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Roadmap for the development of a functional microdiet for *Penaeus vannamei* post-larvae

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Summary

The whiteleg shrimp (*Penaeus vannamei*) is currently the most representative animal species in aquaculture. Despite the vast expansion industrial shrimp farming has experienced, there is still room for further development as production methologies vary considerably, and still require optimization. Additionally, the sector is characterized by inconsistent yields as it is extremely susceptible to severe pathogenic episodes that result in disastrous consequences to production. Shrimp are unable to be vaccinated due to the lack of acquired immunity, and the use of antibiotics in the aquaculture industry is increasingly more restricted, which makes immune modulation through nutrition an extremely important strategy to overcome the constraints of industrial shrimp farming.

This Thesis aimed to develop a microdiet for whiteleg shrimp post-larvae (PL) tailored for production in clear water recirculating aquaculture systems (RAS), yet with potential to be used in all production methods as a functional diet capable of improving the PL health status. Accordingly, a series of experimental trials were performed to determine the dietary protein requirements of whiteleg shrimp PL during the nursery phase in clear water RAS; evaluate the potential of several marine ingredients as the main protein source of the microdiets; test two dietary lipid levels; assess the capability of reducing formulation costs by replacing marine ingredients with soy protein concentrate; compare the microdiets developed in this Thesis with two commercial diets for whiteleg shrimp PL available in the European market; and investigate the potential health-promoting effects of Vitamin C and E, methionine, taurine and *β*-glucans when supplemented in the microdiets.

The results obtained in this Thesis demonstrate that dietary protein requirements of whiteleg shrimp PL in clear water RAS are higher than in traditional production methods. Protein levels around 47 % are suitable for growth and survival, yet levels up to 54 % seem to enhance the PL antioxidant status. Furthermore, marine ingredients and fat levels impact survival, health status and feed conversion. A blend of fish and squid meals as main ingredients and a lipid level of around 14% should be considered when tailoring diets for whiteleg shrimp PL reared in clear water RAS and at this stage of development. Krill meal enhances whiteleg shrimp PL health status but is preferable as a secondary marine ingredient as it is usually more expensive and does not seem to enhance growth performances when used as the main ingredient. Additionally, soy protein concentrate can be used to replace more expensive marine ingredients in the formulation of diets for whiteleg shrimp PL reared in clear water RAS with no adverse effects on growth performances or survival, while possibly improving their health status and increasing their resistance to pathogens. Finally, results from this Thesis demonstrate *β*-glucans improve antioxidant mechanisms of whiteleg shrimp PL while Vitamins C and E, methionine and taurine have modulatory effects on their health status.

Resumo

O camarão-de-patas-brancas (*Penaeus vannamei*) é atualmente a espécie animal mais representativa do sector de aquacultura. Apesar da vasta expansão verificada na produção industrial de camarão, ainda existe margem para desenvolvimento pois as metodologias de produção variam consideravelmente e ainda requerem otimização. Adicionalmente, o setor é caracterizado por resultados inconsistentes devido a uma suscetibilidade extrema a episódios patogénicos severos com consequências desastrosas para a produção. Os camarões não podem ser vacinados dado que não possuem imunidade adquirida e o uso de antibióticos em aquacultura é cada vez mais limitado, o que torna a imunomodulação através da nutrição uma estratégia extremamente importante para ultrapassar os constrangimentos associados à produção industrial de camarão.

Esta Tese teve como objetivo desenvolver uma microdieta para pós-larvas (PLs) de camarão-de-patas-brancas especialmente concebida para produção em sistemas de recirculação em aquacultura (RAS) de água limpa, tendo, no entanto, potencial para ser utilizada noutras metodologias de produção como uma dieta funcional capaz de melhorar a condição de saúde dos animais. Para este efeito, foi realizada uma série de ensaios experimentais para determinar os requisitos proteicos alimentares de PLs de camarão-de-patas-brancas durante a fase de berçário (nursery) em RAS de água limpa; avaliar o potencial de diversos ingredientes de origem marinha como a fonte primária de proteína das microdietas; testar dois níveis de lípidos; avaliar o potencial do concentrado de proteína de soja como substituto de ingredientes de origem marinha, com vista à redução de custos de produção das microdietas; comparar as microdietas desenvolvidas no contexto desta Tese com duas dietas comerciais para PLs de camarão-de-patas-brancas disponíveis para o mercado Europeu; e investigar os potenciais efeitos benéficos para a saúde das PLs da suplementação das microdietas com vitaminas C e E, metionina, taurina e *β*-glucanos.

Os resultados obtidos nesta Tese demonstram que os requisitos proteicos de PLs de camarão-de-patas-brancas em sistemas RAS de água limpa são superiores que em sistemas de produção tradicionais. Níveis de proteína próximos de 47% são adequados para o seu crescimento e sobrevivência, mas níveis até 54% parecem beneficiar a sua condição antioxidante. Para além disso, o tipo de ingrediente marinho e os níveis de lípidos escolhidos têm impactos na sobrevivência, saúde e conversão de alimento das PLs. Uma mistura de farinha de peixe e lula como ingredientes principais e um nível de lípidos de 14% devem ser considerados ao conceber microdietas para este estágio de desenvolvimento e tipo de sistema de cultivo. A farinha de krill tem efeitos benéficos para a saúde das PLs mas é preferível como ingrediente secundário pois é usualmente dispendioso e não parece melhorar as performances de crescimento das PLs quando usado como ingrediente principal. Adicionalmente, o concentrado de proteína de soja pode

ser usado para substituir ingredientes de origem marinha com preços mais elevados sem comprometer a performance de crescimento e sobrevivência das PLs, enquanto parece melhorar a sua saúde e resistência a patógenos. Finalmente, os resultados desta Tese demonstram que os *β*-glucanos melhoram os mecanismos antioxidantes das PLs de camarão-de-patas-brancas, enquanto as vitaminas C e E, a metionina e a taurina têm efeitos modulatórios na sua condição de saúde.

Chapter 1

General Introduction

1.1 The aquaculture industry

The world's human population has reached an impressive 8 billion people in 2022 and it's expected to continue to grow to a total of 10 billion people in the next 30 years (UN, 2022). This growth has put tremendous pressure on the animal-food production sector, which had to expand accordingly to fulfill the increasing alimentary needs of the global population, particularly when it comes to dietary protein. In fact, in the last five decades, the global apparent consumption of aquatic products alone incremented at a rate almost twice that of the annual world population growth, predominantly due to increased supplies, changes in consumers preferences, developments in production technologies and increases in average earnings (FAO, 2022a). The fisheries and aquaculture sectors had a remarkable transformation and yields almost tripled in that time frame, reaching a total of 214 million tonnes in 2020, most of which destined for human consumption. While capture fisheries outputs have remained stagnant since the 1990's, the aquaculture sector has grown exponentially (around 609 % from 1990 to 2020, being the fastest expanding animal food-producing field) and now has a higher preponderance on the total production of seafood. Considering fish, crustaceans, mollusks, other aquatic animals and algae production, yields increased from around 20 million tonnes in 1990 to over 122 million tonnes of live weight in 2020, worth USD 281 billion (FAO, 2022a).

Aquaculture production still has an exceptionally uneven distribution and unbalanced development status. In 2020, Asia and the Pacific region remained the areas where most seafood farming occurred, accounting for 91.6 % of global aquatic animals and 99.5 % of algae yields. China alone surpassed the rest of the world and contributed with over 91 million tonnes, which corresponds to around 58 % of total aquaculture production. Additionally, China is the main exporter of aquatic alimentary products, which have reached values of USD 18 billion in 2020. Indonesia, India, Vietnam, and Bangladesh complete the top five of major producers. The Americas region comes in a far second in terms of contribution, with almost 4.5 million tonnes produced, with Chile being the most substantial contributor (FAO, 2022a).

1.2 The European aquaculture sector

The European region is the third most expressive area in terms of aquaculture, yet it accounts for less than 3 % of global production. Contrarily to global trends, the European aquaculture industry outputs are still a fourth of those of capture fisheries (EUMOFA, 2018). The production of seafood in Europe has been stagnating in recent years due to the increasing restrictions imposed to capture fisheries and the underwhelming development of the aquaculture sector. Although aquaculture has been recognized by the European Green Deal as an industry with a low carbon footprint, the strict requirements to obtain an operative

license in the EU make stakeholders hesitant to invest. Meanwhile, seafood imports have reached 70 % of the total apparent consumption, reflecting Europe's position of dependency on international trade (FEAP, 2019). Therefore, the aquaculture sector in European countries still needs to expand, as the current scenario does not reflect the region's potential to develop a strong industry, capable of providing sustainable seafood for its consumers. Although not an EU member but geographically in the European continent, Norway is the world's major producer of Atlantic salmon, which is the most representative marine finfish species farmed globally, and the second largest exporter of aquatic products, worth USD 11 billion (FAO, 2022a).

Furthermore, new species must inevitably be contemplated, as 95 % of production in terms of weight and 90 % in terms of value originate from only seven species/species groups (blue mussels, Atlantic salmon, gilthead seabream, European seabass, oysters, carpet shell clam and rainbow trout) (FEAP, 2019). This narrowed focus is leading to market saturation and to the decay of the commercial value of most of these species, threatening the economic viability of many European seafood farms. Consequently, the industry and academia have been making efforts to find new species that can improve diversity and boost the development of the industry.

1.3 The whiteleg shrimp (*Penaeus vannamei*, Boone 1931)

The whiteleg shrimp is one of the most produced, traded, and consumed aquaculture species globally. Production in aquaculture has continued to increase steadily over the last decade, and in 2020 a total of 5.8 million tonnes were reached. It is currently the most representative animal species in aquaculture, constituting in 2020 a share of 4.7 % in global production (FAO, 2022a). In fact, its culture generates revenues of over 33 billion USD/year worldwide (FAO, 2022b). Major producers are China, India, Indonesia, Vietnam and Ecuador, accounting for 82 % of global production in 2017. Europe is the third largest market for whiteleg shrimp, following USA and China. Still, whiteleg shrimp production in Europe is very limited and far from a scale capable of reducing the preponderance of imported products. In 2019, imports of Penaeus shrimp to Europe reached 370 thousand tonnes with a total value of almost EUR 3 billion. The focus of European producers is on the supply of freshly harvested shrimp, which is a niche premium market, as currently, they are not able to compete with Asian and South American suppliers in terms of price and quantities (EUMOFA, 2020). Even so, whiteleg shrimp is emerging in the European aquaculture sector and is regarded as a potential species to help revitalize the industry, in a context of raising consumer concerns on carbon footprint, and other sustainability issues.

1.3.1 Main characteristics of biology

The whiteleg shrimp is a tropical marine crustacean species belonging to the Penaeidae family, that inhabits the Eastern Pacific coast (North, Central and South America) in areas where water temperature stays above 20 ºC, preferably over 25 ºC. Adult individuals live in the open sea and are usually sexually mature at 6 months of age, after they reach 20 g (males) - 28 g (females). Spawning occurs in the ocean and eggs hatch 16 hours after fertilization. The newly hatched shrimp then undergo several larval stages: the nauplii stage (2 days), where they feed on their yolk sac reserves, swimming intermittently and with positive phototactic behavior; the zoea (4-5 days) and mysis stages (3-4 days), feeding on phytoplankton and zooplankton and with planktonic behavior, drifting in tidal currents; and lastly the post-larvae (PL) stage (10-15 days), where they start the change to a benthic behavior, feeding on detritus, worms, bivalves and crustaceans and migrating to inshore coastal estuaries, lagoons or mangrove areas where they stay as juveniles and sub-adults (FAO, 2009).

1.3.2 Aquaculture production: stages and methodology

In aquaculture, whiteleg shrimp broodstocks usually consist of wild caught or, more commonly now, specific pathogen free (SPF) or specific pathogen resistant (SPR) adult individuals around 40 g. Female maturation is induced by the ablation of one of its eyestalks and they are spawned in communal or individual tanks. Spawning occurs at night and eggs hatch after approximately 16 hours. The newly hatched nauplii are then condensed in a confined area of the tank by positive phototaxis, collected and stocked in larval rearing tanks, where they are reared up to PL12 before being transferred to ongrowing tanks/ponds. An increasing number of shrimp farms employ an intermediate step of production (nursery) between the early PL stage and the grow out phase. In this instance, shrimp are harvested from the larval rearing tanks after they change to benthic behavior, at around PL 5, and are transferred to separate flat bottom tanks where they are kept at high densities until they reach up to 0.5 g. Nurseries allow farmers to stock their grow out tanks/ponds with larger individuals, which can result in higher survival results. As for feeding, after the nauplii have depleted their yolk sac reserves, a mixture of microalgae and newly hatched *Artemia* nauplii are used in the zoea and mysis stage, while formulated feeds are gradually introduced from the mysis stage onwards and become the predominant feed at the PL stage (FAO, 2009).

Several production methods are used to grow out whiteleg shrimp, that can be categorized as extensive, semi-intensive, intensive, and super-intensive. In extensive systems, the shrimp are farmed in ponds (up to 30 ha) with little to no men-driven water renewals or aeration, usually subject to tidal fluctuation. Formulated diets are used sparingly and shrimp feed predominantly on the primary production of the ponds, stimulated using

fertilizers (FAO, 2009). Additionally, the detrital matter that builds up in the bottom of the ponds is a significant source of nutrients to the shrimp, supplying important amounts of protein from the associated bacterial communities (Gamboa-Delgado et al., 2003; Nunes et al., 1997). Stocking densities are low (4-10 PL/m^2), and annual or biannual yields can range between 150-500 kg/ha/crop (FAO, 2009).

In semi-intensive systems, the shrimp are cultured in ponds (1-5 ha) with regular water exchanges and with aeration. The natural productivity of the ponds and detrital matter are a main source of food for the shrimp, yet inert diets are used more frequently in this production method, typically constituting up to 20 % of the stomachal contents of the shrimp but having a high relative contribution to growth in terms of carbon and nitrogen (Gamboa-Delgado, 2014). Stocking densities are generally low to medium (10-30 PL/m^2) and biannual yields can range between 500-2000 kg/ha/crop (FAO, 2009).

In intensive systems, the shrimp are typically produced in smaller earthen ponds (up to 1 ha) that can be inland and/or in low salinity areas. Water renewals are frequent, and aeration is strong. Formulated diets are the main source of nutrients to the shrimp yet there is still contribution from primary productivity. Stocking densities are high (60-300 PL/m^2), and biannual or triannual yields can reach 20-35 tonnes/ha/crop. This production methodology requires strict pond management and monitoring (FAO, 2009). Moreover, there has been an increase in intensive systems utilizing the biofloc technology. This production method is characterized by purposely stimulating the growth of heterotrophic bacteria in the system to avoid the buildup of nitrogenous compounds in the water, while also serving as a supplemental feed source for the shrimp (Bossier and Ekasari, 2017; Crab et al., 2012). This can decrease associated costs with aquafeeds as the preponderance of inert diets as main protein source for the shrimp is reduced.

In super-intensive systems, the shrimp are grown in little water exchange systems (or RAS), usually using raceway systems, and fed exclusively formulated diets. Stocking densities are extremely high (300-450 small juveniles/m^2), and biannual to quadannual yields can range between 28-68 tonnes/ha/crop (FAO, 2009).

1.4 Constraints of industrial whiteleg shrimp farming

Despite the vast expansion the whiteleg shrimp aquaculture sector has experienced, there is still room for further development. The market value of farmed whiteleg shrimp has been declining gradually due to the accelerated growth the industry has experienced which consequently, led to market saturation. Although the domestic trade has a high preponderance on the sales of major producers, an important part of the inputs come from exports to international markets. However, there has been an increase in the overall consumer's awareness, specially from European consumers to the potential negative

socioecological impacts of traditional industrial shrimp farming (EUMOFA, 2020). Whiteleg shrimp is an exotic species in most of the principal producer countries, which causes concerns regarding the potential of escapes, that could threaten local biodiversity. Furthermore, the expansion of shrimp production led to increases in coastal and inland areas usage for the establishment of farms, that in many cases was associated, among others, to the destruction of natural habitats (particularly mangrove forests), eutrophication of effluent receiving ecosystems and alteration of hydrological patterns (Martinez-Porchas and Martinez-Cordova, 2012). Therefore, there is a growing consumer preference for sustainable shrimp production that is forcing farmers worldwide to adopt more responsible culturing practices. Accordingly, certified organic shrimp originating from several countries have been introduced in the market and its availability is rapidly increasing (EUMOFA, 2020). Furthermore, recent pilot projects in the USA and Europe have been focusing on growing whiteleg shrimp in recirculating aquaculture systems (RAS) and with SPF/SPR individuals. These allow shrimp production to be biosecure and closer to consumption areas, reducing its ecological footprint while also contributing to the expansion and diversification of the aquaculture market in these geographical areas (Browdy et al., 2006; EUMOFA, 2020)

Moreover, industrial shrimp farming is characterized by inconsistent yields as it is extremely susceptible to severe pathogenic episodes that result in disastrous consequences to production. Consequently, economic losses are estimated to reach billions of dollars annually for Asian producers (de La Peña et al., 2015; Shinn et al., 2018). In the last decades, the fluctuation in production outputs from Asian countries has been highly correlated to significant disease events caused by viral (yellow head virus (YHV), Taura syndrome virus (TSV) and white-spot syndrome virus (WSSV)), bacterial (*Vibrio parahaemolyticus* strains responsible for acute hepatopancreatic necrosis disease (VP_{AHPND})) and/or fungal (*Enterocytozoon hepatopenaei* (EHP)) pathogens (Shinn et al., 2018). Shrimp are unable to be vaccinated due to the lack of acquired immunity, and the use of antibiotics in the aquaculture industry is limited due to inherent food safety concerns, environmental issues, and increased antimicrobial resistance (Lulijwa et al., 2020). Selective breeding programs have been devoted to genetically source SPR individuals for most of these infectious agents which attenuated the impacts on production to some extent (Moss et al., 2012), but these are usually time consuming, labor intensive and costly processes (Yang et al., 2021). However, new diseases/pathogens emerge frequently and swiftly become important threats to shrimp production, like the most recently described viral covert mortality disease (VCMD), shrimp hemocyte iridescent virus (SHIV) and translucent post-larva disease (TPD), associated with a *V. parahaemolyticus* strain (Zou et al., 2020).

1.5 The essential role of innate immunity on the shrimp health status

There is increasing evidence that crustaceans, including the whiteleg shrimp, possess innate immune memory. Immune priming through antigens presentation can provide some memory against pathogens and is a promising technique. The studies supporting this new paradigm and that explored the underlying mechanisms behind invertebrate innate immunity have been reviewed extensively (Johnson et al., 2008; Musthaq and Kwang, 2014; Amatul-Samahah et al., 2020; Yang et al., 2021; Zhai et al., 2021). Still, its effectiveness is yet to be demonstrated at farm level and far from being a viable solution to overcome the constraints disease episodes pose to whiteleg shrimp production. Therefore, there is a great reliance on optimal zootechnical conditions and nutrition to ensure the health status of shrimp, as they are highly dependent on their innate immune system to resist infection by opportunistic pathogens (Song and Li, 2014).

Innate immune responses are activated when host pattern recognition receptors (PRRs) identify pathogen associated molecular patterns (PAMPs). PRRs are located in the membrane of innate immune cells, the hemocytes, that can be hyaline (or agranular), semi-granular (small granular) and granular (large granular). Upon recognition of PAMPs, a series of signaling cascades occur and activate cellular and humoral immune defense mechanisms (Musthaq and Kwang, 2014; Tassanakajon et al., 2018; Sánchez-Salgado et al., 2021).

The main cellular immune response in crustaceans is phagocytosis, which is performed by hyaline cells (Musthaq and Kwang, 2014). This process consists in the recognition and ingestion of pathogens and apoptotic cells (Rosales and Uribe-Querol, 2017). In whiteleg shrimp, phagocytic activity is commonly used to assess the immune condition of the animals and their resistance to pathogens (Hou et al., 2015; Khimmakthong et al., 2011, 2013; Wang and Chen, 2005; Xu et al., 2019). When phagocytosis by hyaline cells is not successful, another multicellular response occurs, denominated encapsulation. In this mechanism, semi-granulocytes and granulocytes, recognize and encapsulate pathogens with opsonins that trigger humoral immune responses, such as the pro-phenoloxidase activation system to further deal with the infection (Musthaq and Kwang, 2014). Another cellular defense process is apoptosis, which consists of programmed death of infected cells, hindering pathogenic intracellular proliferation (Bergmann et al., 1998).

The major humoral immune mechanisms include the Toll and IMD pathways, generation of antimicrobial peptides (AMPs), the clotting system and melanization activated by the pro-phenoloxidase system. The Toll and IMD (immune deficiency) pathways are the major signaling mechanisms in invertebrates, that identify specific pathogens, and upon binding, upregulate immune related genes, inducing the synthesis of AMPs and other immune effectors of melanization and the clotting system. The Toll pathway is activated by

Gram-positive bacteria, fungi, and virus, while the IMD pathway is mainly by Gram-negative bacteria (Musthaq and Kwang, 2014; Tassanakajon et al., 2018).

AMPs are involved in the first line of the shrimp defense against host infection and are effectors for other immune responses. In penaeid shrimp, the primary AMPs are penaeidins, crustins, anti-lipopolysaccharide factors, and stylicins, which can have multiple isoforms and antiviral, antibacterial, and antifungal activities (Tassanakajon et al., 2013; Musthaq and Kwang, 2014). AMPs can be gene-encoded, produced as precursor proteins, and stored in semi-granular and granular hemocytes, or cleaved peptides from precursor proteins, like hemocyanin and histones (Tassanakajon et al., 2018).

The clotting system is the series of events that culminate in coagulation during tissue healing, preventing body fluid losses and the dissemination of microbes. It is one of the first immune responses as its timing and efficiency is essential to prevent health related complications derived from the exposure of interior tissues to the environment. The coagulation process is initiated by the activation and lysis of hyaline cells, releasing transglutaminase, which in turn catalyzes clotting proteins involved in hemolymph coagulation and protein post-translational remodeling and polymerization (Musthaq and Kwang, 2014; Song and Li, 2014). Additionally, there is evidence that the clotting system is linked with the activation of AMPs in crustacean species (Iwanaga and Lee, 2005; Zhu et al., 2016), including shrimp (Fagutao et al., 2012, Maningas et al., 2013).

Melanization is the process of melanin deposition around damaged tissue or foreign entities in invertebrates, physically impeding the proliferation of pathogens. Additionally, quinone intermediates are produced during melanization that are responsible for pathogen inactivation (Cerenius and Söderhäll, 2004). The melanization pathway is activated by the pro-phenoloxidase system and includes three significant steps: pathogen recognition by PRRs; the proteolytic cascade; and the activation of pro-phenoloxidase enzymes. In the first stage, PAMPs associated to microbial cell wall components, such as lipopolysaccharides, β-1,3 glucans and peptidoglycans, are recognized and attach to PRRs (Amparyup et al., 2013). Upon binding, serine proteinase cascades are activated, which culminates in the activation of pro-phenoloxidase molecules that initiate the melanization process (Musthaq and Kwang, 2014; Tassanakajon et al., 2018). Melanine formation needs to be highly regulated as quinones are extremely toxic and reactive, capable of breaking DNA strands, and can damage host cells (Dumancas et al., 2022). Therefore, several negative regulators of the melanization cascade have been described and were recently reviewed by Tassanakajon et al. (2018).

1.6 Immune modulation through nutrition

The concept of immune modulation through nutrition consists in the improvement of health status associated parameters mediated by dietary factors and feeding regimes. This view derived from the notion that nutrition is intrinsically connected to immune responses and disease resistance. This paradigm shift in the aquaculture industry occurred due to the increasing association between immunology and nutrition research (Kiron, 2012). Historically, the formulation of aquafeeds focused solely on providing the essential nutrients for growth and to avoid nutritional deficiency symptoms (Kiron, 2012), while currently, feed manufacturers aim to produce “functional or fortified feeds”, capable of supporting optimal growth but also enhancing the health status of farmed animals. The health-related functional properties of an aquafeed can derive from dietary macronutrients, such as proteins/amino acids and lipids/fatty acids; antioxidant micronutrients, namely vitamins A, C and E, carotenoids and minerals; and from additives like polysaccharides, prebiotics and probiotics. The immunomodulatory properties of these nutrients/additives and their uses in aquaculture have been reviewed extensively by several authors (Kiron, 2012; Barman et al., 2013; Encarnação, 2016; Dawood et al., 2018), including the current knowledge and potential applications in the shrimp industry (Emerenciano et al., 2022).

In industrial shrimp farming, the use of functional feeds is of extreme importance as production methodologies (namely, high stocking densities, feeding frequencies and dissolved nutrient loads) can suppress the shrimp immune condition and make them more susceptible to pathogenic outbreaks, and as previously mentioned, the use of antibiotics is increasingly more restricted and shrimp immune primming is yet to be employed at farm level. Examples of some of the most promising additives and their putative effects are summarized in Table 1. In fact, according to Emerenciano et al. (2022), there is a growing trend in the value of the shrimp feed additives market, as farms that employ intensive systems are highly reliant on these to stimulate shrimp immunity and maintain an adequate health status. However, the authors state that most of these additives still require further validation, especially in commercial settings.

Table 1 Examples of some of the most promising dietary additives and their putative effects for whiteleg shrimp (adapted from Emerenciano et al., 2022).

