish meal-free and phosphorus limiting diets

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Abstract

This study investigated the effects of exogenous enzyme supplementation, specifically phytase and protease, in fish meal-free and phosphorus-limited diets for juvenile *Litopenaeus vannamei* through two feeding trials The trials aimed to assess shrimp growth performance and apparent nutrient digestibility simultaneously in a clear-water recirculating tank system (34 ppt, 30°C) employing a continuous feeding regime, with feces being collected on a daily basis throughout the feeding trials. In the first feeding 50-day feeding trial shrimp (3.4 g initial body weight) were fed diets supplemented with phytase (1000 and 2000 FTU/kg) and phytase together with protease (1000 FTU/kg + protease and 2000 FTU/kg + protease), in addition to animals fed a positive control (supplemented inorganic phosphate) and a negative control diet without supplementation. In the second shrimp feeding trial (4.3 g initial body weight), in addition to negative and positive controls, shrimp were fed increasing levels of phytase (1000, 2000, 3000, 4000 and 8000 FTU/kg) over a 42-day experimental period. Both feeding trials showed beneficial effects phytase addition compared to the negative control, with significant improvements ($P < 0.05$) observed at dietary phytase levels of 2000 FTU/kg and above. Gains were obtained in growth performance (observed weekly growth of 1.46 and 1.86 g/week for shrimp fed the negative control and diet supplemented with 3000 FTU/kg, respectively), and apparent phosphorus digestibility increasing from 41.7% in animals fed the negative control diet to 52.9% in animals fed the 3000 FTU/kg supplemented. Results indicated that phytase supplementation yielded significant improvements in shrimp growth performance and phosphorus digestibility compared to the negative control. Notably, the observed benefits were evident at specific dietary phytase levels. However, the addition of protease supplements did not demonstrate any discernible effects on shrimp performance under the experimental conditions. Overall, these findings underscore the potential of phytase supplementation as a means to enhance nutrient utilization and promote optimal growth in *Litopenaeus vannamei*. Further investigations are warranted to explore the full range of benefits and mechanisms associated with protease supplementation in shrimp diets.

Introduction

Fish meal is a highly valued protein ingredient in aquaculture due to its complete nutritional profile (Rolland et al., 2015), but issues such as quality variation and availability of raw materials, sustainability and price (Edwards et al., 2004; Boyd, 2015; FAO, 2018) make necessary the use of alternative protein sources (Tacon and Metian, 2008; Oliva-Teles et al., 2015). Protein sources of plant origin stand out as an alternative to fish meal, due to overall lower prices, uniform quality and composition, global availability, among others (Naylor et al., 2009; Hardy, 2010).

However, plant ingredients may contain anti-nutritional factors, which can limit inclusion in diets for fish and crustaceans (Francis et al., 2001; Bergamin et al., 2013). One of these anti-nutritional factors is phytic acid or phytate, the main form of phosphorus storage in plant ingredients (Cosgrove, 1980; Kumar et al., 2011), which is generally believed to be poorly available to aquatic organisms due to the lack of the enzyme phytase in most aquatic species (Lemos and Tacon, 2017). In addition, phytate is a reactive compound that has affinity to form complexes with different minerals, protein and even enzymes (Kumar

et al., 2011; Samtiya et al., 2020), decreasing the availability of these nutrients (Kies et al., 2001), as well as the role of enzymes, proteases for example, in the digestive process.

Previous studies have shown improvement in the digestibility of plant ingredients with the supplementation of exogenous enzymes, such as phytases and proteases, in diets for fish and shrimp. Moreover, the use of phytase has been reported to reduce the need for inorganic phosphorus supplementation in feeds (Robinson et al., 2002), as well as stimulating improvements in growth, and digestibility of protein, minerals and phosphorus (Kumar et al., 2011; Qiu and Davis, 2017). In addition, the use of exogenous proteases has potential to promote significant benefits, including improvements in weight gain, feed conversion and nutrient digestibility in fish and crustaceans (Dalsgaard et al., 2012; Li et al., 2016; Kemigabo et al., 2017; Saleh et al., 2022).

This study aims to investigate the effects of graded levels of phytase, alone or in combination with protease, in fish meal-free and phosphorus-limited diets on the growth, feed efficiency, survival, nutrient digestibility, and body composition of juvenile Pacific white shrimp (*Litopenaeus vannamei*).

Material and methods

Experimental shrimp

Post-larval Pacific white shrimp, *L. vannamei* (PL 10, Speedline strain) was obtained from a commercial producer in Northeast Brazil (Aquatec Aquacultura Ltda, Barra do Cunhaú, Brazil) and kept in a nursery clear-water tank system with daily cleaning and partial water renewal until reaching about 0.6 g individuals. During this period shrimp was fed a commercial feed (FlashShrimp, Polinutri, Brazil: CP 40.0%, EE 10.0%, Ash 15.0% and P 1.30%) using automatic belt feeders (24 hours, Pentair, Brookfield, USA). Seawater (34 ppt salinity) was pumped from the sea (Flamengo cove, Ubatuba, Brazil), passing through 25 µm and 5 µm cartridge filters, with daily exchanges of 70% of the total tank volume. After this initial growth period shrimp were stocked in a 9,000 L tank in a biofloc system (zero water exchange) and fed a commercial feed (Policamarão, Polinutri, Brazil: CP 40.0%, EE 8.0%, Ash 12.5 and P 1.25%) until reaching proper individual size to be stocked in the experimental feeding trial system (> 3 g shrimp body weight).

Trial system and management

The clear water shrimp trial system was composed of 28 tanks with 500 L each (useful volume, 400 L) containing individual settling columns used to collect feces and removal of solid leftovers. The tank design and collection methodology were previously described in Carvalho et al. (2013). Seawater was filtered and passed through UV filters before entering the tank recirculating system. The tanks had individual water renewal of 4 liters per minute, directed to create a vortex that combined with the cylindrical shape of the tanks facilitated the process of concentrating and decanting feces strands. Throughout the experiment, the number of pellets fed was always adjusted so that there were no leftovers. The daily monitoring of the trial showed that under continuous pellet supply (small amounts

fed on a little and often basis), the shrimp took all the pellets and started the feeding process in a few minutes, with minimum leaching and nutrient losses of pellets. Aeration stones were placed individually in the tanks to ensure additional aeration of the water, in a position that did not interfere with the decanting of solids. The trial system had biological filtration system with extra aeration, UV, and heaters to maintain optimal water parameters for shrimp development.

Shrimps was individually weighed and stocked at 35 individuals per tank (corresponding to 87 animals/m³). Forty shrimps were collected from the same initial stock and sampled to determine the initial body composition and stored in a freezer (-20°) for immediate analysis. Shrimp was acclimated to the experimental diets for five days before the beginning of feces collections for digestibility determination. The volume of feed provided was initially calculated based on the shrimp biomass in the tanks, considering the individual average weight and water temperature (Forster et al., 2003). As shrimp are difficult to handle without mortality, intermittent weight determination was not carried out. However, during the experiment shrimp population was counted periodically (visually), and the supply of dietary treatments was adjusted weekly based on subsequent results of biomass gain per tank in the same experimental system. In case of eventual leftovers detected in the collection tubes the supply of pellets were reduced according to tank replicate. The daily supply of pellets was made continuously for 75% of the ration weight, through automatic feeders (24-h Baby belt feeder, Pentair, Brookfield, USA), and the remaining 25% were divided into three manual feedings at 9 a.m., 11 a.m. and 2 p.m. (feces collection period). The tanks were examined three times a day (08 a.m., 12 p.m. and 04 p.m.) to check for possible mortalities, and whenever dead animals were found, they were immediately removed.

