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Lupin kernel meal as fishmeal replacement in formulated feeds for the Whiteleg Shrimp (*Litopenaeus vannamei*)

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Abstract

Rapidly expanding global aquaculture requires sustainable, local protein sources to supplement the use of fishmeal. Lupin seed meal (*Lupinus angustifolius*) was tested as sustainable diet component for Whiteleg shrimp (*Litopenaeus vannamei*). Controlled feeding experiments were conducted in a recirculating aquaculture system for eight weeks. Juvenile shrimps were provided formulated diets containing various levels of lupin meal inclusion (0, 100, 200 and 300 g kg^{-1}) supplementing the fishmeal component, and a commercial feed as general reference. Shrimp survival, growth, metabolic and immune parameters were analysed. Survival did not differ significantly between groups. Growth performance was significantly impaired in shrimp fed diets containing more than 100 g kg^{-1} lupin meal. Lupin meal supplementation did not affect haemolymph protein content, whereas glucose and acylglyceride concentrations varied between treatments and were highest in animals fed the 100 g kg^{-1} lupin meal diet. Phenoloxidase activity was highest in shrimp fed 100 g kg^{-1} lupin meal diet indicating improved immune status. The present study indicates that low inclusion levels of lupin meal do not cause adverse effects and seem to stimulate the immune system of juvenile *L. vannamei*.

KEYWORDS

alternative protein, haemolymph parameters, immune response, legume, recirculating aquaculture systems (RAS), sustainable production

1 | INTRODUCTION

As wild capture fisheries stagnate or decline the aquaculture sector continues to rapidly expand to meet global fish demand (Waite et al., 2014), more than half of aquaculture species are fed formulated diets and fishmeal and fish oil remain staple ingredients therein. Although inclusion rates have already been strongly reduced in diets, aquaculture still uses the majority of globally produced fishmeal and oil (Tacon & Hasan, 2011). A main environmental and economic concern of aquaculture today is continued dependence on fishmeal as a protein source (Schmidt, Amaral-Zettler, Davidson, Summerfelt, & Good, 2016). Terrestrial plant protein sources have been key to alternative feed protein research for more than a decade (Hardy, 2010). The main interest to date has been in soy products, which have a high protein content with a favourable amino acid profile. The use of soybeans has come under public criticism due to the widespread use of transgenic seeds and deforestation for soy cultivation (Phillips, 2018). Recent studies focus on additional terrestrial protein sources, mainly legumes like field pea, lupin and faba bean (Carter & Hauler, 2000,

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Glencross et al., 2004, Øverland et al., 2009; De Santis, Crampton, et al., 2015; De Santis, Ruohonen, et al., 2015), which are regionally and organically produced. Legumes provide nitrogen for themselves and subsequent plants (Blume et al., 2010; Sulieman & Tran, 2015) and thus reduce the overall fertilization needs during crop rotation.

Several different cultivars of lupin have been tested with different aquaculture species with generally promising results (Bransden, Carter, & Nowak, 2001; Burel, Boujard, Tulli, & Kaushik, 2000; Glencross et al., 2004, 2005; Molina-Poveda, Lucas, & Jover, 2013; Smith, Tabrett, & Glencross, 2007; Smith, Tabrett, Glencross, Irvin, & Barclay, 2007; Sudaryono, Tsvetnenko, Hutabarat, Supriharyono, & Evans, 1999; Zhang et al., 2012). Lupin digestibility has been shown to exceed that of soy (Glencross et al., 2004) in Atlantic salmon (*Salmo salar*). In contrast, when soybean, narrow-leafed lupin and field peas were included as up to 33% fishmeal replacement protein, Atlantic salmon showed reduced weight gain and feed efficiency for lupin feeds (Carter & Hauler, 2000). Extruded lupin seed meal has been found to offer good digestibility coefficients especially for protein, and energy by trout and turbot (Burel et al., 2000) and can heavily substitute fishmeal in European seabass (*Dicentrarchus labrax*) diets (Zeytin, Hoerterer, Weiss, & Slater, 2018). At least 40% of fishmeal protein in diet of the Giant Tiger Prawn, *Penaeus monodon*, can be replaced by lupin kernel meal (used on a protein-equivalent basis), without adverse impacts on growth (Smith et al., 2007). The evaluation of the Andean lupin (*Lupinus mutabilis* Sweet) revealed that at least half of the fishmeal protein (equivalent to a third of the total protein) in the diet for *L. vannamei* can be replaced by this cultivar without negatively influencing growth and feed conversion (Molina-Poveda et al., 2013). Information about the effects of metabolic and immune parameters is missing so far.

In many research studies, diets are formulated with a generous protein supply, which is much higher than the demand of the species studied. This is the common practice to evaluate a general applicability of a new resource. This practice may mask metabolic/immunological effects that arise when diets are formulated less generously, that is tightly aligned to the requirements of a species, which is necessary in aquafeeds not only from the economic but also from the ecological point of view. To evaluate realistic impacts on the physiological status and immune response, feeds are formulated on the lower margin of requirements in the present study.