Type	Effects	References
Nucleotides	Enhanced nonspecific immune activity, survival, growth, and stress resistance	Chowdhury et al., 2021; Murthy et al., 2009; Novriadi et al., 2021, 2022; Yong et al., 2020
Organic acids (lactic, citric, formic, malic, sorbic and succinic acids)	Improve growth, survival, immunity, and resistance to stress	Duan et al., 2018; He et al., 2017; Romano et al., 2015
Amino acids (methionine)	Improved growth, feed efficiency and survival, reduces oxidative stress	Ji et al., 2021; Machado et al., 2023; Niu et al., 2018
Vitamins (vitamins C, D and E)	Enhanced nonspecific immune responses, antioxidant enzymes activity, lipid metabolism	Dai et al., 2022; Liu et al., 2007; Montalvo et al., 2022; Wu et al., 2016;
Trace minerals (copper, zinc, manganese, selenium)	Improved growth, antioxidant enzyme activity, non-specific immunity and upregulation of antioxidant and immune related genes	Jiao et al., 2021; Katya et al., 2016; Wang et al., 2021; Yu et al., 2021 Yuan et al., 2019, 2020
Prebiotic (β-glucans, inulin)	Enhanced nonspecific immune activity, antioxidant mechanisms, survival, growth	Li et al., 2019; Murthy et al., 2009; Wu et al., 2016; Zhou et al., 2020
Probiotic (*Bacillus* spp.)	Improved growth, feed utilization, immune parameters and antioxidant activities, survival	Madani et al., 2018; Tepaamorndech et al., 2019

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1.7 Objectives

This Thesis aspires to contribute to the success of whiteleg shrimp farming by increasing the knowledge on the role of nutrition on the animal's health status, particularly in the PL stage of development. Specifically, this Thesis aims to develop a microdiet for whiteleg shrimp PL tailored for production in clear water RAS, yet with potential to be used in all production methods as a functional diet capable of improving the PL health status. Accordingly, **Chapter 2** determines the dietary protein requirements of whiteleg shrimp PL during the nursery phase in clear water RAS. In **Chapter 3**, several marine ingredients were evaluated as the main protein source of the microdiets. Additionally, two dietary lipid levels were tested. **Chapter 4** assesses the capability of reducing formulation costs by replacing marine ingredients with soy protein concentrate and aimed at further assessing the potential of krill meal as the main ingredient of the microdiets. Moreover, the microdiets developed in this Thesis were compared with two commercial diets for whiteleg shrimp PL available in the European market. **Chapter 5** investigates the potential health-promoting effects of Vitamin C and E, methionine, taurine and *β*-glucans supplemented in inert microdiets for whiteleg shrimp PL.

Chapter 2

Dietary protein requirements of whiteleg shrimp (*Penaeus vannamei*) post-larvae during nursery in clear water recirculating aquaculture systems

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Dietary protein requirements of whiteleg shrimp (*Penaeus vannamei*) post-larvae during nursery in clear water recirculating aquaculture systems

Abstract

This study aimed at assessing the dietary protein requirement of *Penaeus vannamei* post-larvae (PL) in clear water recirculating aquaculture systems (RAS). Six microdiets were formulated to contain 34 %, 44 %, 49 %, 54 %, 58 % and 63 % crude protein (P34, P44, P49, P54, P58 and P63, respectively) and were evaluated in triplicates. Whiteleg shrimp PL (3.2 mg wet weight) were reared for 21 days in a clear water RAS at Riasearch Lda. At the end of the feeding period, the optimal protein requirement was estimated at 47.1 %, 46.4 %, 47.2 % and 44.0 % for weight gain, RGR, FCR and survival, respectively. PL fed the P54, P58 and P63 diets achieved significantly higher final body weights than those fed P34. PL fed P34 showed significantly lower RGR and survival and significantly higher FCR values than those fed the remaining diets, suggesting that low protein diets may not be adequate to be used in this stage of shrimp development and/or for the clear water RAS husbandry conditions. Moreover, diet P34 seemingly reduced the overall antioxidant status of the PL when compared to P44, P49 and P54. On the other hand, the P34 diet seems to have stimulated the PL immune mechanisms when compared to P44, P49 and P54, possibly due to increased levels of fish and algae oil. Similarly, despite the good growth performances, a diet containing 63 % of protein also seemed to have compromised the overall shrimp PL antioxidant status and stimulate their immune system. Shrimp fed diet P54 showed an apparent overall superior antioxidant status when compared to the remaining diets, evidencing that using protein inclusion levels up to 54% in aquafeeds not only potentiates growth performances and survival but also can potentially be beneficial to the health status of whiteleg shrimp PL grown in clear water RAS. Hence, results from this study suggest that a minimum of around 47 % of protein should be considered when tailoring microdiets for whiteleg shrimp PL grown in clear water RAS but inclusion levels up to 54 % can be used with benefits to the PL antioxidant status.

Keywords: *Penaeus vannamei*, whiteleg shrimp, post-larvae, protein requirements, inert microdiets, clear water recirculating aquaculture system

2.1 Introduction

The whiteleg shrimp (*Penaeus vannamei* Boone, 1931) production has increased steadily over the last decade, and in 2020 a total of 5.8 million tonnes were reached, making it the most produced animal species in aquaculture (FAO, 2022a). In fact, the culture of whiteleg shrimp generates profits of over 33 billion USD/year worldwide (FAO, 2022b). Major producers are Asian and South American countries, and in 2019 imports of *Penaeus* shrimp to Europe reached 284.270 tonnes with a total value of 1.98 billion EUR (EUMOFA, 2020). Nonetheless, there has been an increase in the overall consumer's awareness to the potential negative socioecological impacts of traditional shrimp farming and therefore, there is a growing preference for certified sustainable production. Accordingly, recent pilot projects in the USA and Europe have been focusing on growing whiteleg shrimp in recirculating aquaculture systems (RAS) using specific pathogen free (SPF) or specific pathogen resistant (SPR) individuals. These allow shrimp production to be biosecure and closer to consumption areas, reducing its ecological footprint while also contributing to the expansion and diversification of the aquaculture market in these geographical areas (Browdy et al., 2006; EUMOFA, 2020).

Traditionally, a semi-intensive strategy is practiced in shrimp farms, where fertilizers are frequently employed to stimulate the natural productivity of ponds, serving as a supplementary feed source for the shrimp. Additionally, the detrital matter that builds up in the bottom of the ponds is also a significant source of nutrients to the shrimp, supplying important amounts of protein from the associated bacterial communities (Gamboa-Delgado et al., 2003; Nunes et al., 1997). Inert feeds typically only constitute up to 20 % of the stomachal contents of the shrimp but have a high relative contribution to growth in terms of carbon and nitrogen (Gamboa-Delgado, 2014). There is evidence that protein levels in inert diets can be as low as around 25 % in semi-intensive systems without compromising the whiteleg shrimp growth performance, survival, feed conversion ratio and pond productivity (kg of shrimp *per* pond m^3) (Jatobá et al., 2014; Martinez-Cordova et al., 2002). Moreover, there has been an increase in intensive systems utilizing the biofloc technology. This production method is characterized by purposely stimulating the growth of heterotrophic bacteria in the system to avoid the buildup of nitrogenous compounds in the water, while also serving as a supplemental feed source for the shrimp (Bossier and Ekasari, 2017; Crab et al., 2012). Initial reports have shown that, like in semi-intensive systems, dietary protein level can be reduced to 25 % in biofloc systems, without affecting whiteleg shrimp growth, survival, feed conversion ratio or the status of the immune response and antioxidant defense (Xu et al., 2012; Xu and Pan, 2014a, 2014b). However, several authors have recently suggested an optimal dietary protein level of around 35 % when farming whiteleg

shrimp in bioflocs (Jatobá et al., 2014; Olier et al., 2020; Pinho and Emerenciano, 2021; Yun et al., 2016).

Shrimp farming in clear water RAS is relatively distinct from the previously mentioned production methods, particularly regarding nutrition, since all the protein requirements of the shrimp need to be fulfilled by the protein content of inert feeds. This type of production method is relatively recent and consequently, knowledge on the shrimp dietary demands is still insufficient, particularly in the initial stages of development. Despite the availability of several commercial diets for the whiteleg shrimp larval/post-larval phases, there is room for improvements in formulations as these are often not tailored specifically for production type and can result in an under/over estimation of the dietary requirements of the animals. Therefore, determining the optimal protein levels to incorporate in diets for the whiteleg shrimp post-larvae (PL) produced in clear water RAS is of extreme importance since it is the most essential nutrient for growth but also the costliest, greatly influencing the overall production expenditures. Doing so may strengthen knowledge in shrimp farming in clear water RAS by improving feeding efficiency and reducing the overall feed associated costs, reduce environmental impacts, while potentially enhancing shrimp growth performances and health status. Hence, this study aimed at assessing the dietary protein requirement of whiteleg shrimp PL in clear water RAS by evaluating their growth performance, survival and health status when fed diets containing graded crude protein levels ranging from 34 to 63 %.

2.2 Materials and methods

2.2.1 Dietary treatments

Six experimental microdiets were evaluated in triplicates (P34, P44, P49, P54, P58 and P63), which were formulated to contain 34 %, 44 %, 49 %, 54 %, 58 % and 63 % crude protein, respectively. Ingredients with similar percentages of inclusion in the experimental microdiets were the following: fish meal, fish protein hydrolysate, wheat gluten, vitamin and mineral premix, and cholesterol. Different levels of squid meal, wheat meal and fish oil were incorporated to have isolipidic diets. Feed formulation of the experimental diets and their composition are presented in Table 1 and 2, respectively. Composition of the experimental diets was analysed following the Association of Official Analytical Chemists procedures (AOAC, 2000). Briefly, dry matter was determined by drying samples at 105ºC until constant weight; crude protein (N x 6.25), by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit; crude fat, by petroleum ether extraction (Soxtec HT System); ashes, by incineration at 450ºC for 16 h in a muffle furnace; and gross energy, by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). All diets were produced at Sparos Lda facilities (Olhão, Portugal) using

extrusion at low temperature as main production process, as follows: powder ingredient mixing according to target formulation using a double helix mixer; grinding in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany); addition of the oil fraction; humidification and agglomeration through low temperature extrusion (Dominioni Group, Italy); drying of resultant pellets in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 4 h at 60 ºC, crumbling (Neuero Farm, Germany) and sieving to desired size ranges.

Table 1 Feed formulation of the experimental microdiets with graded crude protein levels from 34 to 63 % used to culture whiteleg shrimp (*P. vannamei*) PL in clear water RAS for 21 days.

Ingredients (g kg^{-1})	P34	P44	P49	P54	P58	P63
Fishmeal LT70 [a]	10.0	10.0	10.0	10.0	10.0	10.0
Fish protein hydrolysate [a]	3.0	3.0	3.0	3.0	3.0	3.0
Squid meal [a]	7.5	21.8	29.0	36.2	43.3	50.5
Wheat gluten [b]	5.0	5.0	5.0	5.0	5.0	5.0
Wheat meal [c]	48.3	35.4	28.9	22.3	15.8	9.3
Fish oil [a]	5.8	5.4	5.2	5.0	4.8	4.6
Algal oil [d]	0.6	0.4	0.3	0.2	0.1	0.0
Soy lecithin [e]	6.2	5.8	5.6	5.4	5.2	5.0
Binder & Vit & Min Premix [f]	9.4	9.4	9.4	9.4	9.4	9.4
Cholesterol [g]	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant [h]	0.2	0.2	0.2	0.2	0.2	0.2
Monocalcium phosphate [i]	3.9	3.5	3.3	3.2	3.0	2.8

[a] Sopropêche, France

[b] Roquette Freres Lestrem, France

[c] Molisur - Molinos Harineros del Sur SA

[d] Veramaris, Netherlands

[e] LECICO GmbH, Germany

[f] Sparos Lda, Portugal

[g] Termofisher, UK

[h] Kemin Nutrisurance, Italy

[i] Alltech Coppens, Germany

Table 2 Composition of the experimental microdiets with graded crude protein levels from 34 to 63 % used to culture whiteleg shrimp (*P. vannamei*) PL in clear water RAS for 21 days.

Diet	**P34**	**P44**	**P49**	**P54**	**P58**	**P63**
Dry Matter (DM, %)	93.5 ± 0.0	94.1 ± 0.0	93.7 ± 0.0	93.4 ± 0.0	93.2 ± 0.0	93.7 ± 0.0
Crude protein (% DM)	33.8 ± 0.1	44.2 ± 0.0	48.7 ± 0.0	53.6 ± 0.1	58.3 ± 0.2	63.4 ± 0.1
Crude fat (% DM)	14.0 ± 0.0	14.0 ± 0.1	13.7 ± 0.0	13.5 ± 0.2	13.0 ± 0.1	13.1 ± 0.0
Ashes (% DM)	9.6 ± 0.0	9.7 ± 0.0	9.9 ± 0.0	9.8 ± 0.0	9.9 ± 0.0	10.0 ± 0.0
Phosphorous (% DM)	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.6 ± 0.0
Energy (KJ g^{-1} DM)	18.3 ± 0.1	19.0 ± 0.0	19.3 ± 0.0	19.5 ± 0.0	19.7 ± 0.0	20.1 ± 0.0

Results expressed as mean ± standard deviation (n = 2).

2.2.2 Shrimp rearing and sampling

Whiteleg shrimp post-larvae (PL18), originated from Sea Product Development – Division of Global Blue Technologies (Texas, United States of America), were reared for 21 days at RIASEARCH Lda facilities (Murtosa, Portugal). Shrimp were randomly distributed to 18 tanks with approximately 50 L, part of a clear water recirculating system. Each tank was stocked with 250 individuals (shrimp density of 5000 m^3) averaging 3.2 mg of wet weight. These were kept under a 12 h light:12 h dark photoperiod and were fed close to *ad libitum* with automatic feeders that supplied eight meals a day. Feeders were cleaned daily and charged with adjusted feed quantities based on the observation of the tanks and the presence/absence of remnants from the previous day. Feed size was 400-600 µm for the first week and 600-800 µm for the remaining feeding period. Water temperature was maintained at 28.3 ± 0.6 ºC, dissolved oxygen concentration at 7.23 ± 0.2 mg L^{-1}, salinity at 21.5 ± 0.8 g L^{-1}, pH at 8.05 ± 0.1, NH_3 at 0.02 ± 0.03 mg L^{-1} and NO_2 at 0.05 ± 0.05 mg L^{-1}.

At the start of the trial, a total of 60 PL from the initial stock were randomly selected and group weighed for initial wet weight determination. At the end of the experiment, all shrimp from each tank were counted to determine survival. Additionally, 90 PL were randomly selected and weighed in groups of 10 larvae to determine final body weight, relative growth rate (RGR) and for analysis of feed conversion ratio (FCR). Afterwards, 40 PL were randomly selected from each tank for oxidative stress and immune parameters analysis, 10 for gene expression analysis and 30 for whole-body protein content analysis. These were stored at -80 ºC for subsequent analysis. Shrimp sampled for molecular biology analysis were kept in RNAlater (Sigma) at > 1:5 volume ratio, at 4 ºC for 24 h prior to being stored at -20 ºC. Shrimp were fasted for 12 hours prior to samplings to ensure their guts were empty at collection.

2.2.3 Protein content

A total of 30 whiteleg shrimp PL per tank sampled at the end of the trial were freeze dried and their total nitrogen (N), hydrogen (H) and carbon (C) content was determined using an elemental analyzer (Elementar Vario EL III, Hanau - Germany). Protein contents were then calculated as $\boldsymbol{N \times 6.25}$.

2.2.4 Oxidative stress and immunity-related biomarkers

2.2.4.1 Sample preparation

A total of 40 whole whiteleg shrimp PL from each tank sampled at end of the trial were weighed and homogenized in quadruple groups of 10 individuals for oxidative stress and immune parameters analysis, as described by Peixoto et al., (2021). Briefly, potassium phosphate buffer (0.1 M) was added to each group in a 1/10 (w/v) proportion followed by

homogenization using a high-performance dispersing instrument (SilentCrusher M, Heidolph Instruments). An aliquot for lipid peroxidation (LPO) with butylated hydroxytoluene was reserved prior to centrifugation. After centrifugation (5500 rpm for 20 minutes), sample supernatant was collected and distributed in separate aliquots for oxidative stress parameters and immune parameters. The remaining 10 shrimp sampled for molecular biology analysis were homogenized in NZYol (Nzytech, w/v proportion according to the manufacturer's instructions) using a Precellys 24 tissue homogenizer (Bertin instruments).

2.2.4.2 Determination of oxidative stress biomarkers

Catalase (CAT), superoxide dismutase (SOD), total glutathione (tGSH) and lipid peroxidation (LPO) levels as well as total proteins content were determined in the homogenized samples. Total proteins were measured by using PierceTM BCA Protein Assay Kit, as described by Costas et al. (2014). Samples were diluted in K-phosphate buffer (0.1 M; pH 7.4) and bovine serum albumin (BSA, 2 mg mL^{-1}) was used as standard. Afterwards, 25 µL of each diluted sample and standards were plated in triplicate and read at 562 nm in a Synergy HT microplate reader (BioTek, Winooski, VT, USA). Results were calculated using a standard curve and expressed as mg mL^{-1}.

CAT activity levels were determined measuring the decrease of hydrogen peroxide (H_2O_2 30%, Sigma) concentration, as described by Clairborne (1985). Reaction mixture was composed of K-phosphate buffer (0.05M pH 7.0) and H_2O_2 (30%) as substrate. 10 µL of homogenate sample was added to the reaction mixture effecting a total volume of 300 µL. Absorbance was read at 240 nm in UV microplates for 2 minutes (1 reading every 15 seconds) in the microplate reader and results expressed as enzyme units per milligram of total protein (U mg^{-1} protein). One enzyme unit is the amount of enzyme needed to catalyse one micromole of substrate per minute.

SOD activity levels were determined by measuring the reduction of cytochrome C that occurs in the presence of superoxide radicals and express the amount of enzyme required to inhibit in 50% the rate of reduction of cytochrome C (Almeida et al., 2010; Lima et al., 2007). Briefly, 0.2 mL of the reaction solution, composed by 1 part xanthine solution 0.7 mM (in NaOH 1 mM) and 10 parts cytochrome C solution 0.03 mM (in phosphate buffer 50 mM pH 7.8 with 1 mM Na-EDTA), were added to 0.05 mL of each homogenate sample (0.25 mg mL^{-1} total protein). Absorbance was read at 550 nm in the microplate reader every 20 s interval for 3 min at 25 ºC.

Total glutathione content in the PL homogenate samples was measured based on the oxidation of glutathione by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma) as described by (Baker et al., 1990; Rodrigues et al., 2017; Tietze, 1969). Samples were diluted in K-phosphate buffer (0.1 M pH 7.4) to obtain 0.7 mg mL^{-1} of protein. Thereafter, 50

µL of each diluted sample was added to microplate wells, followed by the addition of 250 µL of a reaction solution, composed by DTNB, K-phosphate buffer (0.1M, pH 7.4), NADPH (ß-Nicotanimide adenine dinucleotide 2'-phosphate reduced tetrasodium salt; Alpha Aesar) and glutathione reductase (Sigma). Absorbance was read at 412 nm for 3 minutes (1 reading every 20 seconds) in the microplate reader and results expressed as nmol mg of protein^{-1}.

Endogenous LPO was assessed by measuring thiobarbituric acid-reactive substances (TBARS) according to Ohkawa et al. (1979) and Bird and Draper (1984), preventing artefactual lipid oxidation by adding butylhydroxytoluene (4 %; Sigma) (Torres et al., 2002). Homogenate samples were incubated for 60 min at 100 ºC with a 100 µL of trichloroacetic acid 100% solution and 1 mL of 2-Thiobarbituric acid 0.73 % (Sigma), trizma hydrochloride (Sigma) and diethylenetriaminepentaacetic acid (Fluka) solution in polystyrene microtubes. Afterwards, these were centrifuged for 5 min at 11500 rpm and supernatant (200 µL) was added to the microplate wells. Absorbance was read at 535 nm and results expressed as nmol g wt^{-1}.

2.2.4.3 Analysis of immune parameters

Lysozyme and pro-phenoloxidase activities were determined in the homogenized samples. Lysozyme activity was measured using a turbidimetric assay, as described by Ellis (1990) with adaptations (Costas et al., 2011). Briefly, a solution of *Micrococcus lysodeikticus* (0.25 mg mL^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was prepared and 40 µL of the homogenized samples and 130 µL of this suspension were added to a microplate, effecting a final volume of 170 µL. The reaction was carried out at 25 ºC, and absorbance (450 nm) measured after 0.5 and 30 min in the microplate reader. Lyophilized hen egg white lysozyme (Sigma) was diluted in sodium phosphate buffer (0.05 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using a standard curve (Fajardo et al., 2022). Lysozyme was expressed as µg mg protein^{-1}.

Pro-phenoloxidase activity was measured spectrophotometrically using L-DOPA (L-3,4- dihydroxyphenylalanine) as substrate, and trypsin (Sigma) as activator following the method described by Ji et al. (2009) with some modifications. Homogenate samples of 50 µL were diluted in 100 µL of trypsin solution (0.1 % in cacodylate solution) in a 96 well microplate and incubated for 30 min at room temperature. Afterwards, 100 µL L-DOPA solution (0.3 % in cacodylate solution) was added. The absorbance was measured every minute during 5 minutes at 490 nm using the microplate reader. Results were calculated using the Beer-Lambert law using the molar extinction coefficient of the L-DOPA (3700). Results were expressed as units of pro-phenoloxidase mL^{-1} of sample.

2.2.5 Gene expression analysis

Extraction of RNA was done using the NZY total RNA isolation kit (NZYTech) according to the manufacturer's instructions. RNA concentration and purity was analysed by spectrophotometry using DeNovix DS-11 FX (Wilmington). RNA concentration varied from 123.9 to 2180.7 ng μL^{-1} and 260:280 ratios between 1.99 and 2.17, respectively. The integrity of the RNA samples was verified through a 2 % agarose gel. The cDNA was obtained using the NZY first-strand cDNA synthesis kit (NZYTech). This step was used to standardize the concentration of the samples. Reverse transcription was carried out in a Veriti DX 96-well thermal cycler (Applied Biosystems), using 4.4 µL of diluted cDNA (20 ng μL^{-1}) mixed with 5 µL of NZYSpeedy qPCR Green Master Mix® (NZYTech) and 0.3 µL (10 µM) of each specific primer in a final volume of 10 µL. Real-time quantitative PCR was performed, in duplicate for each sample, using a CFX384 Touch Real-Time PCR Detection System (Biorad). Nine genes were selected and analysed due to their role in the immune response. Primer efficiency was tested for each gene (Table 3). Cycling conditions were the same between the different genes, consisting on one cycle of 95 ºC for 10 min, followed by 40 cycles of 2 steps of 95 ºC for 15 s and 62 ºC for 1 min, with a final cycle at 95 ºC for 1 min, followed by 35 s at 62 ºC and ending at 95 ºC for 0.5 s. Pfaffl method (Pfaffl, 2001) was used to perform gene expression analyses and target genes were normalized using *bactn* and *rpl-8* as housekeeping.

Table 3 Selected genes and specific primers used to evaluate the immune status of whiteleg shrimp (*P. vannamei*) PL at the end of the experimental period.

Gene	Acronym	Efficiency (%)	Annealing temperature (ºC)	Accession nº	Amplicon length (bp)	Primer Sequence (5´- 3´)
Cytoplasmic-type actin 4	*bactn*	83.2	62	MF627841.1	260	F: CACGAGACCACCTACAACTCCATC R: TCCTGCTTGCTGATCCACATCTG
Ribosomal protein L8	*rpl-8*	90	62	DQ316258.1	219	F: AGCCAAGCAAGATGGGTCG R: TGTAACGATAAGGGTCACGGAAG
Penaeidin-3a	*pen-3*	86.1	62	Y14926.1	137	F: ATACCCAGGCCACCACCCTT R: TGACAGCAACGCCCTAACC
Hemocyanin	*hmc*	92	62	KY695246.1	124	F: GTCTTAGTGGTTCTTGGGCTTGTC R: GGTCTCCGTCCTGAATGTCTCC
Lysozyme C-like	*lys*	73	62	XM_027352857	82	F: CGGGAAAGGCTATTCTGCCT R: CCAGCACTCTGCCATGTACT
C-type lectin 2-like	*lect*	83	62	DQ858899.2	138	F: GCTTCTGTTGGTGCTGTTGGC R: GTTCCCTTCCCGTATGTGGC
Thioredoxin 1	*trd*	85.3	62	EU499301.1	116	F: TTAACGAGGCTGGAAACA R: AACGACATCGCTCATAGA
Glutathione transferase	*gst*	99	62	AY573381	146	F: AAGATAACGCAGAGCAAGG R: TCGTAGGTGACGGTAAAGA
Glutathione peroxidase	*gpx*	86.6	62	XM_027372127.1	117	F: AGGGACTTCCACCAGATG R: CAACAACTCCCCTTCGGTA
Caspase 3	*casp-3*	93.6	62	KC660103.1	182	F: ACATTTCTGGGCGGAACACC R: GTGACACCCGTGCTTGTACA

2.2.6 Data analysis

Relative growth rate (RGR, % weight day^{-1}) was calculated as: RGR = (e^g – 1) x 100, where e = exponential and g = (lnWf – lnWi) x t^{-1}. Wf and Wi correspond to the final and initial weights, respectively. Feed conversion ratio (FCR) was calculated as: FCR = (Fi / Wg), where Fi corresponds to feed given (g) and Wg to the mean weight gain (g). Survival was expressed as percentage and calculated as: S = (Lf / Li) x 100, where Li and Lf correspond to the initial and final number of PL in the tanks, respectively. Differences in growth performance, FCR, survival, oxidative stress, immune condition, gene expression and shrimp proximate composition between dietary treatments were evaluated using One-way ANOVA's, followed by Tukey HSD multiple comparison tests. Kruskal-Wallis one-way analysis of variance tests, followed by Wilcoxon pairwise comparison tests were used when data did not comply with the One-way ANOVA's assumptions. The estimation of the optimal dietary protein level was performed using broken-line models on linear regression analyses of the PL weight gain, RGR, FCR and survival data. Based on the linear regression models for these parameters, new regression models were estimated having broken-line relationships with protein level in the diets, using the "segmented" package for R software (Muggeo, 2003). These produce a straight line with a positive or negative slope that joins the linear model line at a changepoint in the response variable, interpreted as the protein requirement above which there is no significant change. Multivariate linear discriminant analyses (LDA) were performed for the antioxidant and immune status parameters and gene expression to evaluate how these contributed to the dissociation of the diets in the discriminant functions generated. A MANOVA was performed to assess discriminatory significance using Wilk's λ test, after checking data compliance to the statistics assumptions. The distance between group centroids was measured by Mahalanobis distance and its significance inferred by One-way ANOVA's statistics. Results were expressed as means ± standard deviation (SD). In results expressed as percentage, an arcsine transformation was performed prior to any statistical test: T = ASIN (SQRT (value / 100)). The significance level considered was $p < 0.05$ for all tests performed. All statistical analysis were performed using the software R version 4.2.2.