The first trial was conducted over a 50-day period and the second trial over 42-day feeding period. Feces collections were performed throughout the experimental period, five days per week, with six daily collections, divided between morning and afternoon, with 1-hour intervals between each collection. The collection routine was as follows: in the morning the feces and solids accumulated during the night were discarded and the decantation columns were cleaned, after this, feces in tubes were collected every hour, washed gently with distilled water to remove excess salt and vacuum filtered. Possible remains of feed or shrimp exuvia were carefully discarded. The daily volume of feces collected (per replicate tank) was accumulated over the trial and stored at – 20^{o}C.

Water quality parameters were monitored daily for dissolved oxygen, salinity, and temperature (YSI Pro 2030, Yellow Springs, USA) and weekly for ammonia, nitrite, (Marine test kit, RedSea Fish Pharm Ltd, Israel), alkalinity, and pH (Alfakit Florianopolis, Brazil). Mean values remained at adequate intervals for the species during the two trials; in the first trial mean (s.d.) values were: dissolved oxygen 6.11 (0.26) mg/L, temperature 30.2 (0.34) °C, salinity 34.3 (0.25) ppt, total ammonia 0.13 (0.04) mg/L, nitrite 0.21 (0.48) mg/L, pH 7.77 (0.30), alkalinity 154.4 (10.1) mg $CaCO_3$/L, and in the second trial were dissolved oxygen 6.45 (0.54) mg/L, temperature 30.0 (0.37) °C, salinity 34.4 (0.23) ppt, total ammonia 0.13 (0.01) mg/L, nitrite 0.30 (0.14) mg/L, pH 7.66 (0.25), and alkalinity 133.3 (25.2) mg $CaCO_3$/L.

Four replicates were evaluated for each diet (four experimental tanks). As at the start of the feeding trial, shrimp were weighed individually at the end of the feeding trials and counted so as to determine survival, growth, and feed conversion rate, in addition to sampling ten shrimp per tank for the determination of whole-body proximate composition; all calculations were based on the true (practical) value of utilization, which means that mortality can have a negative impact on performance data.

Feed conversion rate (FCR) = total feed consumed (g)/ total weight gain (g)

Specific growth rate (SGR) = (log n mean final weight − log n mean initial weigh)/days x 100

Weight gain (WG, %) = ((mean final weight − mean initial weigh)/mean initial weight) *100

Protein efficiency ratio (PER) = (total final weight − total initial weight) /total protein consumed

Feed ingredients and experimental diets

Ingredients were obtained by local feed suppliers in Brazil, and their analyzed composition is shown in Table 1. Before preparing the diets, ingredients were ground to a particle size of less than 250 µm in a hammer mill (MCS 350 Moinhos Vieira, Tatuí, Brazil). In the first trial, seven different fish meal-free diets were evaluated. Diets had a similar base formulation: a positive control diet (Ctrl+) supplemented with inorganic phosphorus (monocalcium phosphate - MCP), and a negative control without MCP supplementation (Ctrl-) was prepared followed by test diets receiving different levels of phytase (1000 and 2000 FTU/kg, for diets Ph1000 and Ph2000, respectively), and two diets with addition of phytase at 1000 and 2000 FTU/kg combined with protease at 30000 PROT/kg corresponding to 400 ppm (diets PhPr1000 and PhPr2000, respectively), and one more diet, prepared with supplementation of protease *plus* MCP (MCP + Pr), totaling seven test diets in total (Table 2).

Table 1
Proximate and phosphorus composition of feed ingredients (%, as-is) used in test diets for juvenile shrimp (*Litopenaeus vannamei*). Values are mean of duplicate analysis.

	Moisture	Crude protein	Lipid	Ash	Total P	Phytate-P (estimated)*
Wheat Flour[1]	13.1	15.2	2.74	1.40	0.30	0.22
Soybean meal, solvent extracted[2]	12.1	45.0	1.86	6.03	0.55	0.37
Wheat gluten[3]	7.43	76.3	0.94	1.19	0.22	0.16
Squid meal[4]	5.05	84.4	3.17	3.17	1.28	-
Fish hydrolysate, tuna flavor[5]	65.6	10.8	5.50	6.07	1.76	-
Blood meal, ring dried[6]	7.91	88.8	0.90	1.78	0.08	-
Soy protein concentrate[7]	9.07	59.2	0.44	6.20	0.75	0.51
Feather meal[4]	5.73	82.2	7.16	1.84	0.28	-
Dried yeast, molasses cane[8]	4.71	30.4	0.05	7.74	0.67	0.14
Marine fish oil, [4]	0.22	0.01	99.3	0.06	0.01	-
Soy lecithin oil[4]	1.03	0.85	90.8	9.27	1.83	0.05
Monocalcium phosphate[9]	-	-	-	-	22.7	-

(-) not determined.

* Phytate-P estimated from % of total phosphorus based on literature references (Kumar et al., 2011; Selle and Ravindran, 2007).

[1]Coamo, Campo Mourão, Brazil.

[2]Guabi, Campinas, Brazil.

[3]Syral, Aalst, Belgium.

[4]Polinutri, Osasco, Brazil.

5Aquativ, Elven, France.

[6a]&R, Maringá, Brazil.

[7]CJ Selecta, Araguari, Brazil.

	Moisture	Crude protein	Lipid	Ash	Total P	Phytate-P (estimated)*
[8]ICC, São Paulo, Brazil.						
[9]Aliphos, Rotterdam, The Netherlands.						

Table 2
Formulation, proximate and mineral composition of test diets (as-fed) for juvenile shrimp (*Litopenaeus vannamei*), with phytase and/or protease supplementation (Trial 1). For proximate composition and chromium (n = 3) and mean of duplicate analysis for mineral content.

	Diet						
Ingredient (%)	Ctrl +	Ctrl -	Ph1000	Ph2000	MCP + Pr	PhPr1000	PhPr2000
Wheat flour	34.9	35.9	35.9	35.9	34.9	35.9	35.9
Soybean meal, solvent extracted	26.7	26.7	26.7	26.7	26.7	26.7	26.7
Blood meal, ring dried	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Wheat gluten	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Squid meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Soy protein concentrate	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Feather meal	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Dried yeast, molasses cane	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Marine fish oil	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Fish hydrolysate, tuna flavor	4.30	4.30	4.30	4.30	4.30	4.30	4.30
Soy lecithin oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral and vitamin premix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cr_2O_3[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine[3]	0.55	0.55	0.55	0.55	0.55	0.55	0.55
L-Lysine[4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Monocalcium phosphate	1.05	-	-	-	1.05	-	-
Phytase[1] (FTU/kg)	-	-	1000	2000	-	1000	2000
Protease[1] (PROT/kg)	-	-	-	-	30000	30000	30000
Diet composition							
Moisture (%)	5.51	5.15	5.58	5.27	5.46	5.52	5.38