Composition of feed is crucial for growth efficiency and nutritional condition/physiological status, which can be accessed via metabolic parameters such as haemolymph glucose, total haemolymph protein and acylglycerides (Pascual, Gaxiola, & Rosas, 2003). Moreover, feed ingredients influence the shrimp's immune system (Pascual et al., 2006; Rosas et al., 2000, 2001; Sánchez et al., 2001), which can be affected by the protein level in the diet (Pascual et al., 2004). However, information about the influence of dietary ingredients on the shrimp immune system is scarce. There is evidence that dietary ingredients can alter immune activity; for example, the inclusion of soybean meal as protein source reduces non-specific immune responses, but a fermentation of the soybean meal with *Lactobacillus* spp. can reduce negative effects (Lin & Mui, 2017). Dietary ingredients or the supplementation of substances can also strengthen the immune system. A variety of immunostimulants, including terrestrial and marine plants, have been already tested and approved to enhance immune activity and resistance (Huang, Zhou, & Zhang, 2006). Bacterial and viral infections still limit yields in shrimp production and are responsible for collapses in shrimp culture. Improving shrimps immune activity and resistance against diseases, preferably achieved by oral administration of (natural) immunostimulants (Huang et al., 2006), is an important step towards sustainability and a better consumer acceptance of aquaculture-derived products.

The immune system of crustacean consists of a fixed non-specific immune mechanism preventing pathogen entry and spread. On top of that, an immediate multiple innate immune response defends against entering pathogens (Amparyup, Charoensapsri, & Tassanakajon, 2013; Rowley, 2016). The haemocytes play a major role in this innate immune defence system by eliminating foreign particles that penetrate in the haemocoel through phagocytosis and aiding wound healing. The three cell types in penaeid shrimp (Martin & Graves, 1985; Rodriguez, Boulo, Mialhe, & Bachere, 1995; van de Braak, Faber, & Boon, 1996): Hyaline (agranular), semigranular (small-granular) and granular cells (large granular haemocytes), each cover different functions in immunity (Johansson, Keyser, Sritunyalucksana, & Söderhäll, 2000). The characterization of the haemolymph and a differentiated haemocyte analysis are a useful tool for health estimation in shrimps (van de Braak et al., 1996).

A very effective immune mechanism is the cellular melanotic encapsulation of intruder material. The proPO-activating system plays an important role in this innate immune response, as it is involved in different steps, such as induction of phagocytosis or melanin biosynthesis steps. Phenoloxidase is the key enzyme catalysing enzymatic reactions of the melanin synthesis, and it is considered as the rate-limiting step of melanin formation (Amparyup et al., 2013).

Measuring the active phenoloxidase provides a snapshot of the acute immune response, while the determination of the total haemocyte count and the haemogram (differentiated haemocyte count) reflects the immune capacity of the shrimps.

2 | MATERIAL AND METHODS

2.1 | Feed

Four experimental diets:

- Control—fishmeal as main protein source
- L10—10% lupin kernel meal included in the diet in direct replacement of fishmeal
- L20—20% lupin kernel meal included in the diet in direct replacement of fishmeal
- L30—30% lupin kernel meal included in the diet in complete replacement of fishmeal

were formulated to meet the requirements of *L. vannamei* in the grow-out phase, concerning energy content, protein and amino

acid profile, lipid and fatty acid composition, vitamins and minerals (Gonzáles-Félix & Perez-Veazques, 2002; Li et al., 2017; Shao et al., 2017). As necessary, single amino acids (methionine and lysine) were added to balance the amino acid profile. All diets were isonitrogenous and isocaloric (Table 1).

A commercial shrimp diet (Beeskow, Germany; protein 390g/kg lipid 90g/kg ash 90g/kg fibre 15g/kg) without lupin meal was tested in duplicate for comparison.

2.2 | System

Animals were obtained from "Shrimp improvement systems," Islamorada, Florida, on the 11.07.2017 as postlarvae (PL 13 mean weight ca. 3 mg ± 0.5 mg, mean ± standard deviation) and on-grown for 7 weeks in a Recirculating Aquaculture System (RAS) system at the Center for Aquaculture Research belonging to the Alfred Wegener Institute in Bremerhaven, Germany. Shrimp larvae were kept in 200-L HDPE tanks at 26.0 11 ± 0.6°C and fed live Artemia combined with shrimp dry starter feed (PL500, Crevetec, Belgium) followed by commercial pelleted diet (B-Penaeus, Le Gouessant, France) during the grow-out phase. The feeding experiment was conducted in a RAS system, which consisted of 18 separate 50-L tanks combined with a process water treatment system (total system volume 2.6 m^3). The treatment included a mechanical filter, a protein skimmer, a biofilter and ozone treatment. Physical water parameters were measured every day (91.93 ± 4.95% for oxygen, 7.53 ± 0.11 for pH, 26.11 ± 0.69°C for temperature and 15.96 ± 0.53 g L^{-1} for salinity). Twice a week, the concentrations of nitrogen compounds (NH_3/NH_4^+, NO_3^-, NO_2^-) were determined with an autoanalyser (QuAAtro 39 Continuous Segmented Flow Analyzer, SEAL Analytical GmbH, Norderstedt, Germany). Mean concentrations were 0.27 ± 0.245 mg L^{-1} for ammonium, 2.25 ± 3.692 for nitrate and 122.71 ± 96.16 for nitrite.