2.3 Results

2.3.1 Protein content

Increasing levels of protein in the diets did not result in an increasing linear trend in the whole-body protein contents in shrimp PL. No significant differences between treatments were found (Table 4).

Table 4 Whole-body protein content of whiteleg shrimp (*P. vannamei*) PL at the end of the experimental period.

Diet	WBC Protein (% DW)
P34	60.0 ± 0.6
P44	56.7 ± 3.0
P49	61.8 ± 0.6
P54	55.9 ± 4.8
P58	56.3 ± 0.9
P63	58.1 ± 4.6

Results expressed as mean ± standard deviation (*n* = 2 experimental units).

2.3.2 Growth performance

Shrimp PL fed the P54, P58 and P63 diets achieved a significantly higher final body weight than those fed the P34 diet. The P34 dietary treatment resulted in significantly lower shrimp RGR and survival and significantly higher FCR values than all the remaining experimental diets (Table 5).

2.3.3 Dietary protein requirement

The optimal protein requirement was estimated at 47.1 %, 46.4 %, 47.2 % and 44.0 % for weight gain, RGR, FCR and survival, respectively, according to broken line models on linear regression analysis (Figure 1, 2, 3 and 4).

2.3.4 Oxidative stress and immune status related biomarkers and gene expression

Regarding the oxidative stress biomarkers measured: CAT and LPO levels were similar across all treatments; tGSH levels were significantly higher in the P54 diet than in the P34 and P63 diets, with no significant differences between the remaining treatments; and SOD levels were significantly higher in the P54 diet than in the P63 diet, with no significant differences among the remaining treatments. As for the immune parameters, no significant differences between treatments were observed (Table 6). The relative expression of the *hmc* gene was significantly higher in the P34 and P63 dietary treatments than in P44 and P58; transcripts of the *gpx* gene were significantly higher in whiteleg shrimp PL fed the P58 diet than in those fed the P44, P49 and P54; and no significant differences were found between treatments in the relative expression of the remaining genes (Table 7).

Table 5 Initial and final weight, relative growth rate (RGR), feed conversation ratio (FCR) and survival of whiteleg shrimp (*P. vannamei*) PL during the experimental period.

Diet	P34	P44	P49	P54	P58	P63	*p*-value
Initial weight (mg)	3.2 ± 0.0						---
Final weight (mg)	27.5 ± 0.8 ^{a}	45.2 ± 2.6 ^{ab}	52.3 ± 6.2 ^{ab}	60.0 ± 3.0 ^{b}	56.5 ± 10.7 ^{b}	67.5 ± 20.1 ^{b}	0.005
Weight gain (mg)	24.3 ± 0.8 ^{a}	42.1 ± 2.6 ^{ab}	49.1 ± 6.2 ^{ab}	56.9 ± 3.0 ^{b}	53.3 ± 8.8 ^{b}	64.3 ± 20.1 ^{b}	0.005
RGR (% day^{-1})	10.8 ± 0.2 ^{a}	13.5 ± 0.3 ^{b}	14.2 ± 0.6 ^{b}	15.0 ± 0.3 ^{b}	14.6 ± 1.1 ^{b}	15.5 ± 1.5 ^{b}	<0.001
FCR	2.2 ± 0.1 ^{a}	1.5 ± 0.2 ^{b}	1.2 ± 0.2 ^{b}	1.3 ± 0.1 ^{b}	1.1 ± 0.2 ^{b}	1.1 ± 0.3 ^{b}	<0.001
Survival (%)	75.3 ± 5.4 ^{a}	83.1 ± 4.0 ^{ab}	81.1 ± 3.3 ^{ab}	84.1 ± 2.3 ^{ab}	86.8 ± 3.8 ^{b}	86.7 ± 1.0 ^{b}	0.022

Results expressed as mean ± standard deviation. For initial weight n = 60 observational units; for final weight, weight gain, RGR, FCR and survival n = 3 experimental units. Represented are also the p-values for a One-Way ANOVA. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

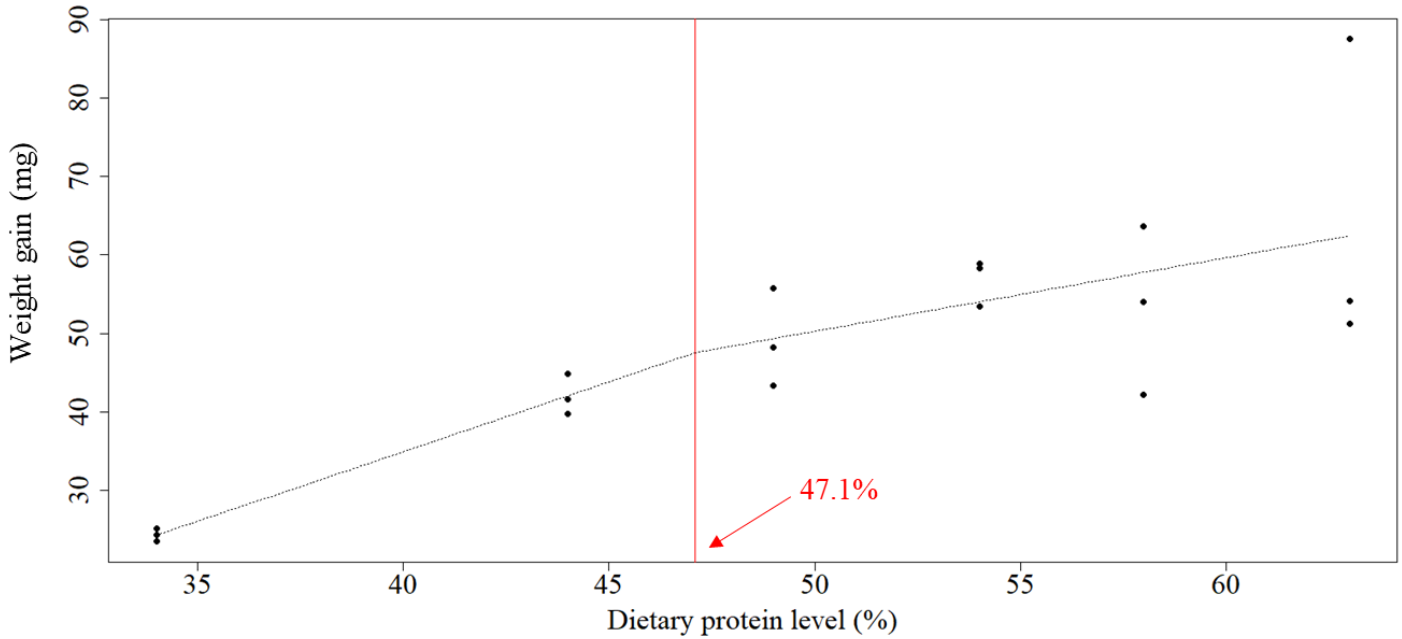

Figure 1 Relationship between dietary protein levels (%) and mean weight gain (mg) of whiteleg shrimp post-larvae. The optimal protein requirement estimated according to the broken-line model, which is given by the abscissa value at the breakpoint, was 47.1 %.

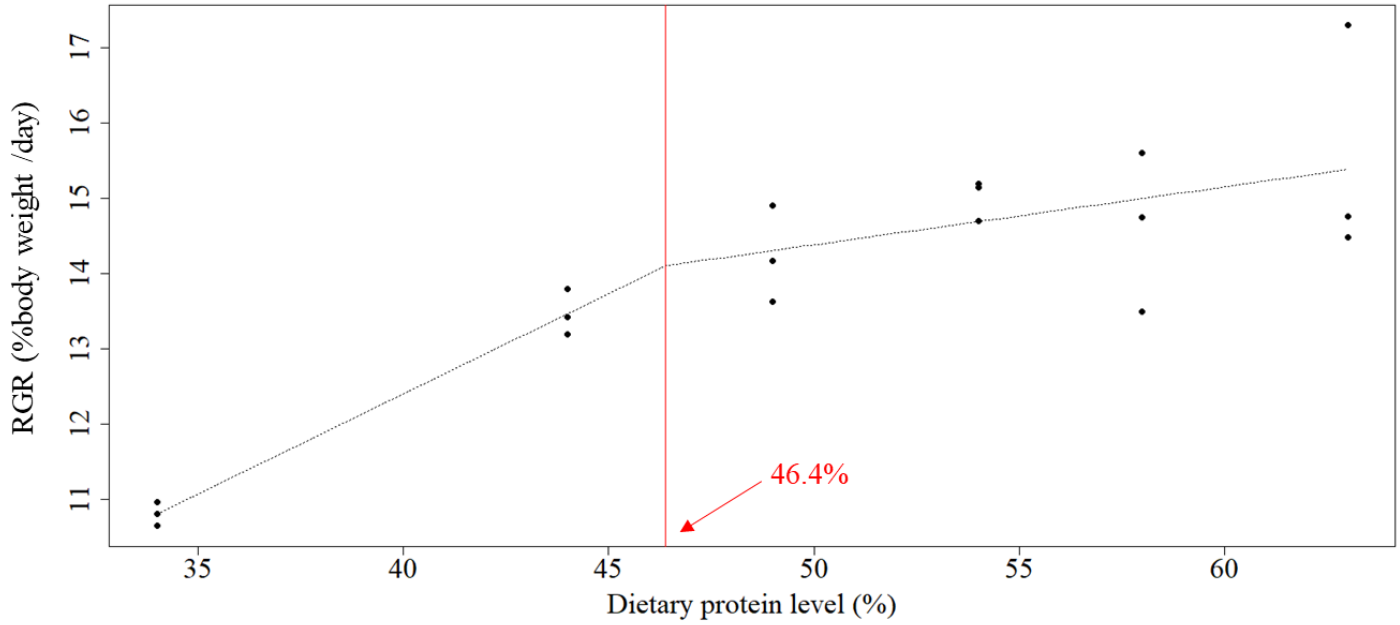

Figure 2 Relationship between dietary protein levels (%) and RGR (% body weight day^{-1}) of whiteleg shrimp post-larvae. The optimal protein requirement estimated according to the broken-line model, which is given by the abscissa value at the breakpoint, was 46.4 %.

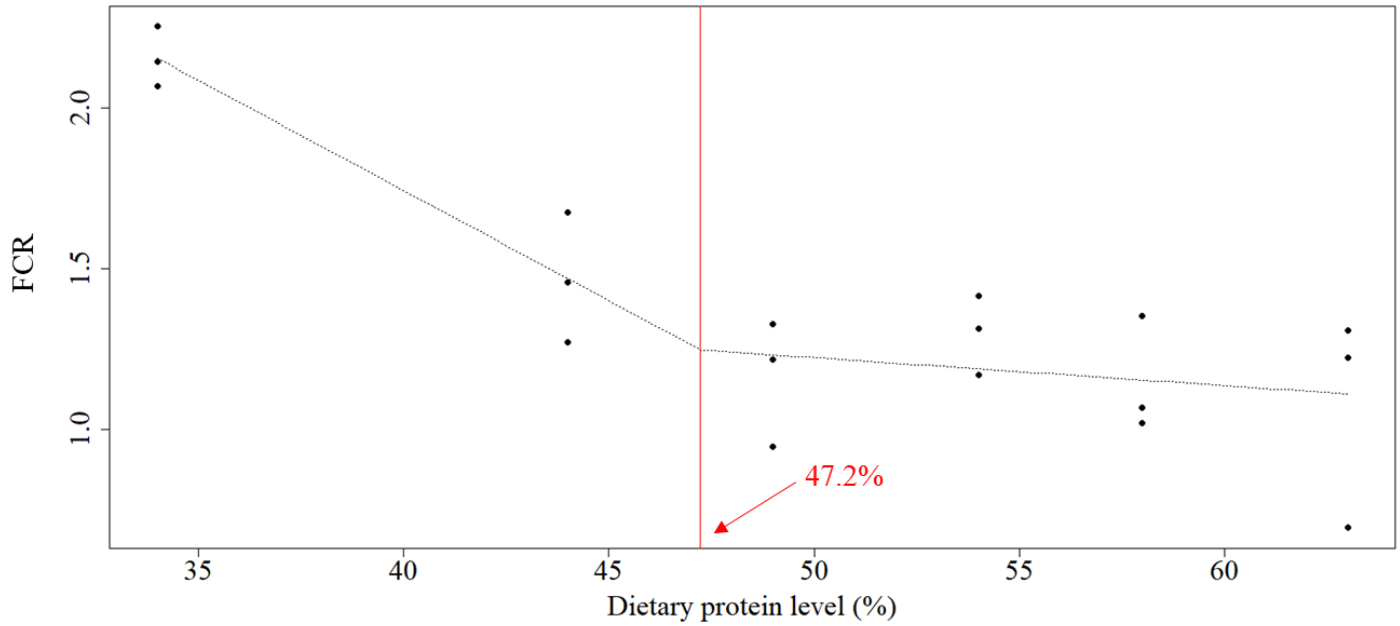

Figure 3 Relationship between dietary protein levels (%) and FCR of whiteleg shrimp post-larvae. The optimal protein requirement estimated according to the broken-line model, which is given by the abscissa value at the breakpoint, was 47.2 %.

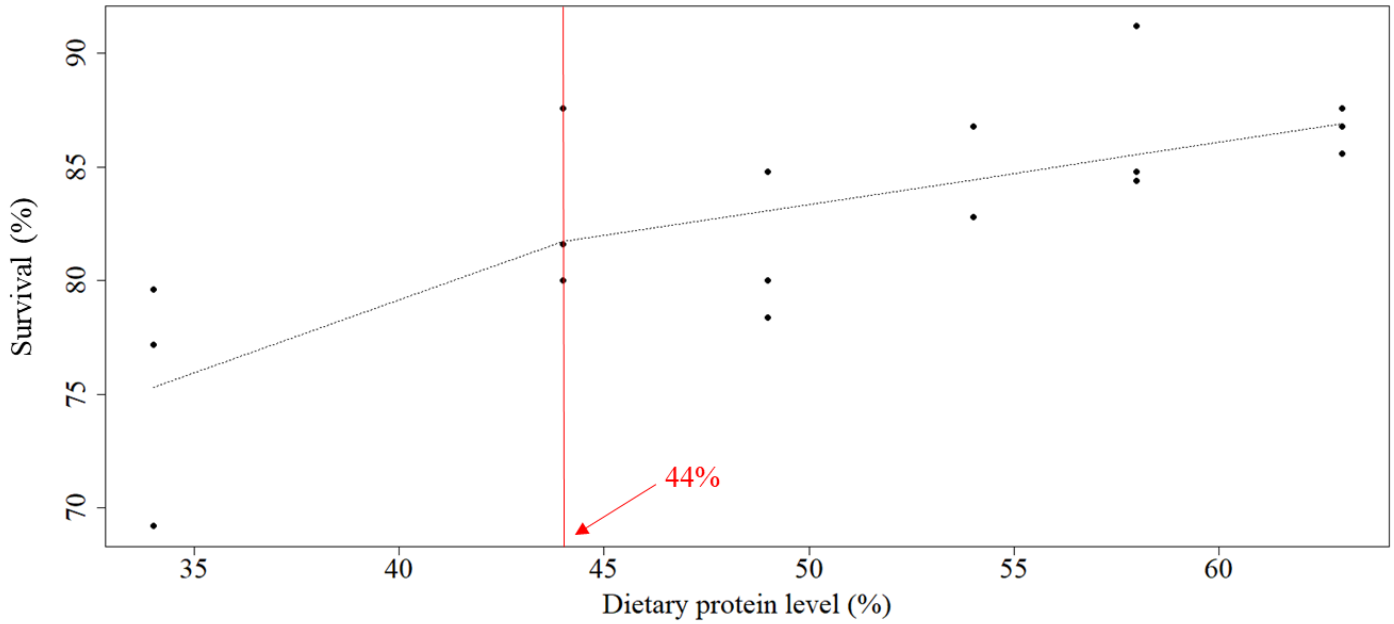

Figure 4 Relationship between dietary protein levels (%) and survival (%) of whiteleg shrimp post-larvae. The optimal protein requirement estimated according to the broken-line model, which is given by the abscissa value at the breakpoint, was 44.0 %.

Table 6 Catalase (CAT), superoxidase dismutase (SOD), total glutathione (tGSH), lipid peroxidation (LPO), lysozyme and pro-phenoloxidase levels in whiteleg shrimp (*P. vannamei*) PL at the end of the experimental period.

Diet	P34	P44	P49	P54	P58	P63	*p*-value
CAT (mg ml^{-1})	5.1 ± 1.0	6.6 ± 1.7	7.2 ± 2.0	8.0 ± 2.4	6.2 ± 1.0	6.8 ± 3.4	0.054
SOD (U mg $protein^{-1}$)	2.2 ± 1.9 ^{ab}	2.0 ± 1.1 ^{ab}	2.8 ± 1.5 ^{ab}	4.1 ± 1.8 ^{b}	1.7 ± 0.5 ^{ab}	1.8 ± 1.8 ^{a}	0.006
tGSH (nmol mg $protein^{-1}$)	5.1 ± 2.3 ^{a}	8.9 ± 2.4 ^{ab}	8.00 ± 2.8 ^{ab}	11.4 ± 4.7 ^{b}	8.7 ± 4.1 ^{ab}	5.5 ± 3.0 ^{a}	<0.001
LPO (nmol g wt^{-1})	8.9 ± 1.7	10.2 ± 2.1	10.2 ± 3.9	8.4 ± 2.1	9.5 ± 3.3	9.9 ± 1.2	0.301
Lysozyme (µg mg $protein^{-1}$)	1.6 ± 0.4	1.4 ± 0.5	1.4 ± 0.3	1.4 ± 0.2	1.8 ± 0.6	1.3 ± 0.1	0.387
Pro-phenoloxidase (*10^{-3} U ml^{-1})	34.8 ± 9.3	30.4 ± 8.4	29.5 ± 13.0	24.7 ± 10.0	28.1 ± 10.0	31.7 ± 8.3	0.389

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a One-Way ANOVA. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

Table 7 Relative expression to housekeeping (*bactn* and *rpl-8*) of target immune related genes of whiteleg shrimp (*P. vannamei*) PL fed the experimental diets.

Gene	Acronym	Relative Expression						*p*-value
		P34	P44	P49	P54	P58	P63	
Penaeidin-3a	*pen-3*	0.9 ± 0.3	1.8 ± 1.0	1.3 ± 0.6	1.4 ± 0.8	1.3 ± 0.7	1.9 ± 1.1	0.115
Hemocyanin	*hmc*	0.9 ± 0.4 ^{b}	0.3 ± 0.4 ^{a}	0.3 ± 0.1 ^{ab}	0.8 ± 0.8 ^{ab}	0.3 ± 0.3 ^{a}	1.4 ± 2.3 ^{b}	0.034
Lysozyme C-like	*lys*	1.1 ± 0.5	0.7 ± 0.3	0.8 ± 0.4	1.2 ± 1.4	1.5 ± 1.8	2.2 ± 3.1	0.411
C-type lectin 2-like	*lect*	1.1 ± 0.7	0.9 ± 1.0	0.6 ± 0.5	0.8 ± 0.7	1.7 ± 3.3	0.8 ± 0.8	0.712
Thioredoxin 1	*trd*	0.8 ± 0.2	1.4 ± 0.8	1.0 ± 0.2	1.1 ± 0.4	1.4 ± 0.7	1.1 ± 0.5	0.304
Glutathione transferase	*gst*	1.4 ± 1.2	0.6 ± 0.3	1.1 ± 1.3	1.5 ± 0.9	0.7 ± 0.5	0.9 ± 0.8	0.119
Glutathione peroxidase	*gpx*	0.9 ± 0.3 ^{ab}	0.7 ± 0.2 ^{a}	0.6 ± 0.1 ^{a}	0.7 ± 0.2 ^{a}	1.3 ± 0.6 ^{b}	0.9 ± 0.3 ^{ab}	<0.001
Caspase 3	*casp-3*	0.9 ± 0.4	1.4 ± 1.4	0.7 ± 0.5	1.2 ± 1.2	1.3 ± 1.7	1.5 ± 1.6	0.953

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a One-Way ANOVA. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in post-hoc Tukey multiple comparison test.

The LDA of the antioxidant biomarkers (CAT, SOD, tGSH and LPO) and transcripts of genes associated with the antioxidant defense (*trd*, *gst* and *gpx*) resulted in five discriminant functions, with the first two accounting for 84.80 % of the data variability (Wilks $\lambda = 0.266$, $p < 0.001$). In the first discriminant function (LDA1, score of 68.95 %) (Figure 5) the analysis revealed a significant group separation between diets P34, P58 and P63 and the remaining diets and between diets P44 and P49 and diet P54. Group discrimination was mainly positively loaded by *gpx* and negatively by CAT and tGSH. In the second discriminant function (LDA2, score of 13.51%) a significant separation of diet P58 from diets P44, P49 and P63 is also shown (Figure 5). This discrimination was mainly positively loaded by *gpx* and negatively by LPO.

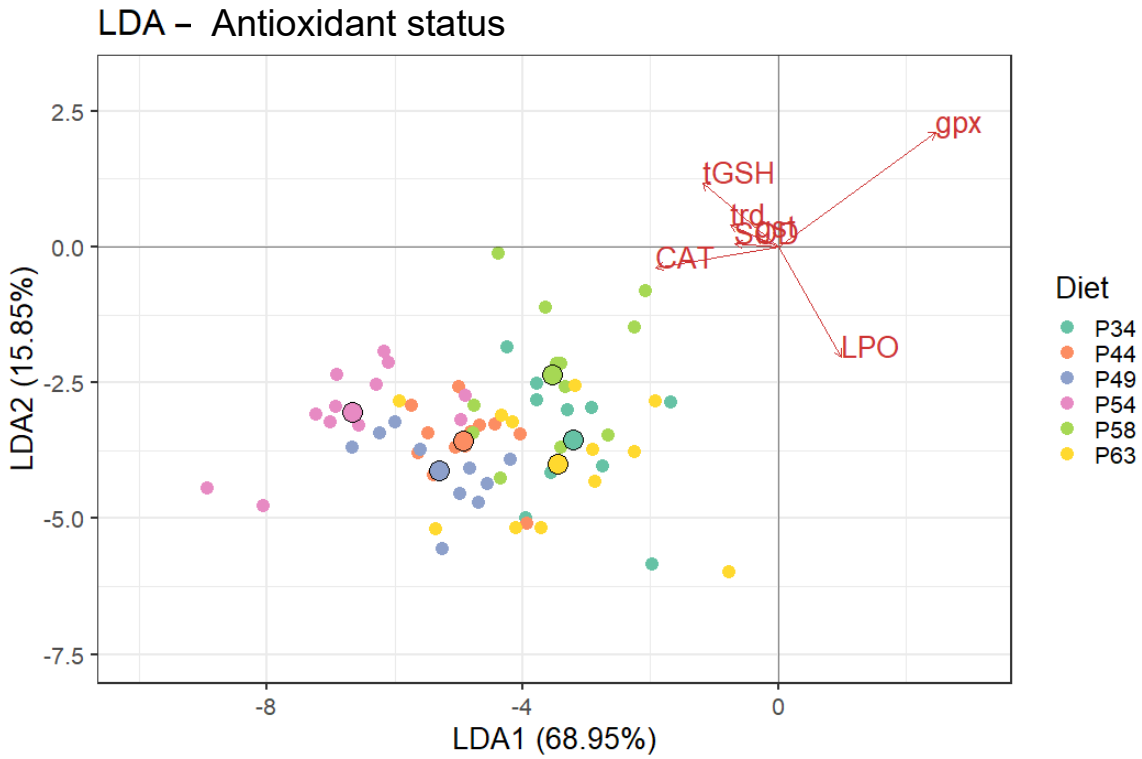

Figure 5 Linear discriminant analysis (LDA) of antioxidant biomakers (CAT, SOD, tGSH and LPO) and relative expression of genes associated with the antioxidant defense (*trd, gst* and *gpx*) of whiteleg shrimp post-larvae fed the different experimental diets. Percentages indicate the main functions (LDA1 and LDA2) discriminant score. Small circles represent diet data distribution while big circles represent diet centroids. Variable loads for both discriminant functions are represented by red arrows. Wilk's lambda test was significant ($p < 0.001$). In LDA1, P34, P58 and P63 diets were discriminated from the remaining diets and diets P44 and P49 from diet P54, mainly due to lower levels of CAT and tGSH and higher transcripts of *gpx*. In LDA2, P58 was discriminated from P44, P49 and P63 mainly due to lower levels of LPO.