	Diet						
Crude protein (%)	39.0	39.8	40.3	40.0 (0.85)	40.2	40.6	40.6
Lipid (%)	7.23	7.41	7.34	7.48	7.54	7.78	7.66
Ash (%)	5.10	4.51	4.31	4.92	5.22	4.65 (0.06)	4.70
Cr (%)	0.39	0.43	0.42 (0.01)	0.39	0.40	0.42	0.42
Total phosphorus* (g/kg)	8.13	5.83	5.84	6.15	8.55	5.99	6.36
Magnesium (g/kg)	1.86	1.81	1.72	1.83	1.91	1.85	1.83
Potassium (g/kg)	9.28	9.61	9.13	9.78	9.77	9.9	10.1
Phytase activity (FYT/kg)	138	183	1229	2155	161	1335	2494
Protease activity (PROT/kg)	-	-	-	-	38050	37350	34530
Abbreviations: Ctrl +: positive control (MCP supplementation); Ctrl –: negative control (no P or enzyme supplementation); Ph1000: phytase supplementation at 1000 FTU/kg; Ph2000: phytase supplementation at 2000 FTU/kg; MCP + Pr: MCP and protease supplementation; PhPr1000: phytase at 1000 FTU/kg and protease supplementation; PhPr2000: phytase at 2000 FTU/kg and protease supplementation plus protease)							
*Phytate-P in test diets was estimated at 1.97 g/kg for all diets, based on % of total phosphorus from data obtained in literature references (Table 1; Kumar et al., 2011; Selle and Ravindran, 2007)							
[1]DSM, Mairinque, São Paulo							
[2]Dinâmica, Indaiatuba, Brazil							
[3]Evonik Industries, Hanau, Germany							
[4]Evonik-Degussa, Castro, Brazil							

For the second feeding trial, seven experimental diets devoid of fish meal and with restricted levels of total phosphorus were tested, using increasing levels of phytase supplementation. Following the same strategy as in the first feeding trial, a positive control diet was formulated supplemented with MCP (Ctrl+) and a negative control diet (Ctrl-) without the addition of MCP supplementation, in addition to the test diets with phytase levels at 1000, 2000, 3000, 4000 and 8000 FTU/kg (named as Ph1000, Ph2000, Ph4000 and Ph8000 respectively, Table 3). Enzymes used during the above feeding trials included a protease enzyme produced by fermentation of *Bacillus licheniformis* (Ronozyme ProAct, DSM, Brazil) and

phytase enzyme in the form of a 6-phytase produced from *Aspergillus oryzae* (Ronozyme HiPhos, DSM, Brazil).

Table 3

Formulation, proximate and mineral composition of test diets (as-fed) for juvenile shrimp (*Litopenaeus vannamei*), with supplementation of graded levels of phytase (Trial 2). Data expressed as mean (s.d.) for proximate composition (n = 3) and mean of duplicate analysis for mineral content (n = 2).

Ingredient %	Ctrl+	Ctrl-	Ph1000	Ph2000	Ph3000	Ph4000	Ph8000
Wheat flour	35.0	36.2	36.2	36.2	36.2	36.2	36.2
Soybean meal, solvent extracted	26.7	26.7	26.7	26.7	26.7	26.7	26.7
Blood meal, ring dried	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Wheat gluten	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Squid meal	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Soy protein concentrate	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Feather meal	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Dried yeast, molasses cane	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Marine fFish oil	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Fish hydrolysate, tuna flavor	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Soy lecithin oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral and vitamin premix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cr_2O_3[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine[3]	0.60	0.60	0.60	0.60	0.60	0.60	0.60
L-Lysine[4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Monocalcium phosphate	1.20	-	-	-		-	-

Abbreviation: Ctrl +: positive control (MCP supplementation); Ctrl -: negative control (no P or enzyme supplementation); Ph1000, Ph2000, Ph3000 Ph4000, Ph8000: phytase supplemented diets at 1000, 2000, 3000, 4000 and 8000 FTU/kg, respectively

*Phytate-P was estimated at 2.13 g/kg for all diets, based on % of total phosphorus from data obtained in literature references (Kumar et al., 2011; Selle and Ravindran, 2007)

[1]DSM, Mairinque, São Paulo

[2]Dinâmica, Indaiatuba, Brazil

[3]Evonik Industries, Hanau, Germany

[4]Evonik-Degussa, Castro, Brazil

Ingredient %	Ctrl+	Ctrl-	Ph1000	Ph2000	Ph3000	Ph4000	Ph8000
Phytase[1] (FTU/kg)	-	-	1000	2000	3000	4000	8000
Diet composition							
Moisture (%)	5.34	5.40	4.98	6.09	5.60	5.77	5.55
Crude protein (%)	39.6	39.9	39.7	39.4	38.8	39.9	38.8
Lipid (%)	6.34	6.33	6.85	6.35	6.55	6.24	6.33
Ash (%)	5.24	4.39	4.37	4.52	4.42	4.44	4.64
Cr (%)	0.35	0.41	0.41	0.41	0.36	0.35	0.36
Total phosphorus* (g/kg)	9.16	5.50	5.54	5.80	5.57	5.36	5.96
Magnesium (g/kg	2.11	2.04	1.91	1.95	1.88	1.89	2.02
Potassium (g/kg)	10.8	10.9	10.3	10.2	9.92	10.4	10.8
Abbreviation: Ctrl +: positive control (MCP supplementation); Ctrl -: negative control (no P or enzyme supplementation); Ph1000, Ph2000, Ph3000 Ph4000, Ph8000: phytase supplemented diets at 1000, 2000, 3000, 4000 and 8000 FTU/kg, respectively							
*Phytate-P was estimated at 2.13 g/kg for all diets, based on % of total phosphorus from data obtained in literature references (Kumar et al., 2011; Selle and Ravindran, 2007)							
[1]DSM, Mairinque, São Paulo							
[2]Dinâmica, Indaiatuba, Brazil							
[3]Evonik Industries, Hanau, Germany							
[4]Evonik-Degussa, Castro, Brazil							

The diets were prepared in the laboratory, for which the dry ingredients were first mixed in a planetary mixer (ES-600, Hobart) for 10 minutes together with chromium oxide, used as an inert marker in digestibility determination. Subsequently, the liquid ingredients, including water, were added to the ingredient mix, and mixed again for the same period. The mixture was then cold pelleted (temperature < 40°C), and pellets of 2 to 4 mm in length and 2 mm in diameter were dried in forced air overnight (temperature between 35 to 45°C, during 18h). The dried pellets were stored in zip-lock bags in a freezer until use.

Analysis and statistics

For the two trials, moisture, crude protein, lipid, and ash contents of ingredients, diets, feces and experimental shrimp were analyzed according to standard AOAC methods (AOAC, 2005). Moisture was determined gravimetrically; samples were dried at 105°C until constant weight. Ash was also analyzed

gravimetrically after ashing samples at 600°C for 4h. Crude protein was determined by Kjeldahl method (N × 6.25) using copper sulphate as catalyzer in acid hydrolysis (FOSS Kjeltec™8200); and lipid was determined gravimetrically after extraction by petroleum ether as solvent (Soxtec™, ST255, FOSS). Phosphorus, calcium and magnesium were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) in samples prepared by microwave-assisted acid digestion (EPA, 2014). Chromium in diets and feces samples was determined by the adjusted spectrophotometric method with 1.5 diphenylcarbazide using 1 cm quartz cuvette and readings at 550 nm (Bremer-Neto et al., 2005). Phytase and protease activity of the test diets was measured by Biopract GmbH according to the DSM Nutritional Products method PHY-101/06E and SOY-101/04E, respectively.