TABLE 1 Ingredient composition and calculated proximate composition of experimental diets to examine the response of *L. vannamei* to different inclusion rates of lupin kernel meal

	Diet			
Ingredient [g kg^{-1}]	**Control**	**L10**	**L20**	**L30**
Fishmeal[a]	250	150	50	0
Shrimp meal[a]	90	90	90	0
Soybean meal (480g/kg CP)[b]	205	205	205	205
Wheat (120g/kg CP)[c]	398	322	260	193
Fish oil[a]	20	20	20	20
Lecithin—soy (700g/kg)[c]	20	20	20	20
Cholesterol[d]	2	2	2	2
Vitamin and mineral premix[e]	5	5	5	5
Gluten (corn)[f]	0	75	130	220
Lupin kernel meal[g]	0	100	200	300
Methionine[h]	0	3	5	10
Lysine[h]	5	3	8	20
TiO_2[i]	5	5	5	5
Calculated nutrient composition				
Dry matter g/kg	901	898	889	872
Ash g/kg	101	81	60	32
Gross energy MJ kg^{-1}	18.3	18.1	17.7	17.5
Digestible energy MJ kg^{-1}	14.4	13.3	12.0	11.3
Crude protein g/kg	365	366	361	358
Digestible crude protein %	32.9	30.7	27.4	24.9
Lipid g/kg	84	80	79	81
Fibre g/kg	24	24	24	12

[a]Bioceval GmbH & Co. KG, Cuxhaven, Germany.
[b]ADM (Archer Daniels Midland) Germany GmbH, Hamburg, Germany.
[c]Bäko Bremerhaven e.G., Bremerhaven, Germany.
[d]Dishman Netherlands B.V., Veenendaal, Netherlands.
[e]Research Diet Services, Wijk bij Duurstede, Netherlands.
[f]Supplied by Cargill Deutschland GmbH, Krefeld, Germany.
[g]Saatzucht Steinach GmbH & Co KG, Steinach, Germany.
[h]Evonik Nutrition & Care GmbH Germany, office Vejle, Denmark.
[i]Kronos Titan GmbH & Co. OHG, Nordenham, Germany.

Prior to the experiments, shrimps were weighed to the nearest ± 0.01 g and length recorded to the nearest ± 0.1 cm. Each compartment was stocked with 25 individuals to a mean biomass per compartment of 90.22 ± 0.86 g. Controlled feeding was maintained for eight weeks, and treatments were maintained in quadruplicate. Weight and length gain were recorded as above at experimental onset, after four weeks and eight weeks.

At the end of the experiment, haemolymph samples were taken for further analyses. Prior to sampling, the moulting stage of each animal was determined (Robertson, Bray, Leung-Trujillo, & Lawrence, 1987) and freshly moulted animals (stages A and B) were excluded from sampling due to known impacts of moulting on various metabolic and immune parameters (Le Moullac, Le Groumellec, Ansquer, Froissard, & Levy, 1997; Liu, Yeh, Cheng, & Chen, 2004). Before puncturing, the shrimp were dried and cleaned with a sterile paper towel to avoid contact of the haemolymph with seawater or contamination from exoskeleton film/biofilm. A minimum of 100 µl haemolymph was taken from the ventral sinus using single-use Sterican gauge needle Gr. 20 (0.40 × 20 mm) and a 1-mL plastic syringe filled with 100 µl cold anticoagulant. The ratio of haemolymph to anticoagulant was adjusted to 1:1 (v/v) immediately. After haemolymph sampling shrimp were weighed, length was measured and frozen at −20°C until further tissue analyses.

For the determination of metabolic parameters (glucose, total protein, acylglycerides), haemolymph was taken from three animals per tank immediately after capture due to fast reaction time of glucose (Aparicio-Simón, Piñón, Racotta, & Racotta, 2010), using an EDTA/HEPES anticoagulant according to Vargas-Albores (1992) in Guzmán, Ochoa, and Vargas-Albores (1993) containing 450 mM NaCl, 10 mM EDTA. Na_2, 10 mM HEPES (Sigma-Aldrich, Germany), pH 7.3. Samples were frozen in liquid nitrogen and stored at −80°C until further analyses. For the determination of phenoloxidase activity, all consumables were autoclaved to avoid pyrogen. Syringes containing 100 µl of sterile EDTA-free buffer according to Huang, Yang, and Wang (2010) (27 mM trisodium citrate, 385 mM sodium chloride, 115 mM glucose, pH 7.5) were used for

TABLE 2 Growth results of the feeding experiments. Initial weight, weight gain and specific growth rate of *Litopenaeus vannamei*. Replicate numbers were 24, 63, 73, 73 and 59 for Com, control, L10, L20 and L30, respectively

	Average biomass start [g]	Average biomass end [g]	Weight gain [g]	Specific growth rate [% BW day^{-1}]*
Com	3.60 ± 0.00	9.50 ± 0.55	5.898	1.731
Control	3.62 ± 0.05	10.37 ± 0.55	6.747	1.878
L10	3.61 ± 0.05	9.66 ± 0.58	6.044	1.756
L20	3.61 ± 0.02	8.53 ± 0.55	4.924	1.537
L30	3.60 ± 0.03	6.24 ± 0.61	2.644	0.984

*Per cent body weight per day.

haemolymph sampling. Samples were diluted 1:2, put on ice, centrifuged 10 min at 4°C and 800 *g*. Supernatant was frozen at −80°C until further analysis. For the determination of the total haemocyte count (THC) and haemocyte type, haemolymph was diluted 1:2 with EDTA/HEPES anticoagulant, fixed with borate-buffered formaldehyde (10% formaldehyde, 0.5% glutaraldehyde, 100 mM sodium borate), diluted 1:100 with anticoagulant and frozen at −80°C until further analysis.