The LDA of the immune parameters (Lysozyme and pro-phenoloxidase) and transcripts of genes associated with the shrimp innate immune system (*casp-3*, *pen-3, hmc, lys* and *lect*) resulted in 5 discriminant functions, with the first two accounting for 81.20 % of the data variability (Wilks λ = 0.532, $p < 0.001$). In the first discriminant function (LDA1, score of 43.87 %) (Figure 6) the discriminant analysis revealed a significant group separation between P34 and diets P44, P49 and P54 and between P58 and P54. Group discrimination was mainly positively loaded by lysozyme and pro-phenoloxidase. In the second discriminant function (LDA2, score of 37.33 %) a significant separation of diet P58 from diets P34 and P63 is also shown (Figure 5). This discrimination was mainly positively loaded by lysozyme and negatively by pro-phenoloxidase and *hmc*.

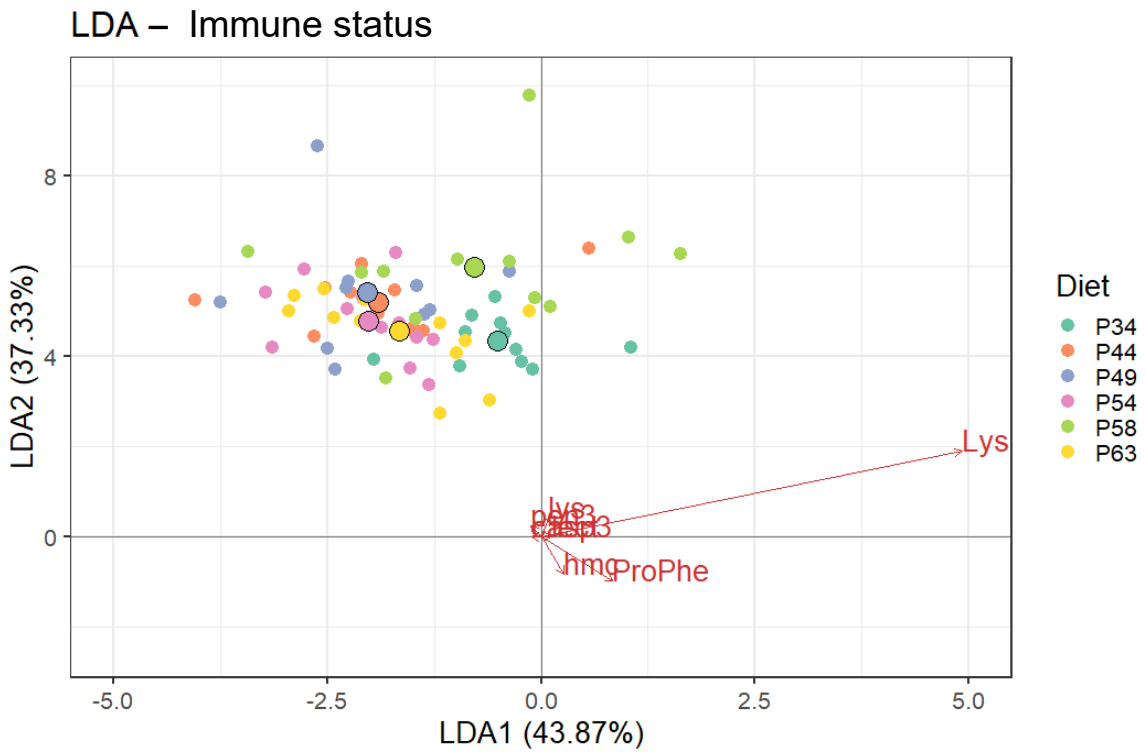

Figure 6 Linear discriminant analysis (LDA) of immune parameters (Lysozyme and pro-phenoloxidase) and relative expression of genes associated with the shrimp innate immune system (*casp-3*, *pen-3, hmc, lys* and *lect*) of whiteleg shrimp post-larvae fed the different experimental diets. Percentages indicate the main functions (LDA1 and LDA2) discriminant score. Small circles represent diet data distribution while big circles represent diet centroids. Variable loads for both discriminant functions are represented by red arrows. Wilk's lambda test was significant ($p < 0.001$). In LDA1, P34 was discriminated from diets P44, P49 and P54 and diet P58 from P54, mainly due to higher levels of lysozyme and pro-phenoloxidase. In LDA2, P58 was discriminated from P34 and P63 mainly due to lower levels of lysozyme, pro-phenoloxidase and transcripts of *hmc*.

2.4 Discussion

Few research has focused on the effects of dietary protein requirements of whiteleg shrimp PL during the nursery stage of production in clear water systems devoid of natural productivity, and studies available have divergent results. Some authors suggest optimal dietary protein levels for whiteleg shrimp PL in clear water RAS to be as low as between 21.4 and 24.5 % (Velasco et al., 2000) or 34 % (Hu et al., 2008). On the other hand, Otoshi et al. (2001) described increases in whiteleg shrimp PL weight gain when fed a diet with 52 % protein over a 45 % protein diet during the nursery phase. Moreover, Xie et al. (2020), evaluated several diets with gradient levels of protein (46 %, 47 %, 49 %, 50 % and 51 %) and estimated 45.6 % and 50.0 % to be optimal, based on the shrimp PL weight gain and survival, respectively. In the current study, six experimental diets were formulated to contain 34 %, 44 %, 49 %, 54 %, 58 % and 63 % of crude protein, covering a wide range of inclusion levels to contemplate several scenarios. The optimal protein level estimated was 47.1 %, 46.4 %, 47.2 % and 44.0 % for weight gain, RGR, FCR and survival, respectively. These results suggest that when tailoring microdiets for whiteleg shrimp PL grown in clear water RAS, a minimum of around 47 % of protein should be considered for optimal growth performances and survival. There have been reports of detrimental effects to whiteleg shrimp growth when levels of dietary protein were higher than 48 % (Kureshy and Davis, 2002), which was not verified in the present study. Although not statistically significant, shrimp PL fed diets containing over the previously stated protein level tended to have higher mean weight gain and RGR values. Moreover, the multivariate analysis performed showed an apparent overall superior antioxidant status of shrimp PL fed the P54 diet compared to the remaining diets, evidencing that using protein inclusion levels up to 54% in aquafeeds not only potentiates growth performances and survival but also can potentially be beneficial to the health status of whiteleg shrimp PL grown in clear water RAS devoid of natural productivity.

A diet containing 34 % protein showed detrimental effects, leading to a decrease in the PL growth performances and survival, when compared with diets with higher protein contents (P54, P58 and P63), suggesting that low protein diets may not be adequate to be used in this stage of shrimp development and/or for the clear water RAS husbandry conditions. Furthermore, tGSH levels were lower in shrimp fed the P34 diet compared with those fed the P54 dietary treatment, indicating a potential decline in their protection against oxidative damage, as tGSH play a key role in the antioxidant defence of the shrimp. These neutralize hydroxyl radicals (OH) against which there is no enzymatic protection (Cardona et al., 2016). In fact, according to the multivariate analysis performed, the P34 diet seemingly reduced the overall antioxidant status of the whiteleg shrimp PL when compared to the P44, P49 and P54 dietary treatments, not only due to lower levels of tGSH but also

CAT activity, an antioxidant enzyme that converts H_2O_2 into molecular oxygen and water (Rodrigues et al., 2017). However, shrimp PL fed P34 showed higher transcripts of the gene *gpx*, responsible for the encoding of glutathione peroxidase, which has an important role in the antioxidant protection by removing lipids and hydrogen peroxides formed during immune and physiological processes (Liu et al., 2007). Although shrimp were reared under standard rearing conditions that should not promote oxidative stress, the accelerated development whiteleg shrimp undergo in the larval/PL stages may result in oxidative damage, as fast growth is likely to produce excess ROS, damaging key physiological structures (Monaghan et al., 2009). Despite having similar LPO levels to the remaining treatments, the apparent compromised antioxidant mechanisms of shrimp fed the P34 dietary treatment may have contributed to the lower survival observed when compared to diets with higher protein contents. On the other hand, the multivariate analysis performed showed that the P34 diet seems to have stimulated the shrimp PL immune system when compared to the P44, P49 and P54 dietary treatments. PL fed P34 showed higher activity levels of lysozyme, an important haemocyte-specific protein with antibacterial properties (de-la-Re-Vega et al., 2006) and pro-phenoloxidase, an enzyme involved in the innate immune system of invertebrates (Kim et al., 2014). These results may be related to the increased proportions of wheat meal in the P34 diet when compared to the higher protein diets. Although wheat is often not the most problematic plant ingredient, these contain anti-nutritional factors that can have adverse impacts on the overall health status of the cultured animals (Francis et al., 2001), and the increased levels of this ingredient may have caused an innate immune response in shrimp PL. Parallelly, the increment of fish and algae oil quantities in diet P34 may have positively influenced the shrimp PL immune mechanisms, as both ingredients have been shown to enhance the health condition of whiteleg shrimp PL (Nonwachai et al., 2010; Xie et al., 2019). The gene expression analysis also showed an increase in transcripts of *hmc* in the P34 dietary treatment when compared to the P44 and P58 diets. It should be noted that hemocyanin is a multifunctional protein involved in several important physiological processes beyond innate immunity such as oxygen transport protein storage, osmoregulation, molt cycle and exoskeleton formation. Therefore, higher expressions of this gene may not be exclusively related to changes in the whiteleg shrimp PL immune status (Zhang et al. 2009; Zheng et al., 2016).

Similarly, a diet containing 63 % of protein also seemed to compromise the overall shrimp PL antioxidant status. The multivariate analysis performed showed a significant separation of P63 from P44, P48 and P54, mainly due to lower levels of CAT and tGSH levels and higher transcripts of the *gpx* gene. These results suggest a lower dependency on these antioxidant molecules to sustain good growth performances. Yet, reduced activity levels of the latter can indicate a decreased antioxidant capacity, particularly important in

more challenging husbandry conditions than those maintained in this study. Still, contrarily to what was observed in the P34 dietary treatment, growth performance and survival were not compromised in P63. Additionally, just like P34, P63 was significantly segregated from P58 due to higher activity levels of lysozyme and pro-phenoloxidase and higher transcripts of the *hmc* gene, suggesting that protein levels as high as 63 % may have originated an innate immune response in the shrimp PL. However, it is unclear what may have caused this response. Hence, additional research should be conducted to clarify the potential detrimental and beneficial effects of microdiets with the highest protein levels tested in the current study, since increases in protein inclusion quantities represent increments in diet prices. These are of extreme importance in large-scale industrial settings, as feeds have a high preponderance on the overall production expenditures and higher priced diets could compromise the economic feasibility of shrimp farms.

2.5 Conclusions

In summary, results from this study confirm the importance of using a high protein aquafeed in the nursery phase if whiteleg shrimp PL are grown in clear water RAS devoid of natural productivity, since inert diets are the exclusive source of nutrients in this type of production system. They also suggest that feeds that work well in semi-intensive or biofloc systems may not fulfil the shrimp PL protein requirements in clear water farming. A minimum of around 47 % of protein was estimated as optimal for weight gain and survival and is recommended when tailoring microdiets for the nursery phase of whiteleg shrimp PL grown in clear water RAS. Moreover, results indicate that protein inclusion levels up to 54% can also seemingly improve the overall antioxidant status of the whiteleg shrimp PL. A diet containing only 34 % protein impaired PL growth performances and survival when compared to higher protein diets. Meanwhile, results also demonstrate that both extreme levels of dietary protein inclusion (34 % and 63 %) impaired the antioxidant mechanisms of PL but stimulated the production of immunity related molecules. Despite the excellent shrimp PL growth performances obtained, using a diet with protein levels as high as 63 % should be considered cautiously, as the current study suggests some putative negative effects on shrimp health, along with predictable higher nitrogen discharges into the environment and higher feed costs.

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Declarations

All activities were undertaken within the clear boundaries of national and EU legal frameworks directed by qualified scientists/technicians and conducted according to the European guidelines on the protection of animals used for scientific purposes (Directive 2010/63/UE of the European Parliament and on the European Union Council) and under strict monitoring and control of DGAV—(Direção Geral de Alimentação e Veterinária), Animal Welfare Division, which is the competent authority responsible for implementing the legislation on the “protection of animals used for scientific purposes”.

AS, WP and LC worked for Sparos Lda during this study. The remaining authors declare that they have no conflicts of interest. The views expressed in this work are the sole responsibility of the authors and do not necessarily reflect the views of any third party.

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Chapter 3

Marine ingredients and fat levels used in inert microdiets for whiteleg shrimp (*Penaeus vannamei*) post-larvae impact survival, health status and feed conversion

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Marine ingredients and fat levels used in inert microdiets for whiteleg shrimp (*Penaeus vannamei*) post-larvae impact survival, health status and feed conversion

Abstract

This study aimed at assessing the effects of several marine ingredients and fat levels in inert microdiets for whiteleg shrimp post-larvae (PL) reared in clear water recirculating aquaculture systems (RAS). Five experimental diets were evaluated: a control diet (CTRL), formulated to contain 54 % of protein and 14 % lipids utilizing a blend of fish and squid meals as main ingredients; diets FISH, SQUID and KRILL, formulated to contain the same levels of protein and lipids but with fish, squid, and krill meals, respectively, as main ingredients; and diet FISHHF similar to FISH but containing a higher lipid content (16 %) through higher inclusion levels of fish oil and reduced wheat meal. Whiteleg shrimp PL (13 mg wet weight) were reared for 21 days in a clear water RAS at Riasearch facilities. At the end of the feeding period, the PL whole body protein contents were similar between treatments, but the fatty acid profile of PL fed FISH and FISHHF differed significantly. Higher n-3 PUFA and lower n-6 PUFA levels were measured in PL fed FISHHF, probably due to the higher fish oil contents compared to FISH. No significant differences were found between treatments in the PL mean wet weight. However, FISHHF resulted in significantly higher FCR values than KRILL and significantly lower survival than diets CTRL and KRILL. Furthermore, results suggest a deterioration in the health status of PL fed the FISH, SQUID and FISHHF diets when compared to CTRL and KRILL (excluding KRILL for SQUID), mainly by lower mRNA transcripts of *trd* and *crus,* and higher of *casp-3*. Still, PL fed the KRILL diet showed lower activity levels of the antioxidant enzyme CAT and higher LPO levels compared to CTRL. On the other hand, PL fed FISHHF showed higher activity levels of CAT, GST and pro-phenoloxidase compared to KRILL. The apparent activation of the antioxidant and immune mechanisms may signal a more stressful condition, which seems corroborated by lower survival values in PL fed FISHHF. In conclusion, improved shrimp PL survival and health status were achieved using diets with a blend of fish and squid meals or krill meal as the main ingredients. Increasing lipid levels from 14 to 16 % in a fish meal-based diet produced similar growth performances but increased FCR and reduced survival. Therefore, a lipid level of around 14% seems to be preferable when tailoring diets for whiteleg shrimp PL reared in clear water RAS and at this stage of development.

Keywords: *Penaeus vannamei*, whiteleg shrimp, post-larvae, inert microdiets, marine ingredients, fat levels

3.1 Introduction

Diets for whiteleg shrimp PL produced in clear water recirculating aquaculture systems (RAS) need high inclusion levels of protein as inert feeds are the only source of these nutrients in a system devoid of natural or artificial productivity. As determined in Chapter 2, a dietary protein level of at least 47% is recommended to ensure maximum growth and survival and even greater proportions up to 54% seem to be beneficial to the antioxidant status of PL, and probably also for other non-growth purposes. Fish meal has predominantly been used as a primary ingredient in shrimp aquafeeds due to its elevated protein content, rich amino acid profile, nutrient bioavailability, feeding stimulating abilities and constant availability (Gatlin et al., 2007; Nunes et al., 2022), and in particular for the early life stages. Nevertheless, fish meal supplies are stagnant, and it is foreseen that they may not be sufficient to cover the necessities of the expanding aquaculture sector (FAO, 2022). Therefore, the industry and academia have been focusing on finding alternative ingredients that can reduce the dependence on fish meal, and replacements either complete or partial, have been successfully performed in diets for whiteleg shrimp, using proteins of plant origin (Chen et al., 2017; Sá et al., 2013; Sookying & Davis, 2012; Xie et al., 2016; Yue et al., 2012); of animal origin (Davis & Arnold, 2000; Hernández et al., 2008); of insect origin (Cummins et al., 2017; Motte et al., 2019); and of microbial origin (Bauer et al., 2012; Chen et al., 2021; Yao et al., 2022; Zhao et al., 2017). However, these ingredients often have lower biological value per kg of product, poorer attractability, unreliable quality and availability, inadequate amino acid profiles and antinutritional factors (Gatlin et al., 2007; Nunes et al., 2022). Other marine protein sources commercially available such as squid and krill meals have nutritional characteristics often more comparable to fish meal and do not present some of the disadvantages of the previously mentioned ingredients. These have been used successfully as partial replacements of fish meal enhancing juvenile shrimp survival, growth performances, health status and/or increase feed palatability and intake (Ambasankar et al., 2022; Derby et al., 2016; Nunes et al., 2014; Soares et al., 2021). Still, studies that focused on the use of these ingredients in diets for the earlier stages of shrimp development are highly limited. Therefore, this study aimed at evaluating the growth performance, survival and health status of whiteleg shrimp PL reared in clear water RAS and fed experimental microdiets formulated with different ingredients of marine origin.

3.2 Materials and methods

3.2.1 Dietary treatments

Five experimental microdiets were evaluated in triplicates. A control diet (CTRL) was formulated to contain 54 % of protein and 14 % lipids utilizing a blend of fish and squid meals as main ingredients. Diets FISH, SQUID and KRILL were formulated to contain the

same levels of protein and lipids but with fish, squid, and krill meals, respectively, as main ingredients. Diet FISHHF composition was similar to FISH but was formulated to contain a higher lipid content (16 %) through higher inclusion levels of fish oil and reduced wheat meal. The composition of the diets can be seen in Table 1. All diets were produced at Sparos Lda facilities (Olhão, Portugal) using extrusion at low temperature as main production process, as follows: powder ingredient mixing according to target formulation using a double helix mixer; grinding in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany); addition of the oil fraction; humidification and agglomeration through low temperature extrusion (Dominioni Group, Italy); drying of resultant pellets in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 4 h at 60 ºC, crumbling (Neuero Farm, Germany) and sieving to desired size ranges.

Table 1 Composition of the experimental diets used to culture whiteleg shrimp (*P. vannamei*) PL for 21 days.

% Wet matter	CTRL	FISH	SQUID	KRILL	FISHHF
Protein	53.5	54.8	54.3	53.5	54.4
Lipids	14.5	14.2	14.3	14.3	16.3
Ashes	9.6	12.0	10.6	12.0	12.1
Phosphorous	1.6	1.7	1.6	1.7	1.7
Energy (KJ g^{-1})	19.7	20.1	20.2	20.1	20.5

3.2.2 Shrimp rearing and sampling

Whiteleg shrimp PL (mean wet weight of 13.0 ± 2.0 mg) originated from Miami Aquaculture (Florida, USA), were reared for 21 days at RIASEARCH Lda facilities (Murtosa, Portugal). These were randomly distributed to 15 tanks with approximately 50 L, part of a 3 m^3 clear water recirculating system, at a larval density of 250 individuals per tank (shrimp density of 5000 m^3). These were kept under a 12 h light:12 h dark photoperiod and were fed close to *ad libitum* with automatic feeders that supplied eight meals a day. Feeders were cleaned daily and charged with adjusted feed quantities based on the observation of the tanks and the presence/absence of remnants from the previous day. Feed size was 400-600 µm for the first week and 600-800 µm for the remaining feeding period. Water temperature was maintained at 27.6 ± 0.5 ºC, dissolved oxygen concentration at 6.6 ± 0.3 mg L^{-1}, salinity at 22.7 ± 1.1 g L^{-1}, pH at 8.1 ± 0.1, NH_3 and NO_2 at 0.0 ± 0.0 mg L^{-1}.

At the start of the trial, a total of 120 PL from the initial stock were randomly selected and weighed in groups of 30 individuals for initial wet weight determination. At the end of the experiment, a total of 50 PL were randomly selected per tank and weighted individually. The remaining PL were counted and weighted in groups to determine tank final biomass and survival. For whole body composition analysis, a total of 10 g of PL were randomly collected from each tank. Additionally, 23 PL were randomly selected from each tank for oxidative stress, energy reserves, immune parameters, and gene expression analysis. These were stored at -80 ºC for subsequent analysis. Shrimp sampled for molecular biology analysis were kept in RNAlater (Sigma) at > 1:5 volume ratio, at 4 ºC for 24 hours prior to being stored at -20 ºC. Shrimp were fasted for 12 hours prior to samplings to ensure their guts were empty at collection.

3.2.3 Oxidative stress-related biomarkers and cellular energy allocation

3.2.3.1 Sample preparation

A total of 9 whiteleg shrimp PL from each tank sampled at end of the trial were weighed and homogenized in triple groups of 3 individuals by sonication on ice using 2800 µL of ultra-pure water. Three aliquots of 300 µL were taken from each sample for the analysis of lipid, sugar and protein contents. An aliquot for lipid peroxidation with butylated hydroxytoluene was reserved prior to centrifugation. The remaining homogenate (~ 700 µL) was diluted with 700 µL of 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 20 min at 9000 g (4 ºC). From the post-mitochondrial supernatant (PMS) aliquots for the measurement of catalase (CAT), glutathione S-transferase (GST), total glutathione (tGSH) and protein content were taken.

3.2.3.2 Determination of oxidative stress biomarkers

Catalase (CAT), glutathione-S-transferase (GST), total glutathione (tGSH) and lipid peroxidation (LPO) levels as well as total proteins content were determined in the homogenized samples following the procedures described in Chapter 2. GST activity was determined following the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) as described by Habig et al. (1974). Absorbance was read at 340 nm and results expressed as nmol mg of protein^{-1}.

3.2.3.3 Determination of cellular energy allocation

Energy available (EA, assessed as the sum of sugars, lipids and proteins) and energy consumption (EC, as electron transport system - ETS activity) were determined by following the methods described by De Coen & Janssen (1997) adapted for microplate

(Rodrigues et al., 2015). All the values obtained were adjusted to PL weight. The final CEA value was calculated as: CEA = EA/EC (Verslycke et al., 2003).

Total lipid contents were determined by adding chloroform (119.38M; ACS spectrophotometric grade, ≥ 99.8%), methanol (32.04M; ACS reagent, ≥ 99.8%) and Mili-Q water in a 2:2:1 proportion to 300 µl of the homogenized samples. These were then centrifuged (1000 g, 5 min, 25 ºC) and the organic phase of each sample transferred to clean glass tubes. 500 µL of sulfuric acid (H_2SO_4) were added and samples incubated for 15 min at 200 ºC. Afterwards, 1500 µL of ultra-pure water were added to each sample and absorbance measured at 375 nm. Tripalmitin was used as a lipid standard. 100 µL of 15 % trichloroacetic acid (TCA) were added to 300 µL of the homogenized samples to determine sugar contents. Samples were incubated for 10 min at -20 ºC and then centrifuged at 1000 g for 10 min at 4 ºC. 200 µL of 5 % phenol and 800 µL of H_2SO_4 were added to the supernatant. Glucose was used as a standard and the absorbance read at 492 nm. The pellet from the previous centrifugation was resuspended in 500 µl of sodium hydroxide (NaOH), incubated (30 min at 60 ºC) and neutralized with 280 µL of hydrochloric acid (HCl). Fractions of energy available were converted into energetic equivalent values using the corresponding energy of combustion: 39500 mJ g lipid^{-1}, 17500 mJ g glycogen^{-1}, 24000 mJ g protein^{-1} (De Coen and Janssen, 1997). The final value of EA was calculated as the sum of lipid, protein and sugar content in mJ mg tissue^{-1}.

ETS activity measurement was performed in 300 µl of the homogenized samples, to which 150 µL of homogenization buffer (0.3 M Tris base; 0.45 % (w/v) Poly vinyl pyrrolidone; 459 µM magnesium sulphate ($MgSO_4$); 0.6 % (v/v) Triton X-100 at a pH of 8.5) were added and then centrifuged (1000 g, 10 min, 4 ºC). 50 µL of supernatant from each replicate were transferred to a microplate and 150 µL of buffered solution B (solution A (0.13 M Tris base; 0.27 % (v/v) Triton X-100); 1.7 mM reduced nicotinamide adenine dinucleotide (NADH); 274 µM NADPH); and 100 µL of INT solution (p-iodonitrotetrazolium; 8 mM) were added to start the reaction. Absorbance was measured at 490 nm for 3 min. Cellular oxygen consumption rate was calculated based on the stoichiometrical relationship of 2 µmol of formazan formed to 1 µmol of oxygen consumed. The quantity of oxygen consumed was determined by the formula of Lambert-Beer: $A = \varepsilon \times l \times c$ (A = absorbance; ε for INT-formazan = 15900/ M.cm; l = 0.9; c = oxygen consumed). EC value was obtained by the conversion to energetic values using the specific oxyenthalpic equivalent for an average lipid, protein and sugar mixture of 480 kJ mol O_2^{-1}.

3.2.4 Immune condition parameters

A total of 9 whiteleg shrimp PL from each tank sampled at end of the trial were weighed and homogenized in triple groups of 3 individuals, as described in Chapter 2.