The proximate and mineral composition of the test diets from the first and second experiments are shown in Tables 2 and 3, respectively. The process of diet preparation was shown not to affect phytase and protease activities in the finished feeds (Table 2).

Apparent digestibility coefficients for dry matter and nutrients in diets (%) were calculated as follows:

ADC (dry matter, %) 100–100 (% Cr in diet / % Cr in feces)

ADC (nutrients, %) 100–100 [(% Cr in diet/ % Cr in feces) x (nutrient in feces / nutrient in diet)]

Nutrient retention efficiency (NRE) for dry matter, crude protein, crude lipid and phosphorus was calculated as:

NRE (%) = 100 [(FW x Nf) - (IW x Ni)] / (feed consumed x N diet), where

FW is the final biomass, IW is the initial biomass, Nf is the final nutrient content, Ni is the initial nutrient content, and N diet is the nutrient content in the diet (Storebakken et al., 1998), in this calculation, the true value of nutrient retention was considered, meaning that tanks that had animal mortality were negatively affected.

All replicate data were submitted to normality and homoscedasticity check prior to application parametric statistics. After this it was applied one-way ANOVA to compare diet performance. Differences between means were analyzed by post hoc Tukey HSD test and considered significant at $P < 0.05$ (Zar, 1984), using the IBM SPSS Statistic package.

Results

Trial 1:

The performance of shrimp fed the experimental diets during trial 1 is shown in Table 4. The results show that shrimp performance was significantly affected by the supplementation of inorganic phosphorus (MCP) in the diet; supplementation with MCP improving growth by more than 25% compared to the non-supplemented diet, and a lower FCR and PER. As observed with the positive control, the diet

supplemented with MCP *plus* protease showed significantly better results compared to shrimp fed the negative control, however, the use of protease did not produce any significant gain in performance in relation to Ctrl+, with a tendency to worsen in some indicators, such as final body weight and protein efficiency ratio, although these differences were not statistically significant ($P > 0.05$). Performance results for the diet Ph2000 (phytase at 2000 FTU/kg) indicate performance gains, with a significantly higher shrimp final body weight ($P < 0.05$) and a tendency to improvement in all other performance parameters compared to negative control (although these differences were not statistically different: $P > 0.05$). The other treatments, supplemented with phytase or phytase in combination with protease, did not show performance any performance gain compared to animals fed the negative control diet. Survival was high for all treatments and was not significantly affected by dietary treatments.

Table 4

Performance of juvenile shrimp (*Litopenaeus vannamei*) fed phytase and/or protease supplemented diets (Trial 1) after 50 days trial, at 30°C, 34 ppt salinity. Values expressed as mean (s.d.) (n = 4). Means in the same row bearing different letters are significantly different ($P < 0.05$).

	Ctrl +	Ctrl -	Ph1000	Ph2000	MCP + Pr	PhPr1000	PhPr2000
Initial body wt (g)	4.63 (0.08)	4.55 (0.10)	4.61 (0.07)	4.66 (0.10)	4.54 (0.52)	4.50 (0.12)	4.68 (0.06)
Final body wt (g)	20.4^{d} (1.00)	16.5^{a} (0.29)	16.3^{a} (0.15)	18.2^{bc} (0.44)	19.5^{cd} (0.97)	17.0^{ab} (1.02)	17.2^{ab} (0.71)
Weight gain (%)	340.8^{d} (15.0)	263.0^{ab} (6.98)	252.2^{a} (5.99)	290.5^{bc} (15.6)	329.2^{d} (22.2)	276.7^{ab} (23.0)	266.7^{ab} (17.1)
Growth (g/week)	2.25^{d} (0.13)	1.71^{ab} (0.03)	1.66^{a} (0.23)	1.93^{bc} (0.71)	2.13^{cd} (0.14)	1.78^{ab} (0.14)	1.78^{ab} (0.10)
FCR	1.66^{a} (0.11)	2.27^{b} (0.14)	2.34^{b} (0.28)	1.99^{ab} (0.13)	1.73^{a} (0.06)	1.96^{ab} (0.13)	2.13^{b} (0.30)
Survival (%)	88.2 (5.88)	84.6 (5.02)	85.3 (5.88)	89.7 (5.63)	88.2 (6.79)	90.4 (7.73)	89.0 (11.10)
SGR	3.02^{c} (0.07)	2.63^{ab} (0.04)	2.56^{a} (0.03)	2.77^{b} (0.08)	2.97^{c} (0.10)	2.70^{ab} (0.11)	2.65^{ab} (0.09)
PER	1.54^{c} (0.10)	1.10^{a} (0.07)	1.06^{a} (0.11)	1.30^{ab} (0.17)	1.43^{bc} (0.05)	1.25^{ab} (0.08)	1.16^{b} (0.15)

Abbreviations: Ctrl +: positive control (MCP supplementation); Ctrl –: negative control (no P or enzyme supplementation); Ph1000: phytase supplementation at 1000 FTU/kg; Ph2000: phytase supplementation at 2000 FTU/kg; MCP + Pr: MCP and protease supplementation; PhPr1000: phytase at 1000 FTU/kg and protease supplementation; PhPr2000: phytase at 2000 FTU/kg and protease supplementation plus protease)

FCR: feed conversion ratio, SGR: specific growth rate and PER: protein efficiency ratio.

Apparent digestibility coefficients (ADC) during trial 1 showed significant differences between treatments ($P < 0.05$: Table 5). The ADC values for dry matter and protein were higher for diets Ctrl + and MCP + Pr in relation to Ctrl-, with some improvement observed in the PhPr2000 diet, though the values were not statistically different compared to Ctrl + and Ctrl- ($P > 0.05$). Phosphorus digestibility ranged from 40.6 to 41.9% in diets Ph1000 and Ctrl- respectively, and from 50.3 to 53.9% for the phytase-supplemented diets at levels of 2000 FTU/kg with or without protease, respectively, the latter significantly superior compared to ADC of diet Ctrl- ($P < 0.05$).

Table 5
Apparent digestibility coefficients (%) of test diets used to feed juvenile shrimp (*Litopenaeus vannamei*, Trial 1). Values expressed as mean (s.d.) (n = 4). Means in the same row bearing different letters are significantly different ($P < 0.05$).