Metabolic parameters, glucose, total protein and acylglycerides, were determined using an automated blood analyser (Fuji DRI-CHEM NX500, Tokyo, Japan).

2.3 | Phenoloxidase (PO)

PO activity in plasma was detected via the formation of dopachrome from L-DOPA analysed kinetically using an Infinite 200 PRO spectrophotometer (Tecan, Männedorf, Switzerland). Activity measurements were performed according to the method described by Huang et al. (2010), which was modified for a 96-well microtiter plate. Therefore, 10 µl of the diluted plasma sample was added to 215 µl L-DOPA solution (3 mg ml^{-1} in 0.1 M potassium phosphate buffer (PPB), pH 6.6). Absorbance at 490 nm was recorded every 20 s for 200 s. One unit of enzyme activity is defined as a linear increase in absorbance of 0.001 per min per ml haemolymph.

2.4 | Total (THC) and differential haemocyte count

Flow cytometric analysis according to Owens and O'Neill (1997) was performed using a BD Accuri C6 (Becton-Dickinson, Mountain-View, CA, USA). Samples were analysed using the parameters forward scatter (FSC), which is proportional to cell size, and side-scatter (SSC), which relates to the granularity or interior structure of the cell. For every sample, between 30,000 and 120,000 events were recorded, depending on the cell density and the dispersal of the cell subpopulations. Before measurement, the haemocyte samples were filtered through a 45-µm syringe filter to exclude larger particles. Scatter plots (FSC vs. SSC) were generated for each sample with FSC data on linear and SSC data on log scale (Xian et al., 2009). Gating was performed using the BD Accuri C6 software (Version 1.0, 2011) to define haemocyte cell types and exclude unwanted events by visual inspection of distinct event clusters in the dot plots. Haemocytes could be divided into three populations (hyaline, semigranular and granular cells). The total haemocyte count was obtained by summing the number of cells per mL of the three population subsets from the differential haemocyte count. Values were expressed as number of haemocytes (×10^5) ml^{-1}.

2.5 | Statistical analyses

All data were analysed by Sigmaplot 11.0 and tested for normality (Shapiro–Wilk) and equal variance before analysis. One-way analysis of variance (ANOVA) was applied to test for tank effects. Where no tank effects occurred, raw data were used for ANOVA to identify differences between groups at a 95% interval of confidence ($p < .05$). For significant differences, a multiple comparison procedure (Holm–Sidak method/Tukey test, significance level 0.05) was conducted. A Kruskal–Wallis ANOVA on ranks was conducted where data were non-normal, followed by a multiple comparison procedure (Dunn's Method). If not stated otherwise, all data are presented as means ± standard deviation (*SD*). As sample numbers were low and premoult stages have been shown to potentially affect haemolymph parameters, a two-way ANOVA was conducted to test for (and exclude) stage effects prior to further statistical analyses.

3 | RESULTS

3.1 | Growth and mortality

All shrimps accepted the feed and showed acceptable growth. The average survival rate of shrimp across all lupin meal supplemented diets was 68.3 ± 7.3%. For the animals receiving the control diet, it was 63.0 ± 5.0%. The survival rates were not statistically different between treatments. After eight weeks, the body weight of the animals differed significantly depending on the diet ($p < .001$, $H = 92.33$, $df = 4$, ANOVA on ranks—Table 2). The animals fed the control feed, the L10 feed and the commercial diet were the heaviest. Shrimps fed the L20 diet were significantly lighter than control and L10 diet but did not differ from the commercial diet. Shrimps fed the L30 diets had significantly lower body weights than all other treatments. These findings are also reflected in the specific growth rate (Table 2), which is above 1.5 for all diets but drops below 1.0 in shrimps fed the L30 diet. The reduced growth trend in the L30 treatment was already evident after four experimental weeks.