Potassium phosphate buffer (0.1 M) was added to each group in a 1/10 (w/v) proportion followed by homogenization using a high-performance dispersing instrument (SilentCrusher M, Heidolph Instruments). After centrifugation (5500 rpm for 20 minutes), sample supernatant was collected and distributed in separate aliquots for lysozyme and pro-phenoloxidase activities determination, following the procedures described in Chapter 2.

3.2.5 Gene expression

A total of 5 PL sampled at the end of the experimental trial for molecular biology analysis were homogenized in NZYol (Nzytech, w/v proportion according to the manufacturer's instructions) using a Precellys 24 tissue homogenizer (Bertin instruments). RNA extraction, analyses of its concentration and purity, attainment of cDNA strands, reverse transcription and RT-PCR were performed for each sample as described in Chapter 2. Genes examined were chosen due to their association with antioxidant or immunity mechanisms (Table 2). The relative expression of target genes was determined according to the Pfaffl method (Pfaffl, 2001), using *gapdh* as housekeeping (Alvarez-Lee et al., 2020).

3.2.6 Chemical analysis

Bromatological analyses for the determination of the PL whole-body composition (protein contents for all treatments; lipids and fatty acid composition for FISH and FISHHF) were performed. For protein determination, whiteleg shrimp PL sampled at the end of the trial were freeze dried and their total nitrogen (N), hydrogen (H) and carbon (C) content was determined using an elemental analyzer (Elementar Vario EL III, Hanau - Germany). Protein contents were then calculated as $\boldsymbol{N \times 6.25}$. For total lipids extraction, samples were homogenized in chloroform/methanol (2:1, v/v) (Folch et al. 1957). Fatty acid methyl esters (FAME) were obtained from total lipids by acid-catalyzed transesterification using 2 mL of 1% H_2SO_4 in methanol plus 1 mL toluene (Christie 1982). Afterwards, FAME extraction and purification was performed by thin layer chromatography (Tocher and Harvie 1988), and separation and quantification by gas-liquid chromatography (Fisons GC8600, Carlo Erba, Milan, Italy) using a 30 m x 0.32 mm capillary column (CP wax 52CB; Chrompack, London, UK) with on-column injection at 50ºC and flame ionization detection at 250 ºC. Individual methyl esters identification was performed by comparison to known standards (Ackman 1980).

Table 2 Selected genes and specific primers used to evaluate the health status of whiteleg shrimp (*P. vannamei*) PL at the end of the experimental period.

Gene	Acronym	Efficiency (%)	Annealing temperature (ºC)	Accession nº	Amplicon length (bp)	Primer Sequence (5´- 3´)
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	104.5	60	MG787341.1	166	F: AAAGGTAGGAATTGCCCCCG R: AGGGATGAGACTAGCACGACT
PvHm117 crustin P	*crus*	101.4	60	AY488497.1	109	F: GAAACCACCACCAACACCTACTCC R: TCTGTGCGGCCTCTTTACGG
Penaeidin-3a	*pen-3*	86.1	60	Y14926.1	137	F: ATACCCAGGCCACCACCCTT R: TGACAGCAACGCCCTAACC
Lysozyme C-like	*lys*	73.4	60	XM_027352857	82	F: CGGGAAAGGCTATTCTGCCT R: CCAGCACTCTGCCATGTACT
Thioredoxin 1	*trd*	85,3	60	EU499301.1	116	F: TTAACGAGGCTGGAAACA R: AACGACATCGCTCATAGA
Glutathione transferase	*gst*	99.3	60	AY573381	146	F: AAGATAACGCAGAGCAAGG R: TCGTAGGTGACGGTAAAGA
Glutathione peroxidase	*gpx*	86.6	60	XM_027372127.1	117	F: AGGGACTTCCACCAGATG R: CAACAACTCCCCTTCGGTA
Caspase 3	*casp-3*	93.8	60	KC660103.1	182	F: ACATTTCTGGGCGGAACACC R:GTGACACCCGTGCTTGTACA

3.2.7 Data analysis

Relative growth rate (RGR, % weight day^{-1}) was calculated as: $RGR = (e^{g} – 1) \times 100$, where $g = (\ln Wf – \ln Wi) \times t^{-1}$. Wf and Wi correspond to the final and initial weights, respectively. Feed conversion ratio (FCR) was calculated as: FCR = (Fi / Wg), where Fi corresponds to feed given (g) and Wg to the mean weight gain (g). Survival was expressed as percentage and calculated as: S = (Lf / Li) x 100, where Li and Lf correspond to the initial and final number of PL in the tanks, respectively. Differences in growth performance, FCR, survival, oxidative stress biomarkers, energy reserves, immune parameters and gene expression between dietary treatments were evaluated using One-way ANOVA's, followed by Tukey multiple comparison tests. Kruskal-Wallis one way analysis of variance tests, followed by Wilcoxon pairwise comparison tests were used when data did not comply with the One-way ANOVA's assumptions. Multivariate linear discriminant analyses (LDA) were performed for gene expression data to evaluate how it contributed to the dissociation of the diets in the discriminant functions generated. A MANOVA was performed to assess discriminatory significance using Wilk's λ test, after checking data compliance to the statistics assumptions. The distance between group centroids was measured by Mahalanobis distance and its significance inferred by One-way ANOVA's statistics. Results were expressed as means ± standard deviation (SD). In results expressed as percentage, an arcsine transformation was performed prior to any statistical test: T = ASIN (SQRT (value / 100)). The significance level considered was $p < 0.05$ for all tests performed.

3.3 Results

3.3.1 Shrimp whole body composition analysis

No significant differences between treatments were found in the PL whole body protein contents at the end of the trial (Table 3). The increased lipid levels in the FISHHF diet did not result in higher lipid and total fatty acid contents in the whole-body composition of the PL when compared with those fed the FISH diet. However, their fatty acid profile differed slightly, with shrimp fed the FISHHF diet showing significantly higher levels of 16:1 monounsaturated fatty acids; 20:5n-3, 21:5n-3 and total n-3 polyunsaturated fatty acids (PUFA); and significantly lower levels of 18:2n-6 and total n-6 PUFA than shrimp fed FISH (Table 4). CTRL

Table 3 Whole body (WBC) protein content of whiteleg shrimp (*P. vannamei*) PL fed the experimental diets at the end of the experimental period.

Diet	WBC Protein (% DW)
CTRL	63.4 ± 0.6
FISH	64.6 ± 1.2
SQUID	64.8 ± 0.2
KRILL	64.2 ± 0.6
FISHHF	64.5 ± 1.7

Results expressed as mean ± standard deviation (n = 3 experimental units). No significant differences between treatments were observed in a One-Way ANOVA (p = 0.070).

Table 4 Lipid contents and fatty acid composition of whiteleg shrimp (*P. vannamei*) PL fed the FISH and FISHHF diets at the end of the experimental period.

Shrimp post-larvae	Diet		*p*-value
Whole body composition	FISH	FISHHF	
Lipids (% DW)	12.83 ± 0.39	12.75 ± 1.24	----
Fatty acids (mg/g DW)			----
14:0	3.49 ± 0.43	3.26 ± 0.36	----
15:0	1.55 ± 0.20	1.74 ± 0.15	----
16:0	114.77 ± 1.37	112.75 ± 1.62	----
17:0	3.73 ± 0.13	3.67 ± 0.19	----
18:0	51.68 ± 1.25	50.75 ± 1.22	----
20:0	1.81 ± 0.11	1.98 ± 0.17	----
22:0	1.65 ± 0.12	1.58 ± 0.09	----
24:0	1.98 ± 0.37	2.01 ± 0.10	----
Total saturated	180.67 ± 2.05	177.74 ± 2.67	----
16:1	10.79 ± 0.60 [a]	11.82 ± 0.75 [b]	0.042
18:1n-9	56.83 ± 4.59	56.79 ± 6.10	----
18:1n-7	32.33 ± 3.92	35.32 ± 1.52	----
20:1	9.58 ± 1.65	8.98 ± 0.87	----
24:1	0.65 ± 0.15	0.64 ± 0.07	----
Total monounsaturated	110.17 ± 6.20	113.55 ± 8.38	----
18:2n-6	89.72 ± 5.28 [b]	79.30 ± 7.22 [a]	0.029
18:3n-6	1.22 ± 0.21	1.32 ± 0.41	----
20:4n-6	11.02 ± 0.39	11.77 ± 1.38	----
Total n-6 PUFA	101.97 ± 5.53 [b]	92.39 ± 5.57 [a]	0.028
18:3n-3	4.10 ± 0.58	3.48 ± 0.33	----
20:4n-3	0.84 ± 0.19	0.93 ± 0.05	----
20:5n-3	63.30 ± 3.65 [a]	68.90 ± 3.27 [b]	0.039
21:5n-3	0.87 ± 0.17 [a]	1.14 ± 0.17 [b]	0.038
22:5n-3	4.22 ± 1.01	5.18 ± 0.61	----
22:6n-3	74.29 ± 6.61	77.41 ± 0.61	----
Total n-3 PUFA	147.61 ± 4.53 [a]	157.04 ± 2.29 [b]	0.005
Total PUFA	249.58 ± 3.91	249.43 ± 3.67	----
Total FA	555.75 ± 8.55	556.82 ± 9.44	----

Results expressed as mean ± standard deviation (n = 3 experimental units). For each parameter, the p-values of a One-Way ANOVA are presented when significant. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

3.3.2 Growth performance and survival

At the end of the experiment, no significant differences between treatments were observed in the whiteleg shrimp PL mean wet weight and RGR (255 ± 26 mg and 15.1 ± 0.5 % day^{-1} treatments average, respectively). The FCR values were similar in all dietary treatments, excluding FISHHF, which had significantly higher values than KRILL. As for survival, the CTRL and KRILL diets resulted in significantly higher values than FISHHF, while no significant differences between the remaining dietary treatments were observed (Table 5).

3.3.3 Oxidative stress related biomarkers and cellular energy allocation

Regarding the oxidative stress parameters measured: CAT activity levels were significantly higher in the CTRL and FISHHF diets than in KRILL (Figure 1); GST activity levels were significantly higher in the FISHHF diet than in KRILL, with no significant differences between the remaining treatments (Figure 2); LPO levels were significantly lower in the CTRL diet than in KRILL, with no significant differences between the remaining treatments (Figure 3). No significant differences between treatments were found in the activity levels of tGSH (15.9 ± 1.1 nmol/min/mg protein treatments average). As for the energy reserves parameters, no significant differences between treatments were found for lipid (511.4 ± 162.5 mJ/mg tissue treatments average), sugar (85.7 ± 29.1 mJ/mg tissue treatments average) and protein (173.8 ± 71.2 mJ/mg tissue treatments average) contents and for EA 770.8 ± 201.8 mJ/mg tissue treatments average), EC (41.3 ± 13.7 mJ/mg tissue treatments average) and CEA (20.7 ± 7.6 mJ/mg tissue treatments average).

3.3.4 Immune parameters

As for the immune parameters evaluated, lysozyme activity levels were similar in all treatments (1.27 ± 0.71 µg/mg tissue treatments average) and pro-phenoloxidase levels were significantly higher in FISHHF than in CTRL, SQUID and KRILL (Figure 4).

3.3.5 Gene expression analysis

The normalized relative expression of the *trd* gene was significantly higher in CTRL than in the remaining treatments, excluding KRILL. As for the normalized relative expression of the remaining genes, no significant differences between treatments were observed. The ANOVA analysis showed marginal significant differences (p = 0.043) between treatments in the normalized relative expression of *casp* gene. However, no differences between treatments were found in the post-hoc Tukey multiple comparison test (Table 6).

Table 5 Initial and final weight, relative growth rate (RGR), feed conversation ratio (FCR) and survival of whiteleg shrimp (*P. vannamei*) PL during the experimental period.

Diet	CTRL	FISH	SQUID	KRILL	FISHHF	*p*-value
Initial weight (mg)			13.0 ± 2.0			----
Final weight (mg)	225.9 ± 58.8	237.5 ± 48.8	265.7 ± 55.0	281.0 ± 14.0	265.0 ± 57.3	0.657
RGR (% day^{-1})	14.4 ± 1.4	14.8 ± 1.1	15.4 ± 1.1	15.8 ± 0.3	15.3 ± 1.3	0.628
FCR	0.7 ± 0.1 ^{b}	1.0 ± 0.1 ^{ab}	0.8 ± 0.1 ^{ab}	0.6 ± 0.0 ^{b}	1.2 ± 0.4 ^{a}	0.034
Survival (%)	76.5 ± 9.4 ^{b}	45.1 ± 11.7 ^{ab}	61.6 ± 14.6 ^{ab}	78.0 ± 4.6 ^{b}	41.5 ± 15.0 ^{a}	0.011

Results expressed as mean ± standard deviation. For initial weight n = 120 observational units; for final weight, FCR, RGR and survival n = 3 experimental units. Represented are also the p-values for a One-Way ANOVA for each parameter. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

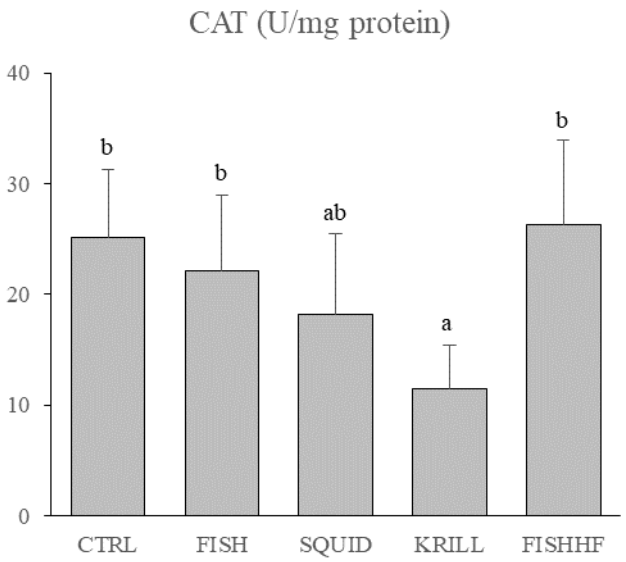

Figure 1 Catalase (CAT) levels (U/mg protein) of whiteleg shrimp post-larvae fed the different experimental diets for 21 days. Results expressed as mean ± standard deviation (n = 3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments.

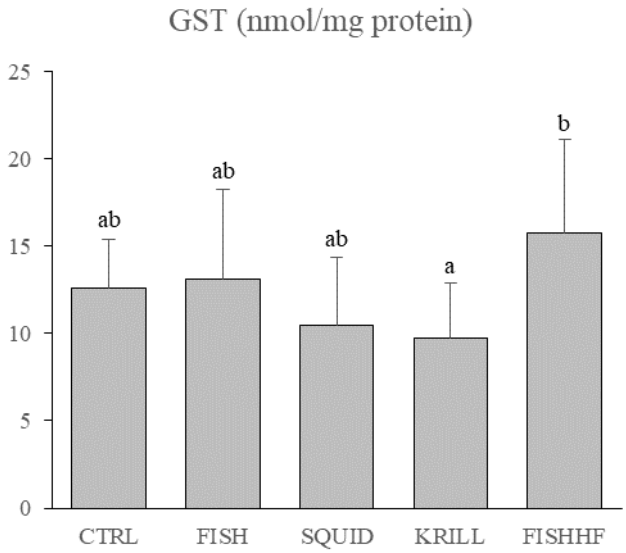

Figure 2 Glutathione S-transferase (GST) levels (nmol/mg protein) of whiteleg shrimp post-larvae fed the different experimental diets for 21 days. Results expressed as mean ± standard deviation (n = 3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments.

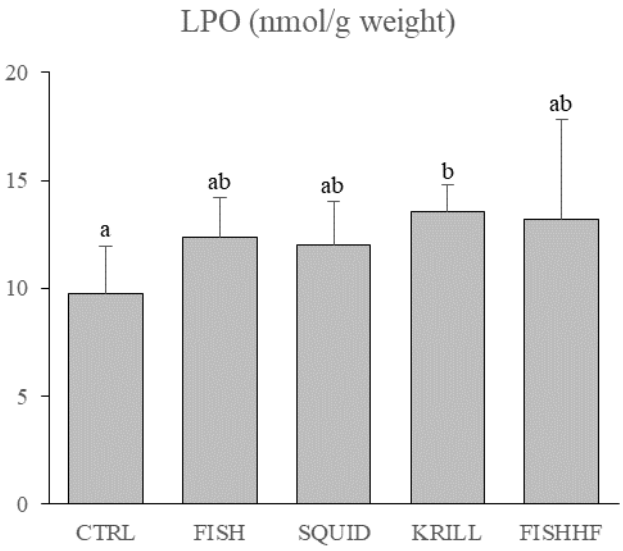

Figure 3 Lipid peroxidation (LPO) levels (nmol/g weight) of whiteleg shrimp post-larvae fed the different experimental diets for 21 days. Results expressed as mean ± standard deviation (n = 3 experimental units). Different superscript letters indicate statistical differences (p < 0.05) between treatments.

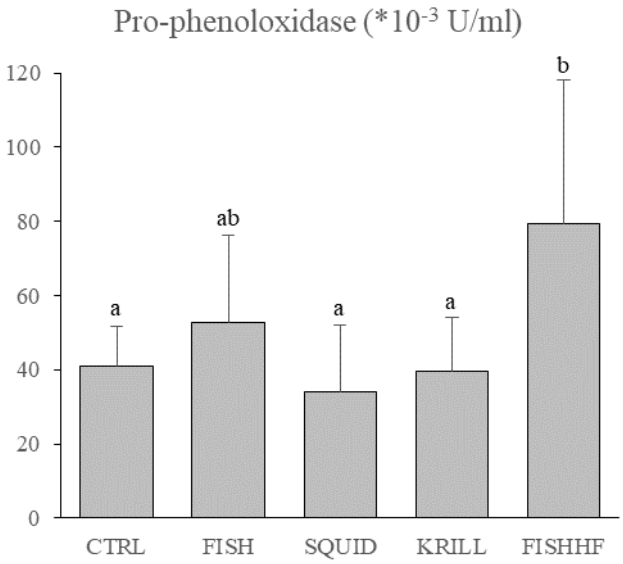

Figure 4 Pro-phenoloxidase levels (*10^{-3} U/ml) of whiteleg shrimp post-larvae fed the different experimental diets for 21 days. Results expressed as mean ± standard deviation (n = 3 experimental units). Different superscript letters indicate statistical differences (p < 0.05) between treatments.

Table 6 Relative expression to housekeeping (*gapdh*) of target immune and antioxidant related genes of whiteleg shrimp (*P. vannamei*) PL fed the experimental diets for 21 days.

Gene	Acronym	Relative Expression					*p*-value
		CTRL	FISH	SQUID	KRILL	FISHHF	
Penaeidin-3a	*pen-3*	0.78 ± 0.43	0.70 ± 0.62	0.56 ± 0.48	1.03 ± 0.75	1.26 ± 1.33	0.354
PvHm117 crustin P	*crus*	1.12 ± 0.93	0.34 ± 0.21	0.58 ± 0.52	0.72 ± 0.49	1.02 ± 1.08	0.129
Lysozyme C-like	*lys*	1.00 ± 0.97	0.80 ± 0.54	0.60 ± 0.52	0.88 ± 0.68	0.60 ± 0.35	0.655
Thioredoxin 1	*trd*	1.03 ± 0.44 ^{b}	0.41 ± 0.19 ^{a}	0.45 ± 0.14 ^{a}	0.68 ± 0.24 ^{ab}	0.64 ± 0.48 ^{a}	<0.001
Glutathione transferase	*gst*	1.09 ± 1.00	0.43 ± 0.27	0.56 ± 0.33	0.61 ± 0.54	0.46 ± 0.33	0.224
Glutathione peroxidase	*gpx*	0.71 ± 0.37	0.58 ± 0.31	0.67 ± 0.53	0.54 ± 0.29	0.56 ± 0.52	0.685
Caspase 3	*casp-3*	0.66 ± 0.49	0.71 ± 0.81	0.77 ± 0.56	0.67 ± 0.38	1.55 ± 1.06	0.043

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a One-Way ANOVA for each gene. Different superscript letters indicate statistical differences ($P < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

The LDA of mRNA transcripts of genes associated with the antioxidant defense and immune condition analyzed (*pen-3, crus, lys, trd*, *gst*, *gpx* and *casp-3*) resulted in four discriminant functions, with the first two accounting for 96.24 % of the data variability (Wilks $\lambda = 0.425$, $p < 0.001$). In the first discriminant function (LDA1, score of 66.25 %) (Figure 5) the discriminant analysis revealed a significant group separation between CTRL and KRILL and FISH and FISHHF, and between CTRL and SQUID. Group discrimination was mainly positively loaded by *trd* and *crus* and negatively by *casp-3*. In the second discriminant function (LDA2, score of 29.99 %) a significant separation of diet FISHHF from diets FISH and SQUID is also shown (Figure 5). This discrimination was mainly negatively loaded by *casp-3*.

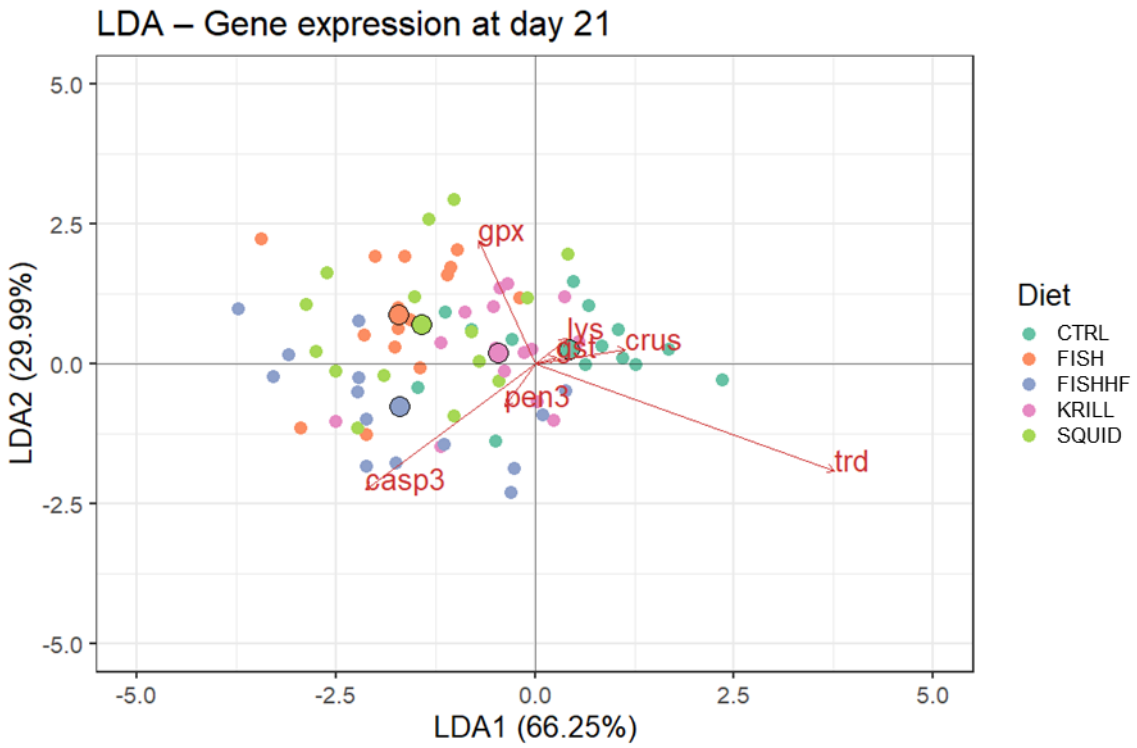

Figure 5 Linear discriminant analysis (LDA) of the relative expression of genes associated with the antioxidant defence and immune condition (*pen-3, crus, lys, trd, gst, gpx* and *casp-3*) of whiteleg shrimp post-larvae fed the different experimental diets for 21 days. Percentages indicate the main functions (LDA1 and LDA2) discriminant score. Small circles represent diet data distribution while big circles represent diet centroids. Variable loads for both discriminant functions are represented by red arrows. Wilk's lambda test was significant ($p < 0.001$). In LDA1, CTRL and KRILL were significantly discriminated from FISH and FISHHF, and diet CTRL from SQUID, mainly due to lower mRNA transcripts of *casp-3* and higher transcripts of *trd*. In LDA2, FISHHF was discriminated from FISH and SQUID mainly due to higher mRNA transcripts of the *casp-3* gene.