	Ctrl+	Ctrl-	Ph1000	Ph2000	MCP + Pr	PhPr1000	PhPr2000
Dry matter	67.0^{c} (3.32)	59.4^{a} (1.93)	59.5^{a} (2.43)	60.2^{a} (1.43)	65.6^{bc} (1.55)	61.1^{ab} (0.91)	63.2^{abc} (1.12)
Crude protein	73.2^{b} (3.34)	67.6^{a} (1.03)	67.1^{a} (0.94)	69.7^{ab} (2.33)	73.7^{b} (1.48)	68.0^{a} (1.21)	71.4^{ab} (1.08)
P	49.1^{ab} (6.17)	41.9^{a} (3.98)	40.6^{a} (5.13)	50.3^{ab} (5.29)	49.4^{ab} (6.19)	46.3^{ab} (3.24)	53.9^{b} (5.13)

Abbreviations: Ctrl +: positive control (MCP supplementation); Ctrl –: negative control (no P or enzyme supplementation); Ph1000: phytase supplementation at 1000 FTU/kg; Ph2000: phytase supplementation at 2000 FTU/kg; MCP + Pr: MCP and protease supplementation; PhPr1000: phytase at 1000 FTU/kg and protease supplementation; PhPr2000: phytase at 2000 FTU/kg and protease supplementation plus protease)

Shrimp whole body and exoskeleton composition showed significant variation in lipid, phosphorus, and ash among dietary treatments ($P < 0.05$: Table 6). The phosphorus content of shrimp fed diets Ctrl + and MCP + Pr was higher in whole body and exoskeleton compared to Ctrl- and phytase treatments. Accordingly, diets supplemented with phytase at 2000 FTU/kg showed a tendency to increase P content, and was significantly higher in whole body composition compared to diet Ctrl- ($P < 0.05$). On the other hand, no difference was found among dietary treatments for moisture, whole body protein, and exoskeleton ash ($P > 0.05$). The whole-body lipid content showed lower values for the protease supplemented dietary treatments, with phytase and protease diets resulting in significantly lower values compared to diet Ctrl+ ($P < 0.05$).

Table 6

Whole body and exoskeleton composition (% or mg/kg, dry matter) of juvenile shrimp (*Litopenaeus vannamei*) after 50 days culture fed phytase and/or protease supplemented diets (Trial 1). Results expressed as mean (s.d.) (n = 4). Different letters in the same row denote significant difference (P < 0.05).

	Ctrl+	Ctrl-	Ph1000	Ph2000	MCP + Pr	PhPr1000	PhPr2000
Whole body							
Crude protein (%)	70.3 (1.81)	69.2 (1.36)	68.3 (1.80)	68.4 (2.17)	68.1 (1.36)	68.3 (1.03)	68.7 (1.72)
Lipid (%)	9.20^{b} (0.93)	8.64^{ab} (0.29)	8.95^{ab} (0.15)	8.42^{ab} (0.46)	8.74^{ab} (0.16)	8.06^{a} (0.22)	8.10^{a} (0.29)
Ash (%)	10.5^{ab} (0.18)	10.4^{a} (0.48)	10.8^{ab} (0.30)	10.2^{a} (0.21)	11.2^{b} (0.49)	10.3^{a} (0.15)	11.2^{b} (0.87)
P (mg/kg)	$10,317^{d}$ (240)	$7,540^{a}$ (270)	$7,689^{ab}$ (150)	$8,481^{c}$ (580)	$9,767^{d}$ (240)	$7,010^{a}$ (400)	$8,382^{bc}$ (130)
Exoskeleton							
Ash (%)	19.2 (0.58)	18.9 (0.62)	18.7 (0.14)	19.3 (0.71)	19.4 (0.39)	19.2 (0.45)	19.4(0.41)
P (mg/kg)	$10,717^{d}$ (80)	$8,020^{a}$ (180)	$8,100^{a}$ (100)	$8,480^{ab}$ (100)	$10,150^{cd}$ (0.93)	$9,233^{bc}$ (550)	$8,631^{ab}$ (280)

Abbreviations: Ctrl +: positive control (MCP supplementation); Ctrl –: negative control (no P or enzyme supplementation); Ph1000: phytase supplementation at 1000 FTU/kg; Ph2000: phytase supplementation at 2000 FTU/kg; MCP + Pr: MCP and protease supplementation; PhPr1000: phytase at 1000 FTU/kg and protease supplementation; PhPr2000: phytase at 2000 FTU/kg and protease supplementation plus protease)

Calculated nutrient retention efficiency during trial 1 (Table 7) also demonstrated the superiority of diets supplemented with inorganic phosphorus, with significantly higher retention efficiency for protein, lipid, phosphorus and ash in relation to diet Ctrl- (P < 0.05). Likewise, it is possible to verify the significant increase in nutrient retention efficiency (except for lipid) caused by enzyme supplementation (P < 0.05), highlighting phosphorus retention efficiency of phytase supplemented diets (2000 FTU/kg) with or without protease, that corresponded to 30 to 45% higher retention efficiency compared to the negative control, respectively.

Table 7
Shrimp whole body nutrient retention efficiency (%) fed phytase and/or protease supplemented diets (Trial 1). Results expressed as mean (s.d.) (n = 4). Different letters in the same row denote significant difference (P < 0.05).

	Ctrl+	Ctrl-	Ph1000	Ph2000	MCP + Pr	PhPr1000	PhPr2000
Crude protein	31.0^{d} (1.79)	20.7^{a} (1.84)	21.0^{ab} (0.38)	24.8^{bc} (1.86)	27.5^{cd} (2.15)	24.6^{abc} (2.06)	24.2^{abc} (1.80)
Lipid	21.9^{c} (1.46)	13.5^{a} (1.59)	15.1^{a} (0.54)	15.8^{a} (0.66)	18.7^{b} (1.11)	14.1^{a} (1.45)	14.1^{a} (1.14)
P	22.9^{d} (1.54)	13.7^{a} (1.85)	15.0^{ab} (0.62)	19.6^{c} (0.86)	19.4^{c} (1.16)	15.4^{ab} (1.75)	18.0^{bc} (1.51)
Ash	30.4^{bc} (2.36)	21.71^{a} (3.13)	25.3^{ab} (1.12)	26.5^{abc} (1.12)	31.6^{c} (2.08)	27.0^{abc} (3.13)	29.1^{bc} (2.6)

Abbreviations: Ctrl +: positive control (MCP supplementation); Ctrl –: negative control (no P or enzyme supplementation); Ph1000: phytase supplementation at 1000 FTU/kg; Ph2000: phytase supplementation at 2000 FTU/kg; MCP + Pr: MCP and protease supplementation; PhPr1000: phytase at 1000 FTU/kg and protease supplementation; PhPr2000: phytase at 2000 FTU/kg and protease supplementation plus protease)

Trial 2:

Shrimp performance during trial 2 (phytase supplemented at graded levels) is presented in Table 8. The results make clear the influence of the digestible phosphorus level in these diets, with the Ctrl + and Ctrl- treatments being the best and the worst in all assessed performance parameters, respectively. The data show a clear improvement of performance in phytase supplemented diets compared to animals fed the negative control diet. Phytase supplementation from 3000 FTU/kg significantly improved final weight, weekly growth, specific growth and protein efficiency ratio in compared to the Ctrl- diet (P < 0.05), with weekly growth showing up to 20% higher values. Survival of shrimp during trial 2 was below that obtained in the previous trials (except for Ctrl + and Ph8000), which ended up reducing FCR values, since this parameter is directly affected by the final biomass.

Table 8

Performance of juvenile shrimp (*Litopenaeus vannamei*) fed diets supplemented with graded levels of phytase after 42 days culture, at 30°C, salinity 34 ppt (Trial 2). Values expressed as mean (s.d.) (n = 4). Means in the same row bearing different letters are significantly different (P < 0.05).