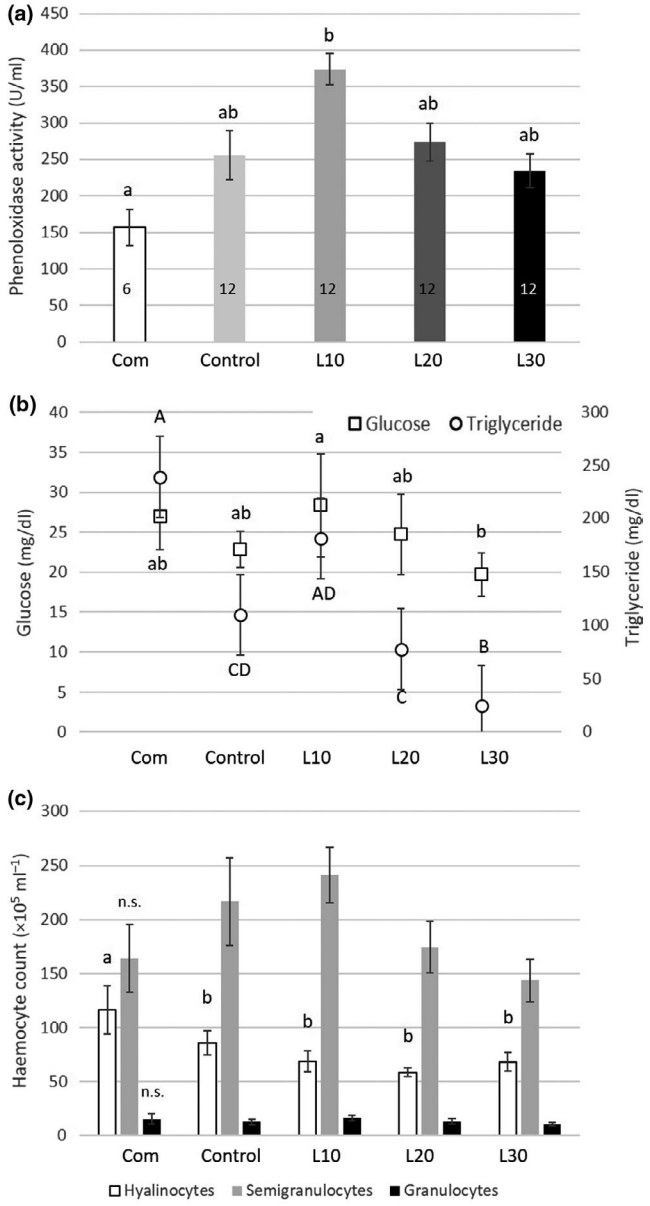

FIGURE 1 *Litopenaeus vannamei*. Results of haemolymph analyses. Com—commercial feed, Control—control feed, L10—10% of the feed is lupin meal, L20—20% of the feed is lupin meal, L30—30% of the feed is lupin meal. Significant differences are indicated by different letters. Replicate numbers are indicated in the bars for a), for b) and c) is Com = 6 individuals (ind.), Control, L10, L20 and L30 = 12 ind., each ind. measured in 3 technical replicates. (a) Phenoloxidase activity in shrimp haemolymph given as mean ± SE. Data were ln transformed to reach normality. (b) Glucose and acylglyceride levels measured in shrimp haemolymph given as mean ± *SD*. (c) Differential haemocyte count

3.2 | Haemolymph metabolic parameters

Two-way ANOVA conducted for each metabolic parameter revealed no impact on significance levels of the different treatments considering the moult stage. Thus, significance levels described here are based on treatment effect only. The glucose level was significantly higher in L10 fed animals (28.36 ± 6.44 mg dl^{-1}) (mean ± *SD*) than in L30 fed animals (19.71 ± 2.73 mg dl^{-1}) (p = .01; df = 4, F = 3.842) (Figure 1b). Glucose levels of shrimps fed the commercial feed (27.0 ± 4.15 mg dl^{-1}), the control feed (22.89 ± 2.26 mg dl^{-1}) and L20 (24.73 ± 5.0 mg dl^{-1}) were intermediate and did not differ significantly from L10 or L30.

Total haemolymph protein showed no significant differences (p = .226, df = 4, F = 1.478) and ranged between 5.73 ± 0.98 g dl^{-1} (mean ± *SD*) in shrimps fed the control feed and 7.13 ± 1.16 g dl^{-1} in shrimps fed the commercial feed (Com). Values for animals receiving the L10 diet were 6.62 g ± 0.65 g dl^{-1}, the L20 diet 6.45 ± 1.37 g dl^{-1} and 5.84 ± 1.64 g dl^{-1} in shrimps fed the L30 diet (Figure 1b).

Acylglyceride levels of animals fed the commercial diet (239.33 ± 60.12 mg dl^{-1}) (mean ± *SD*) and the L10 diet (181.60 ± 84.60 mg dl^{-1}) are significantly higher than in shrimp fed the control diet (110.00 ± 61.37 mg dl^{-1}) and the L20 diet (77.67 ± 51.84 mg dl^{-1}) (p < .001; df = 4, F = 28.244—Figure 1b). Acylglyceride levels are lowest in shrimp fed the L30 diet (24.33 ± 15.08 mg dl^{-1}) and statistically different from all other treatments.

3.3 | Haemolymph phenoloxidase activity

Phenoloxidase activity was highest in shrimps fed the L10 diet (374.0 ± 122.2 U ml^{-1}) (mean ± *SD*) and significantly lower when fed the commercial diet (156.7 ± 103.7 U ml^{-1}) (p < .001; df = 4, F = 10.335) (Figure 1a). The activity in the control, the L20 and the L30 diet was intermediate, without significant differences compared with the Com or L10 diet.

3.4 | Haemocyte counts

The mean total haemocyte count (THC) values were 277.6 (±118.7) (×10^5 cells ml^{-1}). Total haemocytes were higher when shrimp were fed low levels of lupin (L10) (326.6 ± 112.9 ×10^5 cells ml^{-1}) than the THC values of shrimp fed the commercial (272.8 ± 129.7 ×10^5 cells ml^{-1}) and the control feed (315.2 ± 165.6 ×10^5 cells ml^{-1}). THC values gradually decline with higher lupin inclusion levels (L20: (246.6 ± 85.9 ×10^5 cells ml^{-1}, L30: 222.8 ± 63.0 ×10^5 cells mL^{-1})).

Three different morphologic subpopulations of haemocytes were designated as hyaline, semigranular and granular cells based on flow cytometric FSC and SSC values (Sequeira, Vilanova, Loboda-Cunha, Baldaia, & Arala-Chaves, 1995). A mean of 73.8 (±32.8) (×10^5 cells ml^{-1}) hyaline, 191.7 (±99.0) (×10^5 cells ml^{-1}) semigranular, and 13.6 (±8.3) (×10^5 cells ml^{-1}) granular cells were detected per sample.