3.4 Discussion

The RGR values (15.1 ± 0.5 % day^{-1}; treatments average) achieved in this trial are in line with those previously described in Chapter 2 (15.0 ± 0.3 % day^{-1}, diet P54 results). However, the shrimp PL mean initial weight was slightly higher in this study, which help explain the higher final weight in the present study. Slightly superior FCR values were also obtained in the current trial (0.9 ± 0.1, treatments average) than in the previously mentioned study (1.3 ± 0.1, diet P54 results). As for survival, the CTRL and KRILL treatments obtained values close to 80 %, which are only slightly lower than those previously obtained. Even so, survival values of FISHHF (41.5 ± 15.0 %), FISH (45.1 ± 11.7 %, marginally with no statistically significant differences from CTRL and KRILL (p = 0.077 and p = 0.063, respectively)), and even those of SQUID (61.6 ± 14.6 %) were considerably low, suggesting that these diets may not be as adequate as CTRL and KRILL for whiteleg shrimp PL in this stage of development and/or for the clear water RAS husbandry conditions maintained in this study. Additionally, a higher initial shrimp PL density per tank was implemented in this trial, which may have contributed to these results. In fact, the stocking density used (5000 PL m^{-3}) is on the upper end of what is usually employed in the nursery phase of whiteleg shrimp PL (Nunes et al., 2021). Still, the survival results obtained were superior to those reported by Wang et al. (2017a, 2017b) when using graded levels of *Schizochytrium* meal and as a replacement of fish oil in practical diets for whitleg shrimp PL (40.3 – 44.5 % and 42.7 – 45.6 %, respectively). Velasco et al. (2000) reported two experiments testing different inclusion levels of protein and lipids in diets for whiteleg shrimp PL in clear water RAS where survival results were close to 80% in experiment 1 and close to 97% in experiment 2 but weight gain (around 70 and 90 mg in experiments 1 and 2, respectively) and FCR (2.2 and 1.4 in experiments 1 and 2, respectively) were considerably worse to those obtained in the current study. Similar final weight (242 mg) and survival (83 %) of whiteleg shrimp PL (2.1 mg initial weight) fed a diet contain 51 % protein over a 25-day period were described by Xie et al. (2020). Hence, the whiteleg shrimp PL growth performance and survival results obtained in this trial using diets CTRL and KRILL can be considered very satisfactory and comparable or superior to those found in similar studies using individuals of the same size and maintained in similar rearing conditions, demonstrating the adequacy of these experimental diets and of the zootechnical conditions implemented.

Actually, the inclusion of a blend of fish and squid meals has been previously recommended over using either one solely in diets for juvenile whiteleg shrimp reared in clear water RAS (Forster et al., 2010). The same authors reported that krill meal supplementation did not enhance growth and was not included in diets used in a posterior study also described. Accordingly, the combination of fish and squid meals has been designated as the most cost-effective combination of ingredients to be used in feeds for

whiteleg shrimp (Sánchez et al., 2012). However, recent studies have shown that krill meal inclusion in diets does not negatively affect growth and immunity of juvenile whiteleg shrimp (Wei et al., 2022) and even can improve survival, growth performances and/or increase feed palatability and intake (Ambasankar et al., 2022; Derby et al., 2016; Nunes et al., 2014; Soares et al., 2021). Despite the similar growth performances of all dietary treatments in the current trial, the results obtained corroborate the importance of using either a mixture of fish and squid meals or krill meal as main ingredients in diets for whiteleg shrimp PL reared in clear water RAS to ensure high survival values.

In the present study, the multivariate analysis performed on the relative expression of genes associated with the antioxidant defense and immune condition suggests a deterioration in the health status of shrimp PL fed the FISH, SQUID and FISHHF diets when compared to CTRL and KRILL (excluding KRILL for SQUID), mainly by lower mRNA transcripts of *trd* and *crus,* and higher of *casp-3*. The *trd* gene is associated with the redox protein thioredoxin 1, that catalyzes the reduction of disulfide bonds in proteins from different biological systems (Aispuro-Hernandez et al., 2008; Campos-Acevedo & Rudiño-Piñera, 2014); *crus* encodes PvHm117 crustin P, an antimicrobial peptide involved in innate immunity (Vargas-Albores et al., 2004; Li et al., 2019); and *casp-3* gene is related to caspase 3, a protease responsible for programmed cell death and apoptosis in response to over production of ROS and oxidative stress (González-Ruiz et al., 2021). Still, PL fed the KRILL diet showed lower activity levels of the antioxidant enzyme CAT. These results may explain the higher LPO levels when comparing with CTRL, despite the similar levels of tGSH (free radicals scavenger) and GST (multifunctional enzyme involved in the detoxification of xenobiotics, oxidative defense and intracellular transport (Zhou et al., 2009)), as well as the relative expression of genes associated with the antioxidant defense that were evaluated. The lower activity levels of CAT may result in a lower protective capacity against oxidative damage that may occur during these accelerated stages of development where excessive ROS are likely being produced. Nevertheless, this was not reflected in the survival values, similar in both treatments at the end of the trial.

On the other hand, CAT, GST and pro-phenoloxidase activity levels were significantly higher in the FISHHF dietary treatment than in KRILL. The apparent activation of the antioxidant defenses and immune mechanisms may signal a more stressful condition of PL fed FISHHF, what seems corroborated by the lower survival values observed for this treatment. Additionally, the multivariate analysis indicated a significant separation of diet FISHHF from diets FISH and SQUID. Shrimp PL fed FISHHF showed higher mRNA transcripts of *casp-3*, suggesting that the upregulation of this gene, as well as the higher activity levels of the antioxidant enzymes measured in this study, may have been caused by an overproduction of ROS that can occur during immune activity (Aguilera-Rivera et al.,

2018). High fish meal diets have been shown to upregulate immunity related genes, including the pro-phenoloxidase and pro-phenoloxidase activating enzyme genes, in whiteleg shrimp when compared to diets containing krill meal as replacement (Ambasankar et al., 2022). Still, in the current study this was verified only for shrimp PL fed the FISHHF diet but not FISH, suggesting that it may have been caused by the increasing levels of dietary lipids or PUFAs. However, research done on whiteleg shrimp PL dietary lipid requirements is limited. Xie et al. (2019) evaluated the effects of graded dietary lipid levels (9.7 – 15.5%) in the growth performance, stress tolerance and immune response of whiteleg shrimp PL and found that the highest lipid levels tested produced the most shrimp weight gain but also the lowest survival. Levels around 13% upregulated the expression of genes associated with health condition and increased survival of shrimp PL after an hypoxia challenge. Based on these results, the authors indicate 12 % to be the optimal dietary lipid level. In the current study, the expression of immune related genes (*pen-3*, *crus* and *casp-3*), although not statiscally significant, tended to be higher when increasing lipid levels from 14 to 16 % in a fish meal-based diet. Moreover, growth performances were not enhanced and FCR values were higher in shrimp fed FISHHF than those fed diet KRILL. Hence, the results obtained suggest that a lipid level of around 14% seems to be preferable when tailoring diets for whiteleg shrimp PL reared in clear water RAS and at this stage of development.

As for the energy reserves parameters assessed, no significant differences were found between treatments in the PL protein, lipid and sugar content, which reflects the similarities in the proximate composition of all diets and in the whole-body composition of the shrimp PL from all treatments. No significant differences between treatments were found in the PL whole body protein and lipid contents at the end of the trial, despite the increased lipid levels in FISHHF. Total fatty acid contents were similar in both treatments, yet the fatty acid profile of shrimp fed FISH and FISHHF dietary treatments differed to some degree. Higher n-3 PUFA and lower n-6 PUFA levels were measured in the whole-body composition of shrimp PL fed FISHHF. These results can probably be explained by the fact that fish oil is rich in n-3 PUFA (Nunes et al., 2022), and the FISHHF diet was higher in fish oil than FISH.

3.5 Conclusions

In conclusion, improved shrimp PL survival and health status were achieved using diets with a blend of fish and squid meals or krill meal as the main ingredients. Increasing lipid levels from 14 to 16 % in a fish meal-based diet produced similar growth performances but increased FCR and reduced survival. Therefore, a lipid level of around 14% seems to

be preferable when tailoring diets for whiteleg shrimp PL reared in clear water RAS and at this stage of development.

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Declarations

All activities were undertaken within the clear boundaries of national and EU legal frameworks directed by qualified scientists/technicians and conducted according to the European guidelines on the protection of animals used for scientific purposes (Directive 2010/63/UE of the European Parliament and on the European Union Council) and under strict monitoring and control of DGAV—(Direção Geral de Alimentação e Veterinária), Animal Welfare Division, which is the competent authority responsible for implementing the legislation on the “protection of animals used for scientific purposes”.

WP and LC worked for Sparos Lda during this study. The remaining authors declare that they have no conflicts of interest. The views expressed in this work are the sole responsibility of the authors and do not necessarily reflect the views of any third party.

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Chapter 4

Soy protein concentrate and krill meal in microfeeds bring health benefits for whiteleg shrimp (*Penaeus vannamei*) post-larvae

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Soy protein concentrate and krill meal in microfeeds bring health benefits for whiteleg shrimp (*Penaeus vannamei*) post-larvae

Abstract

This study aimed to further analyze the potential of krill meal to be included in microdiets for whiteleg shrimp PL. The inclusion of soy protein concentrate (SPC) to reduce diets cost was also evaluated. Also, the experimental diets were compared to two commercial microdiets for whiteleg shrimp PL available in the European market. Four experimental and two commercial microdiets were evaluated: BASE, as control, formulated with marine (fish meal as first ingredient) and plant proteins (including SPC); KRILL, using krill meal as main ingredient; NKRILL and NSOY, like BASE but formulated to exclude krill meal or SPC in their composition, respectively; ComA and ComB, two available commercial diets for shrimp PL. After 21 days of feeding, growth and survival results indicate that a further optimization of the experimental diets was done properly as most of them outperformed either one or both commercial diets. Results corroborate that SPC can be used to replace marine ingredients with no adverse effects on growth performances or survival. The complete removal of krill meal from the diets did not produce any change in these parameters, yet it seemingly hindered the PL health status, suggesting that it may not be as adequate as fish meal to be used as first ingredient but should be used as a secondary ingredient. At the end of the feeding period, PL were subject to acute ammonia stress. Although no differences in survival were observed, PL fed NKRILL and both commercial diets showed higher activity levels of antioxidant molecules, indicating a higher dependency on these to deal with the stressful stimulus. Parallelly, PL were subjected to a *Vibrio harveyi* challenge. After 24 hours, BASE had significantly higher survival values than the NKRILL, NSOY and ComB dietary treatments. These results agree with those obtained in the growth trial that suggested that the exclusion of krill meal from NKRILL impaired the whiteleg shrimp PL health status. The exclusion of SPC also seems to have been detrimental to the capacity of the PL to resist infection. The gene expression showed an apparent compromised health status of PL fed this diet when compared to those fed the remaining dietary treatments, which was confirmed by the lower survival values when compared to BASE.

Keywords: *Penaeus vannamei*, whiteleg shrimp, early nutrition, disease resistance, immune response, stress response

4.1 Introduction

The aquafeeds market currently has a global value of more than $60 billion USD (Tibbetts et al., 2015) as more than 70% of the aquaculture industry depends on formulated feeds (FAO, 2022). The preponderance of fed aquaculture species in the sector's output has largely surpassed that of non-fed production and is expected to continue to rise, which makes feeds essential for the success of the industry. There has been a growing interest in novel ingredients that can reduce diet formulation costs, as they usually represent more than 50 % of the overall inputs of fish and crustacean farming. To this adds the importance of increasing feeds sustainability since traditionally used ingredients like fish meal and fish oil have been associated with negative environmental repercussions (FAO, 2022). This constitutes a challenge for feed manufacturers as emerging alternatives often do not fulfill the animal's nutritional requirements as well as the previously mentioned ingredients, due to inadequate essential amino acids and fatty acids profiles. Still, many vegetal, animal, insect and microbial-based ingredients have been developed and tested with relative success in several relevant species for the aquaculture industry, including the whiteleg shrimp, as discussed in Chapter 3.

In the previous trial performed under the scope of this thesis, diets with a blend of fish and squid meals or krill meal as the main ingredients resulted in improved whiteleg shrimp PL survival and health status when compared to the other diets evaluated. However, when considering the previously mentioned problems associated with the cost and sustainability of feeds, the use of these ingredients as the main constituents of diets may not be ideal. To tackle these issues, the authors decided to evaluate the potential of using soy protein concentrate (SPC) to reduce the preponderance of marine ingredients in the experimental diets' formulations. Partial replacements of fish meal by SPC in diets for whiteleg shrimp juveniles have been performed successfully in several studies (Chen et al., 2017; Guo et al., 2020; Ray et al., 2020; Xie et al., 2016; Zhu et al., 2021). Moreover, it has been shown by other authors that a complete replacement of fish meal by SPC can be achieved with no negative effects on whiteleg shrimp growth reared in clear water when adjusting dietary fat by using fish oil as a lipid source, ensuring an adequate dietary amino acid and fatty acid profile (Sá et al., 2013) or when coupled with microbial floc meal (Bauer et al., 2012). Accordingly, Sabry-Neto et al. (2017) reported that whiteleg shrimp juveniles fed diets containing SPC and soybean meal as main ingredients and with no fish meal or other marine protein sources achieved similar growth performances and survival to those fed a control diet containing a salmon by-product meal as one of the main protein sources. Even so, knowledge of the potential of using SPC in diets for whiteleg shrimp PL is limited.

Furthermore, although krill meal is a costly ingredient and its production requires harvesting wild stocks (FAO, 2022), its potential to be included in aquafeeds formulations

is high and it has not been extensively tested in diets for whiteleg shrimp PL. Hence, this study aimed to further analyze the potential of krill meal to be included in microdiets for whiteleg shrimp PL produced in clear water RAS. The inclusion of SPC in the diets to reduce their costs was also evaluated. Additionally, the experimental diets adequacy was assessed by comparing them to two commercial microdiets for whiteleg shrimp PL available in the European market.

4.2 Materials and methods

4.2.1 Dietary treatments

Four experimental and two commercial microdiets were evaluated in triplicates. BASE was used as control, formulated with a blend of marine (fish meal, as first ingredient, and krill meal) and plant-based proteins (including SPC). KRILL was formulated with a blend of marine and vegetal protein sources, using krill meal as main ingredient. NKRILL and NSOY were based on BASE but formulated to exclude krill meal or SPC in their composition, respectively. ComA and ComB were commercially available diets for whiteleg shrimp PL in the European market. ComA main ingredients were fish meal, pea meal, fish oil, soy lecithin and fish gelatin while ComB was formulated with fish meal, horsebean, wheat meal, soybean meal, wheat gluten, corn gluten, squid meal, krill meal, and fish oil. Proximate composition of the experimental diets (Table 1) was analyzed following the Association of Official Analytical Chemists procedures (AOAC, 2000). Briefly, dry matter was determined by drying samples at 105ºC until constant weight; crude protein (N x 6.25), by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit; crude fat, by petroleum ether extraction (Soxtec HT System); ashes, by incineration at 450ºC for 16 h in a muffle furnace; and gross energy, by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). With the exception of ComA and ComB, all diets were produced at Sparos Lda facilities (Olhão, Portugal) using extrusion at low temperature as the main production process, as follows: powder ingredient mixing according to target formulation using a double helix mixer; grinding in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany); addition of the oil fraction; humidification and agglomeration through low temperature extrusion (Dominioni Group, Italy); drying of resultant pellets in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 4 h at 60 ºC, crumbling (Neuero Farm, Germany) and sieving to desired size ranges.

Table 1 Proximate composition of the experimental diets used to culture whiteleg shrimp (*P. vannamei*) PL for 21 days.

% Wet matter	BASE	KRILL	NKRILL	NSOY	ComA	ComB
Crude protein	50.3 ± 0.0	49.6 ± 0.0	49.2 ± 0.0	49.8 ± 0.0	60.0 ± 0.0	52.0 ± 0.0
Crude fat	15.0 ± 0.0	14.6 ± 0.0	12.3 ± 0.0	13.7 ± 0.0	15.0 ± 0.0	10.0 ± 0.0
Ashes	13.9 ± 0.0	15.0 ± 0.0	12.3 ± 0.0	14.7 ± 0.0	14.5 ± 0.0	11.2 ± 0.0
Phosphorous	2.1 ± 0.0	2.2 ± 0.0	1.8 ± 0.0	2.0 ± 0.0	2.4 ± 0.0	1.6 ± 0.0
Energy (MJ/Kg)	20.2 ± 0.0	20.0 ± 0.0	19.9 ± 0.0	20.1 ± 0.0	23.5 ± 0.0	19.8 ± 0.0

Results expressed as mean ± standard deviation (*n* = 2 experimental units).

4.2.2 Shrimp rearing and sampling

Whiteleg shrimp PL (mean wet weight of 4.7 ± 0.9 mg) originated from Miami Aquaculture (Florida, USA), were reared for 21 days at Riasearch Lda facilities (Murtosa, Portugal). These were randomly distributed to 18 tanks with approximately 50 L that were part of a 3 m^3 clear water recirculating aquaculture system (RAS), at a larval density of 300 individuals per tank (6000 PL per m^3). These were kept under a 12 hours light:12 hours dark photoperiod and were fed close to *ad libitum* with automatic feeders that supplied eight meals a day. Feed was adjusted daily based on the observation of the tanks and the presence/absence of remnants from the previous day. Feed size was 400-600 µm for the first week and 600-800 µm for the remaining feeding period. Water temperature was maintained at 27.4 ± 0.8 ºC, dissolved oxygen concentration at 5.4 ± 0.3 mg L^{-1}, salinity at 21.6 ± 0.3, pH at 7.8 ± 0.1, NH_3 and NO_2 at 0.0 ± 0.0 mg L^{-1}.

At the start of the trial, a total of 120 PL from the initial stock were randomly selected and weighed in groups of 30 individuals for initial wet weight determination. At the end of the feeding period, all the remaining PL in each tank were counted and weighed in groups of 20 individuals to determine the tank final biomass and survival. Additionally, a total of 27 PL were randomly selected from each tank for oxidative stress, energy reserves, and immune parameters analysis, and 5 for gene expression analysis. These were stored at -80 ºC for subsequent analysis. Shrimp sampled for molecular biology analysis were kept in RNAlater (Sigma, St. Louis, MO, USA) at > 1:5 volume ratio, at 4 ºC for 24 hours prior to being stored at -20 ºC.

4.2.3 Ammonia challenge

At the end of the feeding period, 30 PL were randomly selected from each tank and transferred to a separate clear water RAS with the same configuration as the one used in the feeding trial, where they were subject to acute ammonia stress. An ammonium chloride (NH_4Cl) solution was used to increase the NH_3 values in the water to 1.6 ± 0.1 mg L^{-1} for 24 hours. After the period of exposure, survival was determined and 9 PL per tank were sampled for oxidative status and energy reserves assessment. These were stored until further analysis following the procedures previously described. NH_3 levels were then brought down to 0 mg L^{-1} and PL were kept in standard husbandry conditions for 1 week. At the end of the recovery period, survival was determined in each tank.

4.2.4 *Vibrio harveyi* challenge

Parallelly, at the end of the feeding period, 60 PL were randomly selected from each tank and transferred to CIIMAR facilities (Matosinhos, Portugal) where they were subjected to a *Vibrio harveyi* challenge. Exposure was done by immersion in 10 L tanks with standard

rearing water containing the pathogenic agent in a concentration of $1x10^8$ cells L^{-1}. After 2 hours of exposure, PL were transferred to a clear water RAS and kept in standard husbandry conditions. At 4- and 24-hours post-exposure, survival was determined and the hepatopancreas of 5 PL per tank were collected for gene expression analysis at both sampling points. These were stored until further analysis following the procedures previously described.

4.2.5 Oxidative stress and immunity-related biomarkers and cellular energy allocation

Sample preparation and analyzes of oxidative stress, immune and cellular energy allocation parameters were performed following the procedures described in Chapter 3. Briefly, whole whiteleg shrimp PL from each tank were homogenized in sets of 3 individuals. Catalase (CAT, expressed as U mg $protein^{-1}$), glutathione S-transferase (GST, expressed as nmol mg $protein^{-1}$), total glutathione (tGSH, expressed as nmol mg $protein^{-1}$), lipids peroxidation (LPO, expressed as nmol g $weight^{-1}$), lysozyme (expressed as µg mg $protein^{-1}$), pro-phenoloxidase (expressed as U mL^{-1}), sugars (mJ mg^{-1}), lipids (mJ mg^{-1}), proteins (mJ mg^{-1}), energy available (EA, assessed as the sum of sugars, lipids and proteins and expressed as mJ mg^{-1}), energy consumption (EC, as electron transport system (ETS) activity and expressed as mJ hour mg^{-1}) and cellular energy allocation (CEA, calculated as EA/EC) levels were then determined in the homogenized samples.

4.2.6 Gene expression

Whole whiteleg shrimp PL or their hepatopancreas (samples collected in the *Vibrio harveyi* challenge) were homogenized in NZYol (Nzytech, w/v proportion according to the manufacturer's instructions) using a Precellys 24 tissue homogenizer (Bertin instruments).

RNA extraction, analyses of its concentration and purity, reverse transcription and RT-PCR were performed for each sample as described in Chapter 2. Genes examined were chosen due to their association with antioxidant or immunity mechanisms (Table 2). Pfaffl method (Pfaffl, 2001) was used to perform gene expression analyzes and target genes were normalized using *gapdh* and *act* as housekeeping genes (Alvarez-Lee et al., 2020).

Table 2 Selected genes and specific primers used to evaluate the health status of whiteleg shrimp (*P. vannamei*) PL.

Gene	Acronym	Efficiency (%)	Annealing temperature (ºC)	Accession nº	Amplicon length (bp)	Primer Sequence (5´- 3´)
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	104.5	60	MG787341.1	166	F: AAAGGTAGGAATTGCCCCCG R: AGGGATGAGACTAGCACGACT
Actin	*act*	89.5	60	XM_027364954.1	401	F: CCTCGCTGGAGAAGTCCTAC R: TGGTCCAGACTCGTCGTACTC
PvHm117 crustin P	*crus*	101.4	60	AY488497.1	109	F: GAAACCACCACCAACACCTACTCC R: TCTGTGCGGCCTCTTTACGG
Penaeidin-3a	*pen-3*	86.1	60	Y14926.1	137	F: ATACCCAGGCCACCACCCTT R: TGACAGCAACGCCCTAACC
Lysozyme C-like	*lys*	73.4	60	XM_027352857	82	F: CGGGAAAGGCTATTCTGCCT R: CCAGCACTCTGCCATGTACT
Thioredoxin 1	*trd*	85,3	60	EU499301.1	116	F: TTAACGAGGCTGGAAACA R: AACGACATCGCTCATAGA
Glutathione transferase	*gst*	99.3	60	AY573381	146	F: AAGATAACGCAGAGCAAGG R: TCGTAGGTGACGGTAAAGA
Glutathione peroxidase	*gpx*	86.6	60	XM_027372127.1	117	F: AGGGACTTCCACCAGATG R: CAACAACTCCCCTTCGGTA
Hemocyanin	*hmc*	92.9	60	KY695246.1	124	F: GTCTTAGTGGTTCTTGGGCTTGTC R: GGTCTCCGTCCTGAATGTCTCC
C-type lectin 2-like	*lect*	83.8	60	DQ858899.2	138	F: GCTTCTGTTGGTGCTGTTGGC R: GTTCCCTTCCCGTATGTGGC

4.2.7 Data analysis

Relative growth rate (RGR, % weight day^{-1}) was calculated as: RGR = (e^g – 1) x 100, where e = exponential and g = (lnWf – lnWi) x t^{-1}. Wf and Wi correspond to the final and initial weights, respectively. Feed conversion ratio (FCR) was calculated as: FCR = (Fi / Wg), where Fi corresponds to the feed given (g) and Wg to the mean weight gain (g). Survival was expressed as a percentage and calculated as: S = (Lf / Li) x 100, where Li and Lf correspond to the initial and final number of PL in the tanks, respectively. Differences in growth performance, survival, oxidative stress biomarkers, energy reserves, immune parameters, and gene expression between dietary treatments were evaluated using one-way ANOVA's, followed by Tukey HSD multiple comparison tests. Kruskal-Wallis one-way analysis of variance tests, followed by Wilcoxon pairwise comparison tests were used when data did not comply with the one-way ANOVA's assumptions. Multivariate linear discriminant analyses (LDA) were performed for gene expression data to evaluate how it contributed to the dissociation of the diets in the discriminant functions generated. A MANOVA was performed to assess discriminatory significance using Wilk's λ test, after checking data compliance to the assumptions of the statistics. The distance between group centroids was measured by Mahalanobis distance and its significance inferred by one-way ANOVA's statistics. Results were expressed as means ± standard deviation (SD). In results expressed as a percentage, an arcsine transformation was performed prior to any statistical test: T = ASIN (SQRT (value / 100)). The significance level considered was $p < 0.05$ for all tests performed.

4.3 Results

4.3.1 Feeding period

4.3.1.1 Growth performance and survival

After 21 days, PL fed the NSOY diet showed a significantly higher final body weight and RGR than those fed KRILL and both the commercial diets ComA and ComB (Figure 1 (a) and (b)). Both BASE and NKRILL had significantly higher RGR values than the ComB (Figure 1 (b)). The FCR values of PL fed all experimental diets were significantly lower than those fed the commercial diets except for KRILL (Figure 1 (c)). No significant differences in survival (84.1 ± 6.0 % treatments average; $p = 0.128$) between dietary treatments were observed.