	Ctrl+	Ctrl-	Ph1000	Ph2000	Ph3000	Ph4000	Ph8000
Initial body wt (g)	4.37 (0.26)	4.41 (0.11)	4.36 (0.09)	4.31 (0.13)	4.42 (0.05)	4.31 (0.13)	4.33 (0.07)
Final body wt (g)	18.0^{e} (0.45)	13.2^{a} (0.29)	14.1^{ab} (0.90)	14.3^{abc} (0.23)	15.6^{d} (0.38)	$15.1c^{cd}$ (0.61)	15.4^{cd} (0.22)
Weight gain (%)	311.6^{d} (8.51)	198.3^{a} (4.05)	224.3^{ab} (21.2)	232.5^{bc} (15.1)	252.8^{c} (11.7)	249.1^{bc} (7.99)	256.8^{c} (1.14)
Growth (g/week)	2.27^{e} (0.07)	1.46^{a} (0.03)	1.63^{ab} (0.15)	1.67^{bc} (0.05)	1.86^{d} (0.07)	1.80^{bcd} (0.08)	1.85^{cd} (0.02)
FCR	1.45^{a} (0.11)	2.91^{c} (0.50)	2.59^{c} (0.49)	2.42^{bc} (0.30)	2.23^{bc} (0.20)	2.43^{bc} (0.10)	1.71^{ab} (0.70)
Survival (%)	91.2^{bc} (4.80)	76.5^{a} (7.60)	78.7^{ab} (6.95)	79.4^{ab} (4.15)	78.7^{ab} (5.02)	75.0^{a} (2.94)	93.1^{c} (3.40)
SGR	3.36^{d} (0.5)	2.60^{a} (0.34)	2.80^{ab} (0.16)	2.86^{bc} (0.11)	3.00^{bc} (0.08)	2.98^{bc} (0.51)	3.03^{c} (0.01)
PER	1.74^{d} (0.13)	0.98^{a} (0.05)	1.12^{ab} (0.13)	1.15^{ab} (0.02)	1.30^{bc} (0.04)	1.26^{bc} (0.09)	1.50^{c} (0.06)

Abbreviation: Ctrl +: positive control (MCP supplementation); Ctrl -: negative control (no P or enzyme supplementation); Ph1000, Ph2000, Ph3000 Ph4000, Ph8000: phytase supplemented diets at 1000, 2000, 3000, 4000 and 8000 FTU/kg, respectively

FCR: feed conversion ratio, SGR: specific growth rate, PER: protein efficiency ratio

As verified in shrimp performance, apparent digestibility also showed significantly different results between diets Ctrl + and Ctrl- (P < 0.05), with higher ADC values for dry matter, crude protein and phosphorus observed in animals fed the diet supplemented with inorganic phosphorus (Ctrl+: Table 9). The addition of dietary phytase in diets showed a clear improvement in nutrient digestibility in relation to diet Ctrl-, especially in diets with inclusion level of 3000 phytase FTU/kg or higher.

Table 9

Apparent digestibility coefficients of test diets (%) used to feed juvenile shrimp (*Litopenaeus vannamei*, Trial 2). Values expressed as mean (s.d.) (n = 4). Means in the same row bearing different letters are significantly different (P < 0.05).

	Ctrl+	Ctrl-	Ph1000	Ph2000	Ph3000	Ph4000	Ph8000
Dry matter	67.5^{d} (2.74)	58.1^{a} (2.37)	59.1^{abc} (3.11)	58.2^{ab} (1.83)	64.4^{cd} (0.19)	64.0^{cd} (2.98)	63.6^{bcd} (1.24)
Crude protein	76.9^{c} (2.20)	68.1^{a} (1.94)	69.9^{ab} (1.66)	69.6^{ab} (2.06)	72.8^{bc} (0.75)	72.4^{b} (2.11)	72.2^{ab} (1.24)
P	58.7^{c} (5.28)	41.7^{a} (7.51)	40.2^{a} (4.24)	44.6^{ab} (2.22)	52.9^{bc} (0.45)	49.0^{abc} (3.74)	52.9^{bc} (5.12)

Abbreviation: Ctrl +: positive control (MCP supplementation); Ctrl -: negative control (no P or enzyme supplementation); Ph1000, Ph2000, Ph3000 Ph4000, Ph8000: phytase supplemented diets at 1000, 2000, 3000, 4000 and 8000 FTU/kg, respectively

Whole body and exoskeleton composition were also positively affected by phytase supplementation for some of the assessed nutrients (Table 10). As observed during trial 1, the moisture, crude protein and ash content of shrimp whole body and exoskeleton were not significantly different between dietary treatments in trial 2 (P > 0.05). On the other hand, phytase levels of 3000, 4000 and 8000 FTU/kg significantly increased exoskeleton phosphorus content compared to animals fed the Ctrl- diet (P < 0.05), although values were significantly lower than that observed for animals fed the Ctrl + diet (P < 0.05).

Table 10

Whole body and exoskeleton composition (% or mg/kg, dry matter) of juvenile shrimp (*Litopenaeus vannamei*) fed diets supplemented with graded levels of phytase after 42 days culture (Trial 2). Values expressed as mean (s.d.) (n = 4). Different letters in the same row bearing different letters are significantly different (P < 0.05).

	Ctrl+	Ctrl-	Ph1000	Ph2000	Ph3000	Ph4000	Ph8000
Whole body							
Crude protein (%)	70.4 (1.61)	71.8 (2.04)	72.3 (1.91)	73.4 (1.67)	72.5 (2.02)	73.3 (1.50)	72.4 (0.82)
Lipid (%)	6.14^{bc} (0.51)	5.33^{a} (0.22)	5.55^{ab} (0.17)	5.73^{abc} (0.47)	5.66^{ab} (0.38)	6.09^{abc} (0.24)	6.48^{c} (0.17)
Ash (%)	10.4 (0.19)	10.7 (0.22)	10.4 (0.49)	11.9 (1.43)	11.3 (0.60)	10.3 (0.60)	10.5 (0.87)
P (mg/kg)	$9,590^{c}$ (650)	$7,130^{a}$ (90)	$7,520^{ab}$ (390)	$7,420^{ab}$ (310)	$7,510^{ab}$ (120)	$7,800^{ab}$ (140)	$8,030^{bc}$ (490)
Exoeskeleton							
Ash (%)	17.7 (1.97)	17.6 (2.20)	17.4 (0.91)	19.2 (1.44)	19.7 (0.90)	19.1 (0.58)	19.8 (1.35)
P (mg/kg)	$10,400^{c}$ (170)	$7,720^{a}$ (230)	$7,860^{a}$ (130)	$7,840^{a}$ (540)	$8,250^{bc}$ (190)	$8,940^{b}$ (510)	$8,770^{b}$ (530)

Abbreviation: Ctrl +: positive control (MCP supplementation); Ctrl -: negative control (no P or enzyme supplementation); Ph1000, Ph2000, Ph3000 Ph4000, Ph8000: phytase supplemented diets at 1000, 2000, 3000, 4000 and 8000 FTU/kg, respectively

The nutrient retention efficiency (NRE) in juvenile shrimp showed significant differences for all assessed nutrients (P < 0.05), with diet Ctrl + being superior to diet Ctrl- with > 100% higher efficiency for lipid and phosphorus, and also elevated retention values for protein and ash (Table 11). As observed in other parameters in the present study, phytase supplementation at inclusion levels ≥ 3000 FTU/kg significantly improved NRE in juvenile shrimp (P < 0.05). Lipid and phosphorus retention increased more than 100% in shrimp fed diet Ph8000 in comparison to diet Ctrl-. Accordingly, phytase supplementation level of ≥ 3000 FTU/kg improved phosphorus and ash retention efficiencies in shrimp and treatments found significantly superior to treatment Ctrl- (P < 0.05), exception to ash NRE with diet Ph4000 (Table 11).