No significant differences between the dietary treatments occurred. However, there is a tendency that semigranular cells are promoted in animals fed the L10 diet but are reduced with further increasing lupin content in the feed (Figure 1c).

4 | DISCUSSION

Sustainable and viable alternative proteins remain high priorities for future aquaculture development. The results of the current study clearly indicate the applicability of lupin seed meal as a replacement for fishmeal in diets for *L. vannamei* aquaculture. Inclusion is, as with many terrestrial alternative sources, recommendable but only within clear limitations. The growth of *L. vannamei* indicates that increasing inclusion rates of lupin meal exceeding 100 g kg^{-1} (replacing 40% of fishmeal) in the feed cause a progressive decline in shrimp performance.

Similar replacement ratios of dehulled lupin seed meal (*Lupinus angustifolius*) were applied by Smith, Allan, Williams and Barlow (2000) in feeds of Black Tiger shrimps (*P. monodon*). No significant decrease in weight gain was evident up to an inclusion level of 200 g kg^{-1} when shrimp were raised in clear water aquaria. In raceway ponds, even higher (lupin meal) supplements of up to 250 g kg^{-1} feed did not negatively affect growth. The weekly weight gain of 1.4–1.6 g was about twice as high as in the present study. However, Smith et al. (2007) reported for juvenile *P. monodon*, grown in 100-L tank flow-through aquariums feeding on diets containing lupin kernel meal of different cultivars, a weekly weight gain of 0.7–0.9 g. These growth rates are similar to the ones observed in the present study for juvenile *L. vannamei*. Overall, the SGR of 1.7–1.9 of shrimps in the present study is in the range of the SGRs of other studies (Tacon, 2002; Xu & Pan, 2012).

Sudaryono et al. (1999) demonstrated that different lupin species can replace 30% of soybean meal (350–400 g kg^{-1} lupin meal) as protein source in the diets of Black Tiger shrimp (*P. monodon*). When testing *Lupinus mutabilis* meal in diets for juvenile *L. vannamei*, Molina-Poveda et al. (2013) found a threshold for equal survival and growth compared with fishmeal at 50% substitution of fishmeal (182.5 g kg^{-1} lupin meal, respectively). For *P. monodon*, substitution of up to 75% (300 g kg^{-1}) of fishmeal by lupin meal (*Lupinus albus* cultivar) was reported to be suitable (Sudaryono et al., 1999). Smith et al. (2007) reported good growth of *P. monodon* juveniles with lupin seed meal (*Lupinus angustifolius*) replacing at least 40% of the fishmeal protein (supplementation level of 351–396 g kg^{-1} lupin meal in the diets). Unfortunately, no general prediction of lupin meal supplementation effects on growth in relation to dietary protein level or shrimp species can be made as different shrimp species, lupin species, culture techniques and different levels of crude protein in the diets were used. Therefore, Li et al. (2017) demonstrated good growth of *L. vannamei* fed diets containing 300–360g/kg protein, offering a protein reference level for plant-based diets. The other important factors determining growth are the lupin species and cultivars used for supplementation in the diets. Nutrient levels and anti-nutritional factors, such as alkaloid levels, seem to depend strongly on plant species and growing conditions and have to be determined to estimate the suitability of regional grown lupin cultivars (Glencross, 2001; Smith et al., 2007).

4.1 | Haemolymph metabolic parameters

New ingredients for aquafeeds, especially plant-derived products, can have impacts on the metabolism of the animal that might not be expressed on the growth level but in metabolic parameters. Pascual et al. (2003) stated that the type of feed is the dominant factor affecting shrimp haemolymph metabolites. Additionally, metabolic parameters might be used as a "reference for evaluating the physiological status of *L. vannamei*" (C. Pascual et al., 2003; Pascual et al., 2004). The metabolic data from the current study show that while inclusion of 10% lupin meal has no negative influence, increasing supplementation with lupin meal (20% and 30%) progressively deteriorates the physiological status of shrimps towards lower metabolite content in the total haemolymph.

Glucose haemolymph level is a relatively fast reacting parameter used for measuring acute responses after stress treatment. Stress may be induced by variations in temperature and salinity, exposure to ammonia (Rodríguez & Le Moullac, 2000), sulphide (Hsu & Chen, 2007) and nitrite (Tseng & Chen, 2004), as well as captivity (Sánchez et al., 2001; Pérez-Jar, Rodríguez-Ramos, Ramos, Guerra-Borrego, & Racotta, 2006), handling (Aparicio-Simón et al., 2010) or injurious physical procedures (e.g. eyestalk ablation in females and spermatophore extirpation in males) (Perazzolo, Gargioni, Ogliari, & Barracco, 2002). In the present study, samples were taken immediately upon handling, faster than an acute stress reaction can occur (Aparicio-Simón et al., 2010). Hence, glucose results represent the nutritional status of the shrimps rather than a stress response. The glucose levels of shrimp fed the Com, control and L10 and L20 diet are comparable to the results for shrimps maintained in indoor tanks reported by Pascual et al. (2003). The lower levels were found in shrimp fed the L30 diet, in a similar range to the glucose content reported by Racotta and Palacios (1998) (~14 mg dl^{-1}). Contrary to the current study, both studies mentioned above measured the glucose concentration in haemolymph plasma only. Values obtained here represent the glucose content in the plasma combined with the glucose possibly leaking from lysed haemocyte cells after freezing and thawing. Thus, plasma glucose levels might even be a little lower than in Pascual et al. (2003) and Racotta and Palacios (1998). However, a stress reaction would implement higher glucose values (Aparicio-Simón et al., 2010; Mercier et al., 2009).