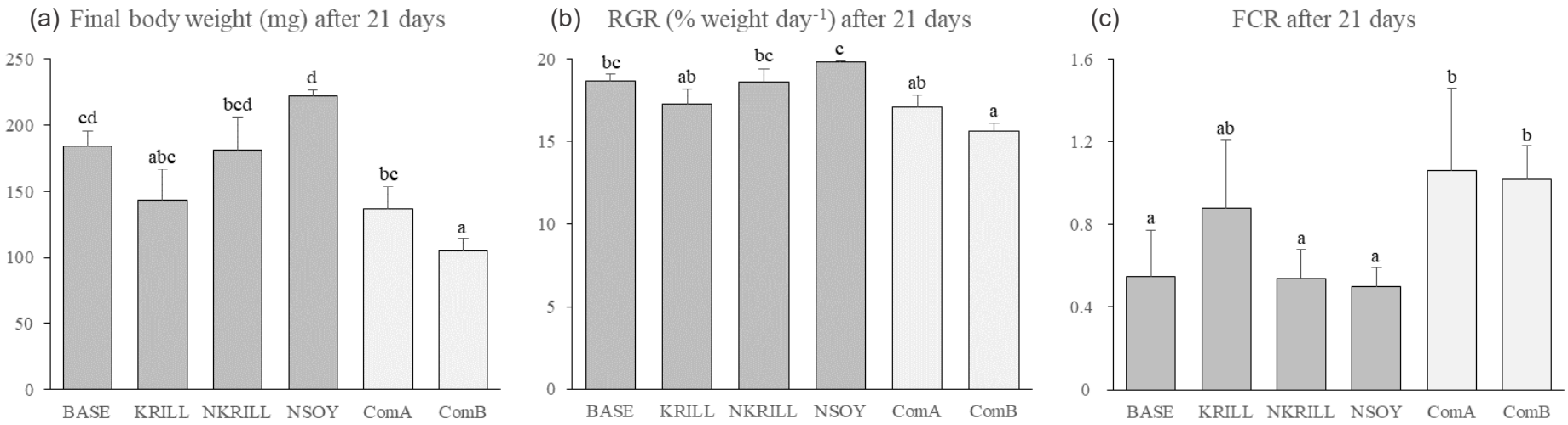

Figure 1 Mean wet final body weight (a), relative growth rate (RGR) (b) and feed conversion ratio (FCR) (c) of whiteleg shrimp post-larvae fed different experimental and commercial microdiets at the end of the experimental period. Results expressed as mean ± SD (n=3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a One-Way ANOVA, followed by a post-hoc Tukey multiple comparison test.

4.3.1.2 Oxidative stress and immunity-related biomarkers and cellular energy allocation

Regarding the oxidative stress parameters measured after 21 days of feeding: CAT activity levels were significantly higher in KRILL than in ComA; GST and tGSH levels were similar between treatments; and LPO levels were significantly higher in the ComA dietary treatment than in all the remaining treatments (Figure 2). As for the immune parameters evaluated, activity levels were marginally similar in all treatments for lysozyme (2.5 ± 2.1 µg mg^{-1} treatments average; p = 0.051) and pro-phenoloxidase (48.3 ± 13.2 $*10^{-3}$ U ml^{-1} treatments average; p = 0.056) (Figure 2). Concerning the energy reserves; sugar levels were significantly higher in ComB than in all the remaining treatments, while lipids and proteins were similar; EA was significantly higher in BASE and ComB than in NKRILL; no significant differences between treatments were found in EC; and CEA was significantly higher in BASE and KRILL than in NKRILL (Figure 3).

4.3.1.3 Gene expression

At the end of the feeding trial, the normalized relative expression of the *crus* gene decreased significantly in NKRILL compared to BASE and NSOY; the *lys* gene was significantly lower in most experimental treatments than in BASE, excluding NSOY; the *hmc* gene was significantly lower in NKRILL than in all the experimental diets, being similar to the commercial diets; the *lect* gene was significantly lower in NKRILL and ComA than in BASE; the *gst* gene decreased significantly in most experimental treatments compaed to BASE and NSOY, excluding KRILL; and the *gpx* gene was significantly lower in NKRILL and both commercial diets than in BASE (Table 3).

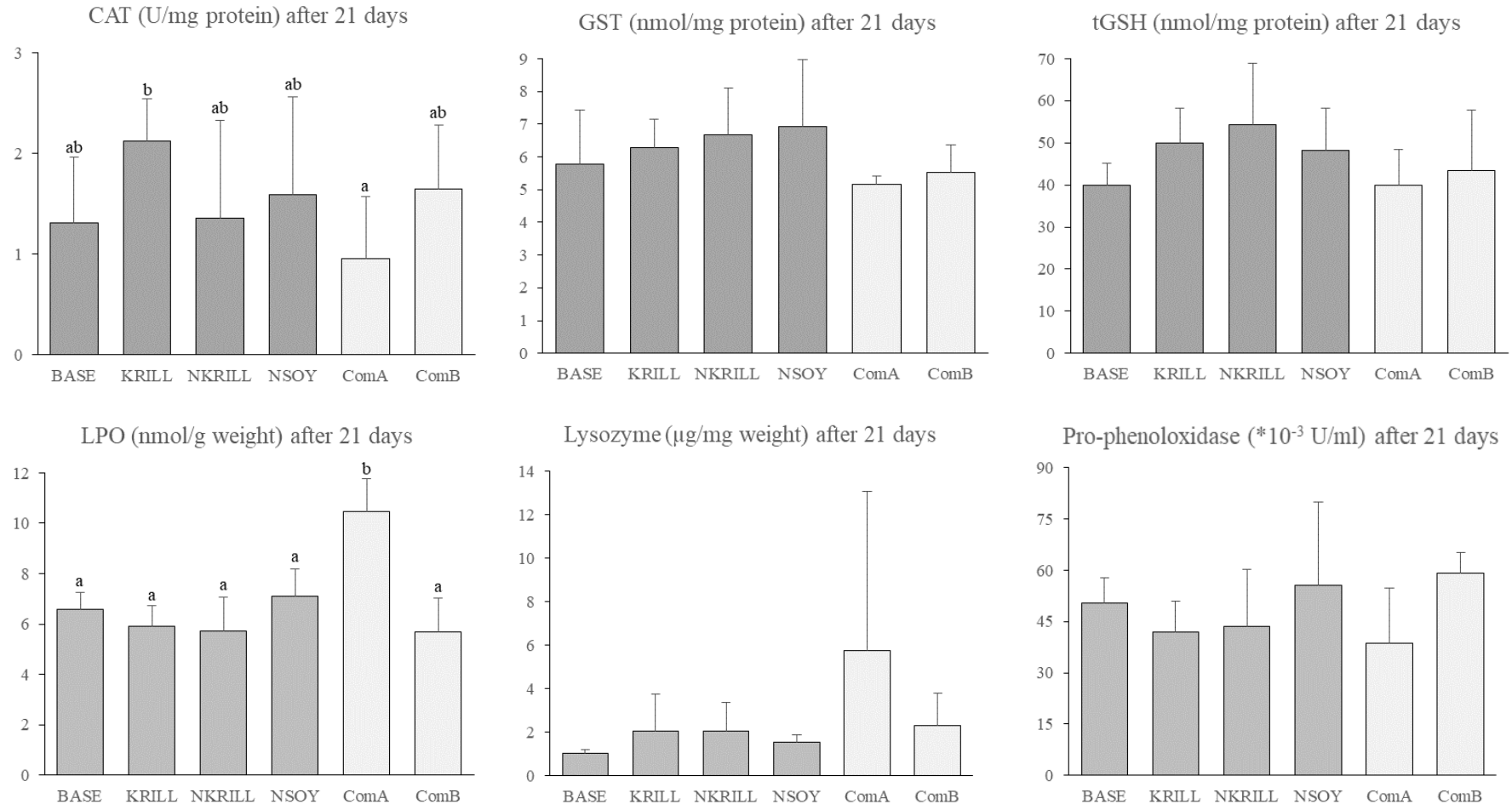

Figure 2 Catalase (CAT), glutathione S-transferase (GST), total glutathione (tGSH), lipid peroxidation (LPO), lysozyme and pro-phenoloxidase of whiteleg shrimp post-larvae fed different experimental and commercial microdiets at the end of the experimental period. Results expressed as mean ± SD (n=3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a One-Way ANOVA, followed by a post-hoc Tukey multiple comparison test.

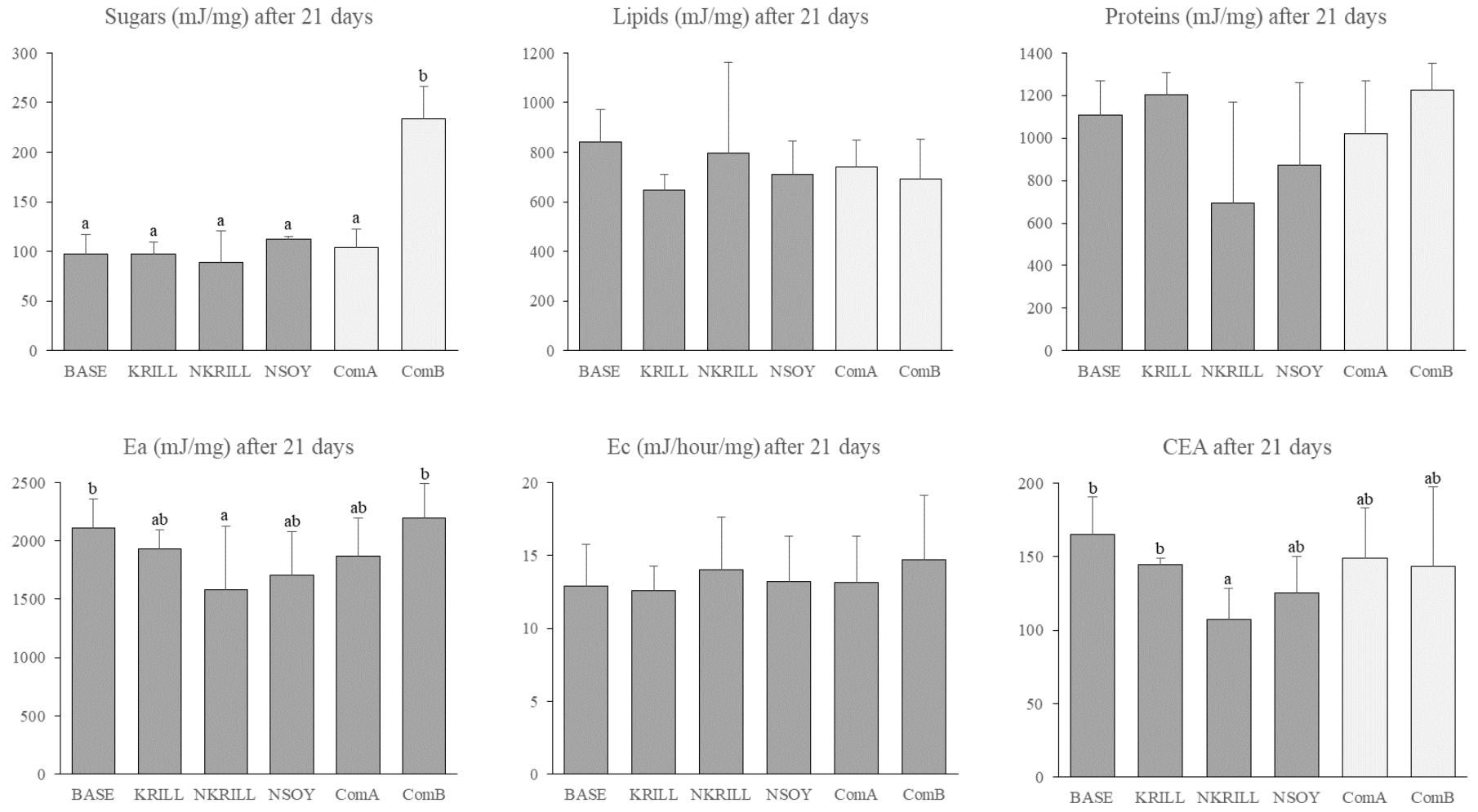

Figure 3 Energy reserves of whiteleg shrimp post-larvae fed different experimental and commercial microdiets at the end of the experimental period. Ea – Energy available; Ec Energy consumption; CEA – Cellular energy allocation. Results expressed as mean ± SD (n=3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a One-Way ANOVA, followed by a post-hoc Tukey multiple comparison test.

Table 3 Relative expression to housekeeping (*gapdh* and *act*) of target antioxidant and immune defence related genes of whiteleg shrimp (*P. vannamei*) PL after 21 days.

Gene	Acronym	Relative Expression						*p*-value
		BASE	KRILL	NKRILL	NSOY	ComA	ComB	
Penaeidin-3a	*pen-3*	0.68 ± 0.32	0.39 ± 0.15	0.45 ± 0.28	2.64 ± 4.64	2.70 ± 4.07	0.40 ± 0.15	0.088
PvHm117 crustin P	*crus*	1.06 ± 0.58 ^{b}	0.74 ± 0.43 ^{ab}	0.47 ± 0.37 ^{a}	0.95 ± 0.50 ^{b}	0.58 ± 0.38 ^{ab}	0.51 ± 0.28 ^{ab}	0.002
Lysozyme C-like	*lys*	1.28 ± 0.90 ^{b}	0.36 ± 0.31 ^{a}	0.41 ± 0.40 ^{a}	0.53 ± 0.35 ^{ab}	0.19 ± 0.11 ^{a}	0.22 ± 0.08 ^{a}	<0.001
Thioredoxin 1	*trd*	1.25 ± 0.91	0.90 ± 0.55	0.65 ± 0.60	0.90 ± 0.66	0.97 ± 0.76	0.53 ± 0.26	0.106
Hemocyanin	*hmc*	1.18 ± 1.52 ^{c}	1.16 ± 1.22 ^{bc}	0.06 ± 0.06 ^{a}	0.71 ± 0.86 ^{bc}	0.42 ± 0.62 ^{ab}	0.29 ± 0.16 ^{abc}	<0.001
C-type lectin 2-like	*lect*	1.89 ± 1.57 ^{c}	0.47 ± 0.49 ^{bc}	0.04 ± 0.02 ^{a}	0.79 ± 0.72 ^{bc}	0.36 ± 0.41 ^{ab}	0.29 ± 0.35 ^{bc}	<0.001
Glutathione transferase	*gst*	1.12 ± 0.59 ^{b}	0.76 ± 0.24 ^{ab}	0.39 ± 0.28 ^{a}	1.15 ± 0.83 ^{b}	0.47 ± 0.37 ^{a}	0.43 ± 0.23 ^{a}	<0.001
Glutathione peroxidase	*gpx*	1.45 ± 1.02 ^{b}	0.62 ± 0.59 ^{ab}	0.33 ± 0.44 ^{a}	0.61 ± 0.28 ^{ab}	0.25 ± 0.20 ^{a}	0.17 ± 0.09 ^{a}	<0.001

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a One-Way ANOVA for each gene. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

The LDA of the relative expression of all genes resulted in five discriminant functions, with the first two accounting for 73.63 % of the data variability (Wilks λ = 0.323, $p < 0.001$). In the first discriminant function (LDA1, score of 44.20 %) (Figure 4) the discriminant analysis revealed a significant group separation between BASE and NSOY and the remaining diets (excluding KRILL for NSOY). Additionally, KRILL was significantly separated from NKRILL. Group discrimination was mainly positively loaded by *gpx* and *gst* and negatively by *trd*. In the second discriminant function (LDA2, score of 29.43 %) a significant separation of diet NKRILL from the remaining diets, excluding BASE, is also shown. Furthermore, BASE was significantly separated from ComA (Figure 4). This discrimination was mainly postively loaded by *lys* and negatively by *pen-3* and *trd*.

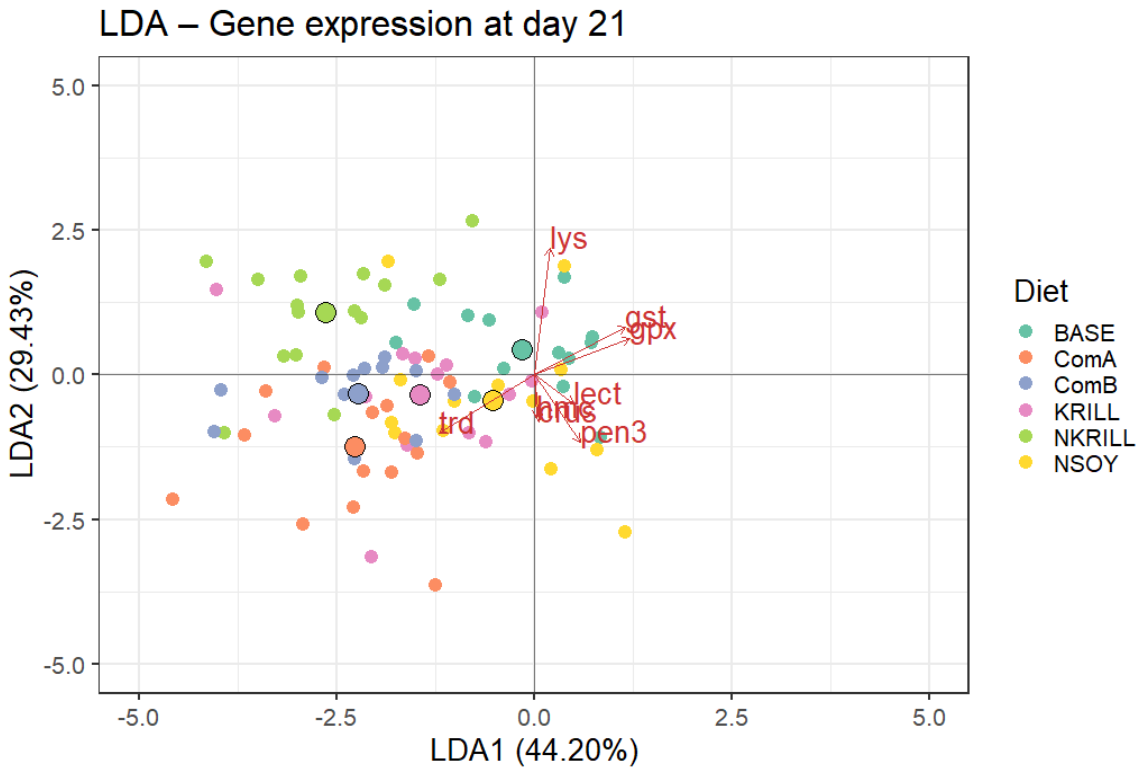

Figure 4 Linear discriminant analysis (LDA) of the relative expression of genes associated with the antioxidant and immune mechanisms (*pen-3*, *crus*, *lys*, *trd*, *hmc*, *lect*, *gst* and *gpx*) of whiteleg shrimp post-larvae fed the different experimental diets for 21 days. Percentages indicate the main functions (LDA1 and LDA2) discriminant score. Small circles represent diet data distribution while big circles represent diet centroids. Variable loads for both discriminant functions are represented by red arrows. Wilk's lambda test was significant ($p < 0.001$). In LDA1, BASE and NSOY were significantly discriminated from the remaining diets (excluding KRILL for NSOY), while KRILL was from NKRILL, mainly due to higher mRNA transcripts of *gpx* and *gst*. In LDA2, NKRILL was discriminated from the remaining treatments, excluding BASE, while BASE was significantly separated from ComA, mainly due to differences in the mRNA transcripts of *lys*, *pen-3* and *trd*.

4.3.2 Ammonia challenge

No significant differences between treatments were observed in shrimp survival after 24 hours of exposure to raised NH_3 levels, with values ranging between 91 and 97% (p = 0.488). Shrimp mortality occurring in the week following the exposure was also not significantly different between treatments, with the final survival values ranging between 90 and 96% (p = 0.519).

4.3.2.1 Oxidative stress-related biomarkers and cellular energy allocation after ammonia challenge

CAT activity levels were significantly higher in ComB than in BASE and NSOY; GST levels were significantly higher in NKRILL and ComA than in BASE; tGSH levels were significantly higher in NKRILL and ComA than all the remaining dietary treatments excluding ComB, whose levels were significantly higher than BASE and KRILL; and LPO levels were similar between treatments (Figure 5). Sugar levels were significantly higher in ComB than in all the remaining treatments; lipids were similar in all treatments; proteins were significantly higher in ComB than in NKRILL and NSOY, while NSOY levels were significantly lower than KRILL, ComA and ComB; EA was significantly higher in ComB than in BASE and NSOY; no significant differences between treatments were found in EC and CEA (Figure 6).

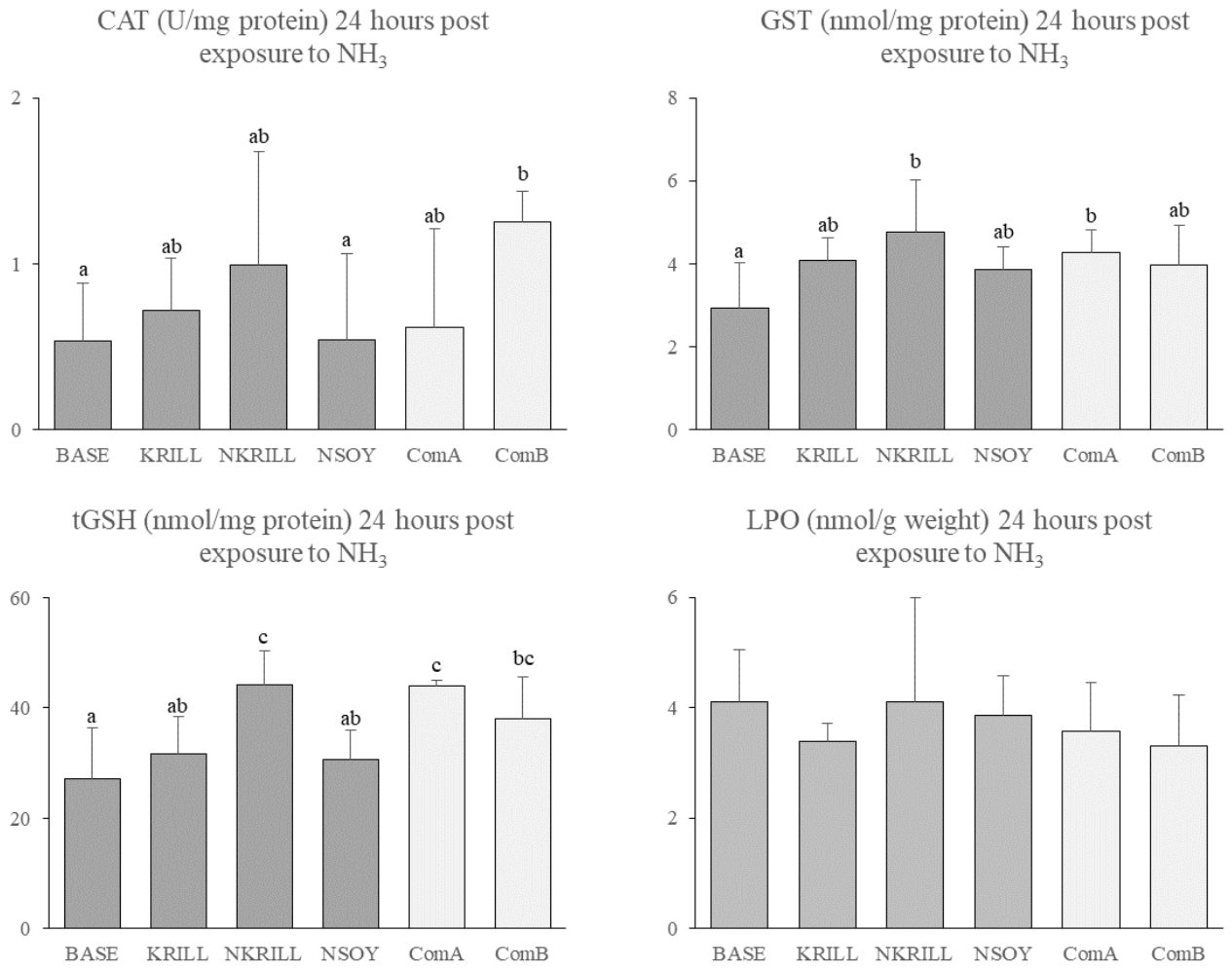

Figure 5 Catalase (CAT), glutathione S-transferase (GST), total glutathione (tGSH) and lipid peroxidation (LPO) of whiteleg shrimp post-larvae fed different experimental and commercial microdiets after 24 hours of exposure to raised levels of NH_3. Results expressed as mean ± SD (n=3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a One-Way ANOVA, followed by a post-hoc Tukey multiple comparison test.