Table 11
Whole body nutrient retention efficiency (%) of juvenile shrimp (*Litopenaeus vannamei*) fed diets supplemented with graded levels of phytase (Trial 2). Values expressed as mean (s.d.) (n = 4). Different letters in the same row bearing different letters are significantly different (P < 0.05).

	Ctrl+	Ctrl-	Ph1000	Ph2000	Ph3000	Ph4000	Ph8000
Crude protein	32.4^{c} (5.43)	17.4^{a} (1.30)	19.2^{a} (3.18)	21.9^{a} (1.93)	22.0^{ab} (1.18)	23.0^{ab} (3.22)	28.8^{bc} (0.64)
Lipid	22.9^{c} (3.76)	8.64^{a} (0.70)	9.70^{a} (1.65)	12.8^{ab} (1.80)	10.6^{ab} (1.00)	15.2^{b} (2.00)	21.4^{c} (0.55)
P	19.4^{c} (3.14)	8.27^{a} (0.73)	11.5^{ab} (2.14)	12.5^{ab} (1.94)	13.4^{b} (1.30)	15.9^{bc} (2.30)	19.3^{c} (0.51)
Ash	29.8^{d} (5.60)	15.5^{a} (1.43)	17.3^{ab} (3.60)	25.2^{bcd} (3.78)	23.7^{bcd} (2.33)	21.3^{abc} (3.46)	27.7^{cd} (0.76)

Abbreviation: Ctrl +: positive control (MCP supplementation); Ctrl -: negative control (no P or enzyme supplementation); Ph1000, Ph2000, Ph3000 Ph4000, Ph8000: phytase supplemented diets at 1000, 2000, 3000, 4000 and 8000 FTU/kg, respectively

Discussion

Dietary phytase supplementation

Phytate molecules constitute most of the phosphorus present in plant ingredients (Selle et al., 2010; NRC, 2011; Kumar et al., 2011), with the bulk of the phosphorus present in plant ingredients being biologically unavailable to shrimp and most monogastric animals due to the absence of the enzyme phytase (Jackson et al., 1996; Ramseyer et al., 1999). In addition, the unavailability of phosphorus, phytate also has other negative effects by reducing the digestibility and assimilation of other nutrients, such as protein, lipid, calcium, magnesium, and individual trace elements (Urbano et al., 2000; Sugiura et al., 2001; Cao et al., 2007; Kumar et al., 2011). Phytase acts as a catalyst by promoting the hydrolysis of phytate (Greiner and Konietzny, 2006) and thereby releasing inorganic phosphate for assimilation by the animal during digestion. Moreover, complete degradation of phytate may also release the vitamin myo-inositol, which has numerous beneficial effects on animal metabolism (Laird et al., 2018; Moran et al., 2019).

The use of phytases within compound aquafeeds has been increasing, with benefits linked primarily to phosphorus nutrition, by converting plant phytate-phosphorus into available phosphorus (Morales et al., 2018), but also including the increased digestibility and retention of other dietary nutrients (Morales et al., 2018); in addition to reducing dietary phosphorus losses to the aquatic environment (Kumar et al., 2011; Lemos and Tacon 2017; Yang et al 2022). Apparent phosphorus digestibility may be considered a sensitive and clear criterion to assess the effect of phytase supplementation in diets (Qiu and Davis, 2017). Present results showed phytase supplementation in phosphorus limited plant-based diets positively affected the performance of *L. vannamei* juveniles, apparent digestibility of phosphorus, body

composition and phosphorus retention. Similar results were found in fish species fed diets based on plant ingredients, such as Nile tilapia (Portz and Liebert, 2004; Silva et al., 2005), channel catfish (Eya and Lovell, 1997) and Atlantic salmon (Sajjadi and Carter, 2004). Previously, Qiu and Davis (2017) reported significant increase in body phosphorus content of juvenile *L. vannamei* in phytase-supplemented diets, though this influence did not affect the dietary retention of this mineral, which is of great importance to crustaceans in general, due to their use in the molting process (Lemos and Weissman, 2020), with phosphorus limitation leading to losses in growth and increased mortality (Yang et al., 2022).

Although phosphorus is an indispensable mineral in shrimp diets, as it is often not found in sufficient levels in the water to be absorbed, the amount of phosphorus required by *L. vannamei* is not yet fully established, ranging from < 0.3 to more than 2.2% of total phosphorus in diets (Davis et al., 1993; Huang and Wang, 2004); the dietary requirement depending on other factors such as calcium concentration and dietary Ca:P ratio (Cheng et al., 2006). Studies show that in diets with low Ca content (Ca:P < 0.6), 0.3 and 0.4% available phosphorus appear to meet species requirement (Lemos et al., 2021). In the current study, the Ca:P ratio in the positive control was below 0.6 with estimated available phosphorus above 0.4% (based on diet digestibility), thus apparently satisfying species needs (Davis et al., 1993; Lemos et al., 2021). In the negative control, despite the Ca:P being low (< 0.5), the available phosphorus values were found below 0.25%, possibly lower than required by the species, which resulted in reduced growth. These results suggest that the inclusion of phytase in diets increased dietary available P to values above 0.30% (calculated from digestibility coefficient), even in diets with lower total phosphorus and higher phytate phosphorus (trial 2), improving the performance of shrimp, despite not at the same efficiency as checked in the positive control supplemented with inorganic phosphorus (MCP).

In addition to beneficial effects of phytase supplementation on phosphorus availability, this enzyme may be also responsible for the so-called extra-phosphoric effects (Walk et al., 2013; Lu et al., 2019), related to improved digestibility of energy, amino acids and minerals, through the dissociation of complexes formed by the phytate molecule together with these components (Zanella et al., 1999; Sugiura et al., 2001; Selle and Ravindran, 2008). In addition, phytate in plant ingredients may combine with endogenous digestive enzymes (Ravindran et al., 1995), such as amylases and proteases, reducing their action in animal digestive process. The extra-phosphoric effects of phytase have been reported for terrestrial monogastrics such as swine (Nortey et al., 2007; Hill et al., 2009) and broilers (Singh, 2008; El-Hack et al., 2018), and also aquatic species as carp (Baruah et al., 2005; Roy et al., 2014) rainbow trout (Vielma et al., 1998; Sugiura et al., 2001; Vielma et al., 2004; Wang et al., 2009), Atlantic salmon (Storebakken et al., 1998), sea bass (Oliva-Teles et al., 1998), among other species (Kumar et al., 2011; Lemos and Tacon, 2017). As observed for these species, phytase supplementation provided gains in addition to digestibility of phosphorus in the present study, mainly the increase in digestibility and retention of protein and minerals, in addition to the increase in the content of body ash in juvenile *L. vannamei*.