The content of carbohydrates in the haemolymph depends on carbohydrates provided in feed and the gluconeogenic pathway. Wheat meal can serve as a carbohydrate source for shrimp (Cuzon, Rosas, Gaxiola, Taboada, & Van Wormhoudt, 2000). In the present study, it was used for the mass adjustment in our diets (see Table 1). L30 has a lower wheat content than the other diets, which might cause a deficiency of easily digestible carbohydrates. Generally, shrimp are able to maintain glucose levels in the haemolymph by converting dietary proteins into glucose via gluconeogenesis. A sufficient supply of carbohydrates facilitates spare proteins for growth instead using of them for energy (Cuzon et al., 2000; Rosas et al., 2001). This might explain that the glucose content in the haemolymph of shrimp fed the L20 diet with lower wheat content is not significantly different from the control group. If insufficient carbohydrates are supplied

with the diet, conversion of dietary proteins to carbohydrates via gluconeogenesis may not be sufficient or fast enough, resulting in reduced haemolymph glucose levels in the animals fed the L30 diet. As gluconeogenesis takes part in the midgut gland (Cuzon et al., 2000), protein values in the haemolymph may remain unaffected. This may explain the stable total protein levels in the haemolymph with a lower glucose content found in the animals fed the L30 diet.

In general, total protein levels in the haemolymph of shrimps fed the different diets are in the range reported for animals maintained under similar conditions (Pascual et al., 2003). However, similar to the glucose values, protein concentration measured in the current study contains the protein content of haemolymph plasma and in addition of lysed haemocyte cells. For pond-raised shrimp, higher protein levels then measured here have been reported as well (Mercier et al., 2006; Pascual et al., 2003). Decreasing levels of protein have been reported to occur in crustaceans of all life stages due to elevated energy usage reacting to stressful conditions like temperature stress, suboptimal nutrition (Anger, 2001; Mercier et al., 2006; Weiss, Heilmayer, Brey, & Thatje, 2009) and after moulting (Hagerman, 1983). Current results show low but stable haemolymph protein levels for all diets, indicating limited but sufficient protein supply not affected by the rate of lupin meal inclusion in the diets.

Acylglyceride (AG) levels in the haemolymph of shrimp fed the commercial diet (240 mg dl^{-1}) and the L10 diet (180 mg dl^{-1}) are much higher than levels from other studies (Apún-Molina et al., 2015; Mercier et al., 2006). This might be partly explained by the acylglyceride content measured including the acylglyceride content of lysed haemocyte cells, as for the other heamolymph metabolites due to sample treatment. Though, the lower AG values of the L20 and L30 treatment group are in the same range as AG values reported for similar sized Whiteleg shrimp kept in indoor plastic tanks (control treatment: 35.2 ± 5.4 mg dl^{-1}) (Mercier et al., 2006). The highest AG value measured for animals feeding on the commercial diet might be related to the higher lipid content in the diet. The commercial feed contains 90g/kg lipids, while the formulated diets and the diets supplied by Mercier et al. (2006) were set to have 79g/kg crude lipids. Hu et al. (2008) found a positive correlation between triglyceride values in the serum of *L. vannamei* and the dietary lipid levels.

Higher values in the formulated diet containing low lupin meal compared with the control diet might indicate an improved lipid synthesis and stimulated lipolysis. Stimulated lipid synthesis is not seen in shrimps fed medium (L20) and high (L30) lupin meal diets, since suboptimal nutritional conditions and energy supply might overcome enhancement of lipid synthesis. Pascual et al. (2003) stated that plasma acylglyceride levels are directly affected by the quantity and quality of lipids in the feed. This effect may be seen for total haemolymph acylglyceride content as well. Since the diets are formulated to have rather similar lipid content, the quality of the supplied lipids in the medium and high lupine diets might not be ideal for shrimp.

Antinutrient compounds, such as trypsin inhibitor, saponins, phytoestrogens, fibre or phytic acid in soybean meal, are suggested to interfere with feed intake, digestion and growth in several aquacultured shrimp species as well as disturbing the metabolism determining haemolymph triacylglyceride and cholesterol content in European seabass (Bulbul, Koshio, Ishikawa, Yokoyama, & Kader, 2015; Dias et al., 2005; Hulefeld et al., 2018). Those plant-associated factors can occur in lupine meal as well and might also interfere in shrimp metabolism and decrease haemolymph acylglyceride content in shrimp. Yet, more information on the cholesterol level in the haemolymph and lipid content of the shrimps' midgut gland is necessary to make detailed inferences on changes in the shrimps' lipid metabolism.

4.2 | Immune response

Nutritional condition, blood metabolites and immunological condition are closely related in shrimp (Pascual et al., 2006; Rosas et al., 2000, 2001; Sánchez et al., 2001). There is evidence that dietary ingredients can alter immune activity. The inclusion of soybean reduces non-specific immune response in *L. vannamei* (Lin & Mui, 2017); despite, the authors showed that fermentation of the soybean meal with *Lactobacillus* spp. can reduce these negative effects (Lin & Mui, 2017). To foster disease resistance by improving shrimps immune activity, stress resistance (preferably achieved by oral administration of immunostimulants) while feeding a sustainable feed (Huang et al., 2006) is an important step towards sustainability and a better consumer acceptance of aquaculture-derived products. The sustainability of the shrimp industry depends on disease control (Rodríguez & Le Moullac, 2000). A variety of immunostimulants, including terrestrial and marine plants, have been already tested and approved (Huang et al., 2006) to enhance immune activity and resistance.