Previous studies suggest the effects of phytase are more pronounced in diets in which the levels of phosphorus, calcium and other minerals are limited (Zeng et al., 2014; Laird et al., 2018); This may be one of the factors that led the present study to obtain a greater number of beneficial effects of phytase

supplementation if compared to a previous study with the species in which dietary phytase supplementation was carried out in elevated phosphorus diets, at levels potentially above that required for the species (Qiu and Davis, 2017). On the other hand, Rachamawati and Samidjan (2018), using phytase supplementation in diets rich in fishmeal for juvenile *L. vannamei* (phosphorus content not reported), obtained extra phosphoric gains, mainly in protein digestibility and retention. Present results show that phytase supplementation promoted an increase in the availability of important nutrients to the growth of *L. vannamei*, and this was sufficiently beneficial to support improvement in the general performance of shrimp, in parameters including final weight, weekly growth, protein efficiency and FCR. Studies on phytase supplementation in *L. vannamei* are recent and still limited (Suprayudi et al., 2012; Qiu and Davis, 2017; Rachmawati and Samidjan, 2018), and although reporting some beneficial effects, these studies show wide variation, which may be related to different dietary approach and composition of test diets.

Literature shows phytase inclusion from 500 to 2000 FTU/kg to be the ideal range for positive results in growth, digestibility and reduction of phosphorus excretion in studies with fish species (Kumar et al., 2011; Lemos and Tacon, 2017; Liang et al., 2022). The efficiency of phytase is closely linked to some factors such as processing of diets, culture temperature and digestive system features of target species (Kumar et al., 2011; Lemos and Tacon, 2017). The enzyme activity determined in the trial 1 diets showed diets retained phytase and protease activity, as well as the culture temperature was within the parameters used for tropical fish, in which the current phytases seem to act more efficiently. However, the digestive system of shrimp without a true stomach and acid digestion may be a limitation to most of the phytases currently used, with optimal activity at pH between 4.0 and 6.0 (Greiner and Konieztny, 2010), that may have been reason to best results found with phytase supplementation above 2000 FTU/kg in the present study. Accordingly, the use of acid phytases did not result in positive effects for *L. vannamei* (Davis et al., 1998) while, on the other hand, supplementation with neutral phytase produced increased performance and nutrient digestibility in the same species (Cheng et al., 2013). New generations of phytase with wider ranges of pH performance and more resistant to manufacturing processes may further improve results in farmed shrimp species.

Dietary protease supplementation

In the first trial, protease supplementation did not result in significant effect upon performance, digestibility and nutrient retention in juvenile *L. vannamei*. As with phytase, the use of proteases has been better established and benefits reported for terrestrial monogastrics such as poultry and swine (O'doherty and Forde, 1999; Ghazi et al., 2002; Ghazi et al., 2003; Yu et al., 2007). However, recent studies with fish indicate the potential for the positive effects of protease supplementation in species such as carp (Leng et al., 2008; Chen et al., 2009; Shi et al., 2016), salmonids (Drew et al., 2005; Zhang et al., 2012), tilapia (Dalsgaard et al., 2012; Liet al., 2015), and also shrimp (Li et al., 2016; Yao et al., 2019).

Previous studies have shown that the efficiency of the use of proteases may be dependent upon a diverse range of factors, including the potential digestibility of the amino acids in the diet used, the structure of the protein and its characteristics, the occurrence of anti-nutritional factors in the diets, and

manufacturing processes and the handling of proteases, among others (Cowieson and Ross, 2016; Yang et al., 2022). For example, a study with rainbow trout reported significant gains in growth and feed conversion rate with the use of proteases in diets formulated with canola seed flour, while similar diets formulated with flaxseed flour did not result in positive effect of addition of exogenous proteases (Drew et al., 2005). Similarly, positive and null results for the use of proteases were obtained with gibel carp by alternating the manufacturing process of diets with the same formulation, with positive effects reported for pelleted diets (low temperature) and no positive effect checked for extruded diets (high temperature: Shi et al. 2016). Nevertheless, studies have reported positive effects of dietary protease supplementation for *L. vannamei*, with results demonstrating the possibility of fish meal replacement in diets with the addition of exogenous proteases, without loss of performance (Li et al., 2016). In another study with the same species, supplemented protease in diets with 50% fish meal replacement improved performance compared to the diet without supplementation, especially when protease was used together with carbohydrase and organic salt acids, though similar results were not found as in the positive control (fish meal diet: Yao et al., 2019).

Several factors are cited as having potential negative effects on the functioning of dietary exogenous enzymes, such as type of ingredient, type of enzymes, manufacturing process, breeding conditions, stage of life of the cultivated animal and characteristics of its digestive system (Yang et al., 2022). In the present study, diet manufacturing (cold pelleting) did not give any indication of having been determinant for the lack of beneficial effects of protease addition. On the other hand, the present study formulation had only a limited number of protein ingredients, which may have been a determining factor due to the potential specificity of the protease. Furthermore, the highly satisfactory performance of diet Ctrl + may have leveled the values to very high, with not much margin for improvement from enzyme supplementation.

Dietary phytase plus protease supplementation

In most plant ingredients, phytates are usually encased in layers of proteins, forming inseparable and indigestible complexes (Chow and Schell, 1980). The breakdown of phytate by phytase may release portions of substrate that may become more susceptible to the action of proteases, both endogenous and exogenously added to diets (Kemigabo et al., 2017). Previous studies with some fish species, such as tilapia, grass carp and gibel carp, have reported that the addition of combined phytase and protease may have a greater effect than the individual addition, in a complementary way (Li et al., 2019; Zheng et al., 2020; Xu et al., 2022), though the results are not repeated in all studies. For example, whereas rainbow trout diets supplemented with these enzymes had no positive effects on growth and nutrient digestibility, beneficial effects were reported when the enzymes were added separately (Yigit et al., 2018). Similarly, *in vitro* digestibility studies using catfish intestinal enzyme extracts with diets based on plant ingredients supplemented with phytase and protease reported no positive effects of combined supplementation compared to beneficial effects when enzymes were individually added to diets (Kemigabo et al., 2017). In addition to reporting the lack of synergism between these two enzymes, the possibility that fungal protease added to the diet could degrade phytase was further suggested, resulting in negative effect on

digestibility (Novelli et al., 2017). Our results are similar to these findings, in which the combination of protease and phytase did not bring beneficial effects on shrimp performance, and a slight worsening was observed when combining the protease in the PhPr2000 treatment compared to the same treatment without protease (although these differences were not statistically significant).

Conclusions

The study showed that phytase is a potential feed additive for juvenile *L. vannamei* fed phosphorus limited diets, although supplementation did not achieve the same performance as diets supplemented with inorganic phosphorus (MCP), possibly by the lower level of available phosphorus in phytase supplemented test diets. Addition of phytase into plant-based diets allowed an increase in phosphorus nutrition for shrimp, with positive effects on growth, feed efficiency, digestibility and nutrient retention, in addition to decreasing phosphorus discharge in culture water. Based on present data, supplementation levels of the phytase type used for benefit of juvenile *L. vannamei* were in the range of 2000 and 3000 FTU/kg. The study also suggests supplementation of the protease type used was not effective for shrimp performance, requiring further investigation according to origin enzyme and type.

Declarations

Ethical Approval

Not applicable

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions

Rafael Coelho: Ideas; formulation; development or design methodology; application of statistical; conducting a reserch inverstigation, Preparation, creation and/or presentation of the published work, Management and coordination responsibility for the research activity planning and execution.

Albert Tacon: Preparation, creation and/or presentation of the published work, versight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.

Daniel Lemos: Preparation, creation and/or presentation of the published work, versight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team, Acquisition of the financial support for the project leading to this publication, Management and coordination responsibility for the research activity planning and execution

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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