Phenoloxidase (PO) is the key enzyme catalysing enzymatic reactions of the melanin synthesis, and it is considered as the rate-limiting step of melanin formation (Amparyup et al., 2013). The present study gives evidence that the inclusion of lupin meal in the feed has a modulating effect on the shrimps' immune system with positive enhancement of the haemocytes and the phenoloxidase system when the lupin is included in moderate levels (10%) (Figure 1a,c).

Most of the designed diets resulted in slightly higher PO activity, but the L10 diet-treated animals showed a significant increase in PO activity compared with the commercial diet. Higher values of PO activity were also found in Whiteleg shrimp when other immune-stimulating and probiotic ingredients were included in the feed (Chang, Su, Chen, & Liao, 2003; Chang et al., 2012; Tseng et al., 2009; Wu et al., 2015). Previous studies showed an enhanced protection against pathogenic infection that could be correlated with an increment in PO activity (Chang et al., 2003).

Yu et al. (2016) found a similar immune-promoting effect of low-to-medium diet inclusion levels of the red algae *Gracilaria lemaneiformis* (up to 2%–3% inclusion) but not higher. The authors suggested the immune-stimulating effect was limited by the

presence of anti-nutritional and anti-physiological factors in the algae material. Lupin meal may also stimulate the immune system up to a certain level in the diet. Above that threshold-negative impacts of other factors associated with lupin meal inclusion, such as anti-nutritional factors or low energy supply, do not improve the immune system in shrimp. Some immune activating components like algae extracts (Huang et al., 2006) or terrestrial plant extracts (Wu et al., 2015) have an enhancing effect on the total haemocyte count (THC) and also enhance disease resistance towards bacterial and viral infections. Our results show the tendency of THC promotion with low inclusion levels of lupin meal (L10) and a decrease at higher inclusion levels (L20, L30). Although these results could not be confirmed statistically, due to high standard deviations the power of the conducted ANOVA is weak (0.28) and implies cautious interpretation. The present trend in THC would fit many other parameters of this study like growth, phenoloxidase activity and glucose levels.

Within the immune system, different types of haemocytes fulfil specialized tasks. Commonly, it is accepted that three different types of circulating haemocyte cells can be distinguished (Martin & Graves, 1985). These cell types have different morphological criteria and different functions, and can be described as hyaline, semigranular and granular cells. The haemocyte profile in terms of absolute numbers and percentage of total haemocytes varies considerably between different species and phyla (Cheng & Chen, 2001). In *Penaeus japonicus*, Sequeira et al. (1995) found about 40%–45% of hyaline cells, about 30% semigranular cells and 25% granular cells. The cell numbers found for *L. vannamei* in the current study are very different from *P. japonicus* with a maximum of 30% hyaline, predominant numbers of semigranular cells (about 60%–70%), and 5%–10% granular cells. This picture corresponds very closely to the haemogram generated by Rodríguez et al. (2000) for *L. vannamei* fed a commercial diet with 220g/kg protein.

There is an unexplained tendency of promotion of semigranular cells in shrimp fed with the L10 diet. With increasing lupin content in the diet, lower numbers of semigranular cells along with higher numbers of hyaline cells were observed. Semigranular and granular haemocytes are the main location where prophenoloxidase is produced and stored (Lanz, Tsutsumi, & Aréchiga, 1993; Söderhäll & Smith, 1983). Although granular cells are the main producers of proPO, semigranular cells are the most active in immune defence against invading cells (Johansson & Söderhäll, 1985). Therefore, the trend of a higher semigranular and granular haemocyte content in the L10 treatment may yield higher PO activity. Due to large standard deviations occurring, differences were not statistically significant. Haemocyte amounts in the shrimp haemolymph vary depending on individual, sex, and stage in the moult cycle (Sequeira et al., 1995). This complicates the verification of occurring differences and might mask distinct patterns between the dietary treatments. In future experiments, replicate numbers should be as high as possible and the stage selection should be reduced to one exact point of time in the moult cycle to further minimize overlying effects.

5 | CONCLUSION

The present study demonstrates successful inclusion of dehulled lupin seed meal in feeds for Whiteleg shrimp without adverse effects on survival, growth performance or metabolic parameters for inclusion rates of up to 100 g kg^{-1} feed. High inclusion rates (300 g kg^{-1} lupin seed meal) resulted in reduced growth performance and nutritional status (expressed as reduction in haemolymph glucose and acylglyceride content). An immune-stimulating effect on the shrimps was detected for 100 g kg^{-1} lupin meal based on an increase in phenoloxidase activity as seen with other immunostimulant additives. The results demonstrate that dehulled lupin seed meal is a suitable, regional alternative protein source for aquaculture feeds that can supply good quality protein to Whiteleg shrimp and can replace significant amounts of diet fishmeal. For future diet developments, higher substitution rates might be achieved by supplementing a mix of lupin meal and other regional plants, such as faba bean. This might provide a more balanced nutritional supply and make use of the immuno-stimulating effect of moderate lupin inclusion rates. Additionally, further research is required to assess methods for lupin pretreatment to enhance digestibility.

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