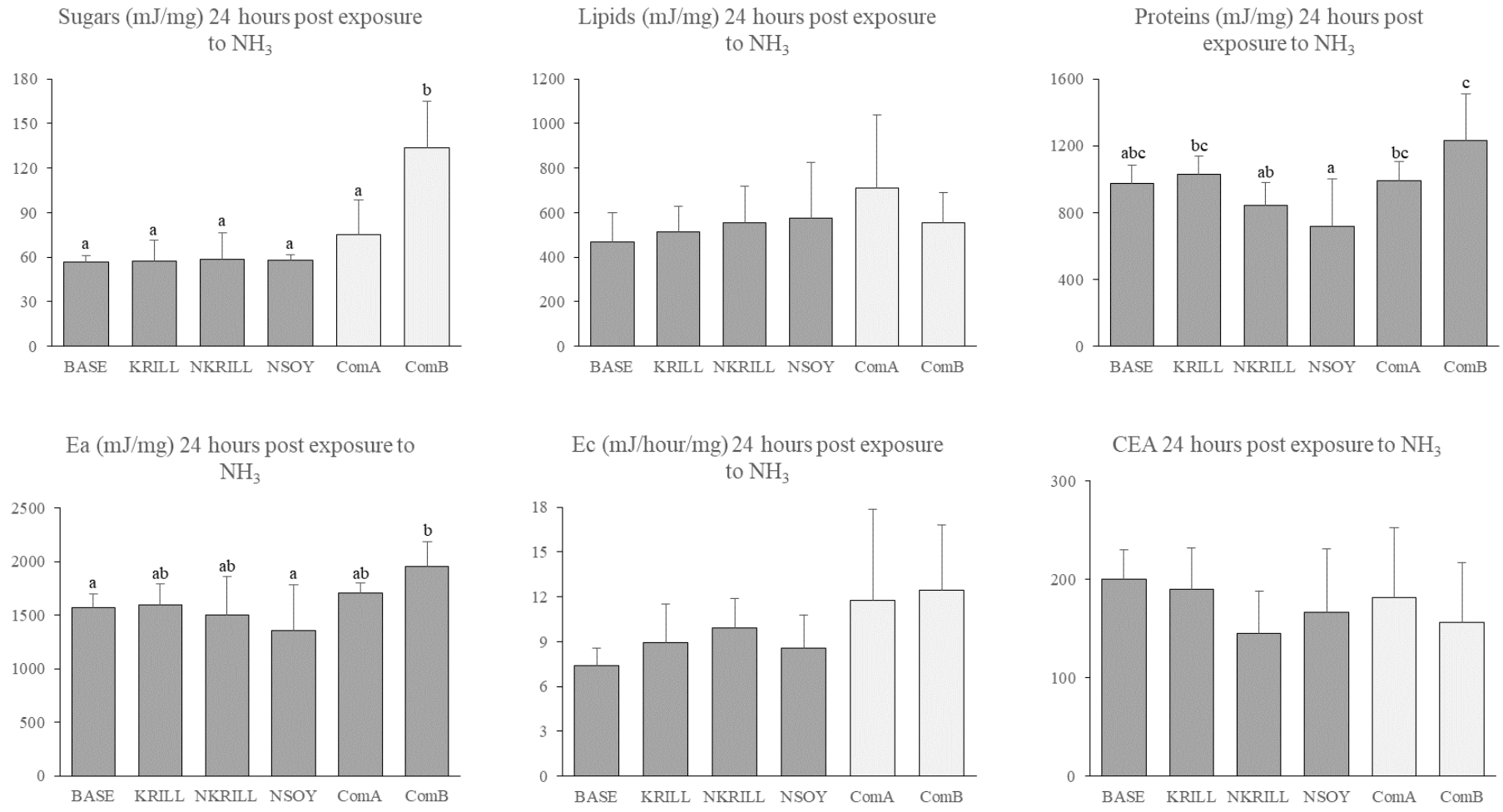

Figure 6 Energy reserves of whiteleg shrimp post-larvae fed different experimental and commercial microdiets after 24 hours of exposure to raised levels of NH_3. Ea – Energy available; Ec Energy consumption; CEA – Cellular energy allocation. Results expressed as mean ± SD (n=3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a One-Way ANOVA, followed by a post-hoc Tukey multiple comparison test.

4.3.3 *Vibrio harveyi* challenge

After 4 hours of exposure, PL survival was similar between treatments (99.2 ± 1.2 % treatments average; $p = 0.089$). However, PL survival was significantly lower in NKRILL, NSOY and ComB than in BASE at 24 hours after the challenge with *V. harveyi.* KRILL also showed a significantly higher survival than ComB, with no significant differences between the remaining treatments (Figure 7).

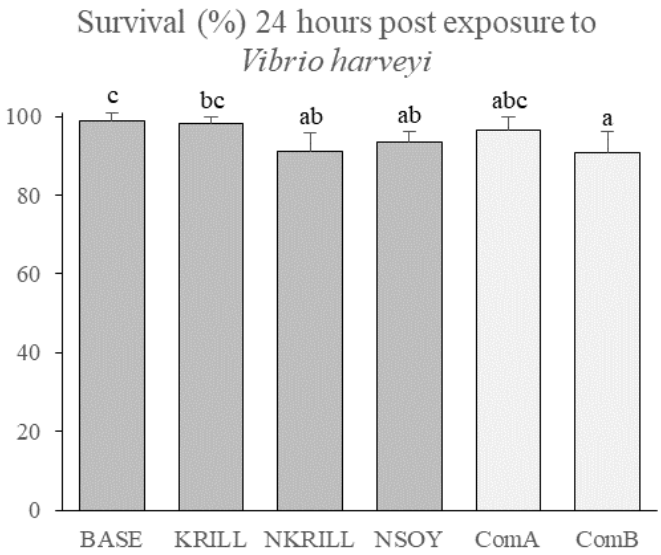

Figure 7 Survival of whiteleg shrimp post-larvae fed different experimental and commercial microdiets after 24 hours of exposure to *Vibrio harveyi*. Results expressed as mean ± SD (n=3 experimental units). Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a One-Way ANOVA, followed by a post-hoc Tukey multiple comparison test.

4.3.3.1 Gene expression after *Vibrio harveyi* challenge

At 4 hours of exposure, the normalized relative expression of the *crus* gene was significantly higher in NKRILL than in KRILL and both commercial diets, while transcripts levels of ComA and ComB were significantly lower than those of BASE and NSOY; the *lys* gene was significantly higher in NKRILL and ComA than in ComB; the *lect* gene was significantly higher in NKRILL and NSOY than in KRILL and both commercial diets; the *gst* gene was significantly higher in NKRILL and NSOY than in both commercial diets; the *gpx* gene was significantly lower in NSOY than in BASE and NKRILL; the *pen-3* gene was significantly higher in NSOY than in NKRILL and both commercial diets, while transcripts levels of ComB decreased significantly compared to BASE, KRILL and NKRILL; and the *trd* gene was significantly lower in NSOY than in BASE, NKRILL and ComB (Table 4).

Table 4 Relative expression to housekeeping (*gapdh* and *act*) of target oxidative and immune defence related genes of whiteleg shrimp (*P. vannamei*) PL after 4 hours post exposure to *Vibrio harveyi*.

Gene	Acronym	Relative Expression						*p*-value
		BASE	KRILL	NKRILL	NSOY	ComA	ComB	
Penaeidin-3a	*pen-3*	0.56 ± 0.39 ^{bc}	0.54 ± 0.43 ^{bc}	0.32 ± 0.26 ^{b}	3.41 ± 5.69 ^{c}	0.25 ± 0.15 ^{ab}	0.10 ± 0.05 ^{a}	<0.001
PvHm117 crustin P	*crus*	1.32 ± 1.17 ^{bc}	0.42 ± 0.19 ^{ab}	1.80 ± 1.55 ^{c}	0.83 ± 0.70 ^{bc}	0.23 ± 0.12 ^{a}	0.28 ± 0.22 ^{a}	<0.001
Lysozyme C-like	*lys*	1.18 ± 0.66 ^{ab}	0.79 ± 0.51 ^{ab}	1.63 ± 1.45 ^{b}	1.06 ± 0.81 ^{ab}	1.33 ± 0.86 ^{b}	0.56 ± 0.65 ^{a}	0.019
Thioredoxin 1	*trd*	1.07 ± 0.39 ^{b}	0.67 ± 0.22 ^{ab}	1.37 ± 0.92 ^{b}	0.43 ± 0.17 ^{a}	0.69 ± 0.44 ^{ab}	1.34 ± 0.71 ^{b}	<0.001
Hemocyanin	*hmc*	1.28 ± 0.93	2.21 ± 2.31	1.99 ± 1.38	0.92 ± 0.56	0.90 ± 0.63	1.43 ± 0.80	0.065
C-type lectin 2-like	*lect*	1.10 ± 0.61 ^{ab}	0.64 ± 0.38 ^{a}	1.62 ± 1.05 ^{b}	1.75 ± 0.90 ^{b}	0.69 ± 0.46 ^{a}	0.58 ± 0.33 ^{a}	<0.001
Glutathione transferase	*gst*	1.09 ± 0.55 ^{ab}	0.95 ± 0.50 ^{ab}	1.52 ± 0.54 ^{b}	1.40 ± 0.62 ^{b}	0.81 ± 0.49 ^{a}	0.76 ± 0.42 ^{a}	0.001
Glutathione peroxidase	*gpx*	1.12 ± 0.75 ^{b}	0.70 ± 0.26 ^{ab}	1.21 ± 0.86 ^{b}	0.43 ± 0.17 ^{a}	0.74 ± 0.48 ^{ab}	0.85 ± 0.41 ^{ab}	0.002

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a One-Way ANOVA for each gene. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

The LDA of the relative expression of all genes resulted in five discriminant functions, with the first two accounting for 83.73 % of the data variability (Wilks λ = 0.183, $p < 0.001$). In the first discriminant function (LDA1, score of 52.91 %) (Figure 8) the discriminant analysis revealed a significant group separation between NSOY and the remaining diets. Additionally, ComB was significantly separated from the remaining treatments, excluding NKRILL. Group discrimination was mainly positively loaded by *gpx*, *trd* and *hmc* and negatively by *pen-3* and *gst*. In the second discriminant function (LDA2, score of 30.82 %) a significant separation of diet NKRILL and BASE from the remaining diets (excluding NSOY for BASE), is also shown. Furthermore, NSOY was significantly separated from ComA (Figure 8). This discrimination was mainly positively loaded by *crus, trd* and *gst*.

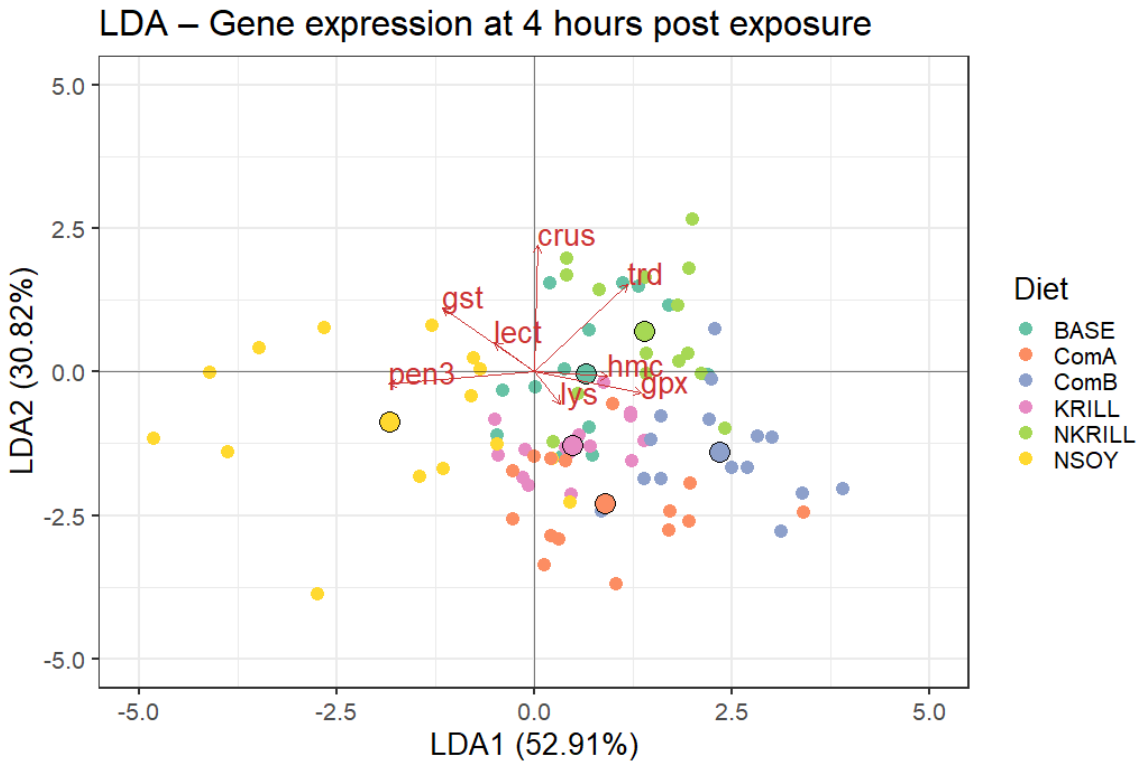

Figure 8 Linear discriminant analysis (LDA) of the relative expression of genes associated with the antioxidant defense and immune condition (*pen-3*, *crus*, *lys*, *trd*, *hmc*, *lect*, *gst* and *gpx*) of whiteleg shrimp post-larvae fed the different experimental diets after 4 hours of exposure to *Vibrio harveyi*. Percentages indicate the main functions (LDA1 and LDA2) discriminant score. Small circles represent diet data distribution while big circles represent diet centroids. Variable loads for both discriminant functions are represented by red arrows. Wilk's lambda test was significant ($p < 0.001$). In LDA1, NSOY was significantly discriminated from the remaining diets, while BASE, KRILL and ComA were discriminated from ComB, mainly due to lower mRNA transcripts of *gpx*, *trd* and *hmc* and higher transcripts of *pen-3* and *gst*. In LDA2, NKRILL and BASE were discriminated from the remaining diets (excluding NSOY for BASE), while NSOY was significantly separated from ComA, mainly due to higher mRNA transcripts of the *crus, trd* and *gst* genes.

After 24 hours of exposure to virulent *V. harveyi*, the normalized relative expression of the *lys* gene was significantly higher in NKRILL than all the remaining treatments; the *lect* gene was significantly lower in both commercial diets than in BASE, NKRILL and NSOY; the *gst* gene was significantly higher in ComB than in KRILL and NSOY; the *gpx* gene was significantly lower in NKRILL and NSOY than in BASE and ComB, while transcripts levels of KRILL were significantly higher than those of NSOY; the *hmc* gene was significantly higher in ComB than in all the remaining treatments, excluding ComA, while transcripts levels of NSOY were significantly lower than all the remaining dietary treatments; and the *trd* gene was significantly lower in NSOY than in BASE, KRILL and ComB (Table 5). The LDA of the relative expression of all genes resulted in five discriminant functions, with the first two accounting for 87.82 % of the data variability (Wilks $\lambda = 0.098$, $p < 0.001$). In the first discriminant function (LDA1, score of 59.86 %) (Figure 9) the discriminant analysis revealed a significant group separation between NSOY and the remaining diets. Additionally, BASE and NKRILL were significantly separated from the remaining treatments. Group discrimination was mainly positively loaded by *lect* and negatively by *hmc* and *trd*. In the second discriminant function (LDA2, score of 27.96 %) a significant separation of diet NKRILL from the remaining diets, is also shown (Figure 9). This discrimination was mainly positively loaded by *gpx* and negatively by *lys.*

Table 5 Relative expression to housekeeping (*gapdh* and *act*) of target oxidative and immune defense related genes of whiteleg shrimp (*P. vannamei*) PL after 24 hours post exposure to *Vibrio harveyi*.

Gene	Acronym	Relative Expression						*p*-value
		BASE	KRILL	NKRILL	NSOY	ComA	ComB	
Penaeidin-3a	*pen-3*	0.55 ± 0.36	0.40 ± 0.22	0.47 ± 0.24	0.45 ± 0.25	0.35 ± 0.27	0.45 ± 0.24	0.561
PvHm117 crustin P	*crus*	1.25 ± 0.65	1.36 ± 0.53	1.48 ± 0.89	1.16 ± 0.51	0.93 ± 0.42	1.20 ± 0.62	0.401
Lysozyme C-like	*lys*	1.00 ± 0.60 ^{a}	0.73 ± 0.60 ^{a}	3.06 ± 1.35 ^{b}	0.81 ± 0.51 ^{a}	0.62 ± 0.31 ^{a}	0.82 ± 0.37 ^{a}	<0.001
Thioredoxin 1	*trd*	1.50 ± 1.14 ^{b}	1.36 ± 0.72 ^{b}	0.77 ± 0.42 ^{ab}	0.57 ± 0.33 ^{a}	1.51 ± 1.33 ^{ab}	1.46 ± 0.60 ^{b}	0.002
Hemocyanin	*hmc*	1.14 ± 0.75 ^{b}	1.89 ± 0.82 ^{b}	1.66 ± 0.65 ^{b}	0.43 ± 0.25 ^{a}	2.00 ± 0.91 ^{bc}	2.98 ± 1.25 ^{c}	<0.001
C-type lectin 2-like	*lect*	1.02 ± 0.44 ^{b}	0.63 ± 0.18 ^{ab}	0.93 ± 0.41 ^{b}	1.09 ± 0.55 ^{b}	0.47 ± 0.20 ^{a}	0.56 ± 0.22 ^{a}	<0.001
Glutathione transferase	*gst*	1.40 ± 0.68 ^{ab}	1.38 ± 0.75 ^{a}	1.43 ± 0.41 ^{ab}	1.37 ± 0.63 ^{a}	1.39 ± 0.58 ^{ab}	2.11 ± 0.61 ^{b}	0.024
Glutathione peroxidase	*gpx*	1.46 ± 0.89 ^{c}	1.03 ± 0.64 ^{bc}	0.56 ± 0.23 ^{ab}	0.50 ± 0.21 ^{a}	0.85 ± 0.30 ^{abc}	1.30 ± 0.63 ^{c}	<0.001

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a One-Way ANOVA for each gene. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post-hoc Tukey multiple comparison test.

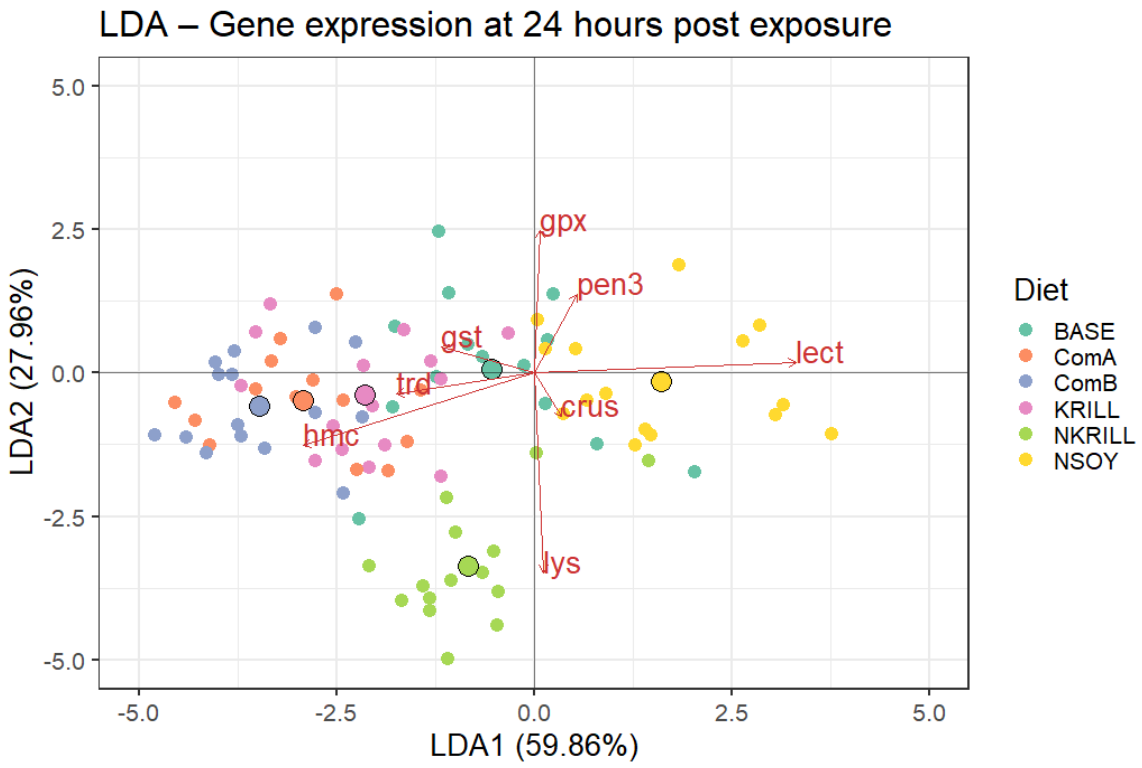

Figure 9 Linear discriminant analysis (LDA) of the relative expression of genes associated with the antioxidant defense and immune condition (*pen-3*, *crus*, *lys*, *trd*, *hmc*, *lect*, *gst* and *gpx*) of whiteleg shrimp post-larvae fed the different experimental diets after 24 hours of exposure to *Vibrio harveyi*. Percentages indicate the main functions (LDA1 and LDA2) discriminant score. Small circles represent diet data distribution while big circles represent diet centroids. Variable loads for both discriminant functions are represented by red arrows. Wilk's lambda test was significant ($p < 0.001$). In LDA1, NSOY was significantly discriminated from the remaining diets, while BASE and NKRILL were discriminated from KRILL, ComA and ComB, mainly due to higher mRNA transcripts of *lect* and lower transcripts of *hmc* and *trd*. In LDA2, NKRILL was discriminated from the remaining diets, mainly due to lower mRNA transcripts of *gpx* and higher transcripts of *lys.*

4.4 Discussion

The overall zootechnical results obtained for PL fed diet NSOY (final weight - 222 ± 5 mg; RGR - 19.8 ± 0.1 % day^{-1}; and FCR - 0.5 ± 0.1) can be considered an improvement over those described in Chapter 2 trial for PL fed diet P54 (final weight - 60 ± 3 mg; RGR - 15.0 ± 0.3 % day^{-1}; and FCR - 1.3 ± 0.1,) and in Chapter 3 trial for PL fed KRILL diet (final weight - 281 ± 14 mg; RGR - 15.8 ± 0.3 % day^{-1}; and FCR - 0.6 ± 0.0). Although higher mean final weight values were achieved in Chapter 3, the PL initial weight was also higher. As for survival at the end of the feeding period, results are in line with previous trials (75 - 91%), which can be deemed as an improvement when bearing in mind that the initial density employed (6000 PL m^{-3}) was the highest amongst trials performed under the scope of this Thesis. Furthermore, the lowest survival value obtained in this trial with an experimental diet (KRILL – 79 ± 3 %) was considerably higher than that of Chapter 3 trial (FISHHF – 42 ± 15 %). The whiteleg shrimp PL growth performances achieved with the experimental diets tested can be considered very satisfactory as most of them outperformed either one or both commercial diets used for comparison in this study. The FCR values obtained with the experimental diets were all very positive (0.6 ± 0.2 treatments average) and significantly lower than those of the commercial diets (1.0 ± 0.3 treatments average), with only diet KRILL being statistically similar to ComA and ComB. These results indicate that a further optimization of the experimental diets was done properly and demonstrate their adequacy for whiteleg shrimp PL in this stage of development and for the clear water RAS husbandry conditions maintained in this study.

Given the good results obtained in the previous trial (Chapter 3) with diets CTRL and KRILL, the latter was used in the current study, while BASE was mainly based on CTRL, keeping the same nutritional premises but employing a more economical approach when formulating. In CTRL, the main protein sources were a blend of fish meal and squid meal, while in BASE, fish meal and SPC were used, which allowed for a significant reduction in the overall cost of the diet. The inclusion of SPC had no alleged negative effects on shrimp PL growth performances or survival, as results of BASE were comparable to those of CTRL in Chapter 3 trial and some of the highest in this trial. In fact, whiteleg shrimp PL fed diets BASE and NKRILL achieved similar final weights and RGR values to those fed NSOY, in which SPC was entirely removed, suggesting that the inclusion of this ingredient was not detrimental to the PL development. Hence, results obtained in this trial corroborate that SPC can be used to replace more expensive/less sustainable marine ingredients in the formulation of diets for whiteleg shrimp PL reared in clear water RAS with no adverse effects on growth performances or survival.

Still, whiteleg shrimp PL fed the NSOY diet achieved the highest mean final body weight and RGR values amongst all dietary treatments, being significantly higher than those

fed KRILL and both commercial diets, which had fish meal as first ingredient. In fact, diet ComB had significantly inferior growth performances than all the experimental diets developed under the scope of this thesis, excluding KRILL. These results can potentially be related to the relatively low lipid contents of diet ComB. Although knowledge on whiteleg shrimp PL dietary lipid requirements is unsubstantial, Xie et al. (2019) reported increases in weight gain when dietary lipid levels were closer to those used in the experimental diets of this study (12.3 – 15.0%) than to those of ComB (10 %). ComA had comparable dietary lipid levels to the experimental diets and even higher energy and protein contents and still produced lower FCR values and similar (BASE, KRILL, NKRILL) or significantly lower (NSOY) mean final body weights and RGR values. These results suggest that higher energy diets may not be necessary for this stage of whiteleg shrimp PL development, even when reared in a clear water RAS devoid of natural productivity where inert feeds are the only energy source. As for protein content, as discussed in Chapter 2, inclusion levels of over 47 % do not seem to further enhance whiteleg shrimp PL growth performances.

The complete removal of krill meal from the diet composition of NKRILL did not produce any change in the whiteleg shrimp PL growth performance or survival when compared to KRILL that contained krill meal as main protein source. In fact, experimental diets that contained fish meal as main ingredient and krill meal as secondary marine protein source resulted in similar (BASE) or even significantly higher (NSOY) shrimp final body weight and RGR values than KRILL. Additionally, both commercial diets had fish meal as first ingredient and resulted in similar growth performances to KRILL. Hence, growth results obtained in the current trial suggest that using fish meal as first ingredient may be preferable over krill meal as the latter is usually more expensive. Furthermore, the exclusion of SPC in a fish meal-based diet (NSOY) seems to accentuate the benefits to whiteleg shrimp PL growth when compared to a krill meal-based diet. In Chapter 3, a krill meal-based diet resulted in similar whiteleg shrimp PL growth performances to diets using fish meal as the main protein source but improved their survival. In this trial, similar survival results were obtained with both types of formulations, which may be attributed to the potential differences in the shrimp PL genetic background. Despite having been obtained from the same supplier in both trials, their origin might have been distinct, as the animals are acquired from different hatcheries depending on their availability.

The gene expression analysis performed at the end of the feeding period shows that the NKRILL diet seemingly hindered the whiteleg shrimp PL health status when compared to the remaining experimental diets. The relative expression of *crus*, *lys*, *hmc*, *lect*, *gst,* and *gpx* genes, associated with the whiteleg shrimp innate defense and cellular antioxidant mechanisms, were significantly lower in NKRILL than in either BASE, NSOY, KRILL, or a combination of the previous. The multivariate analysis confirmed a significant separation of

BASE and NSOY from NKRILL in the first discriminant function, mainly by lower transcripts of genes *gpx*, *gst* and *trd*, related to the antioxidant defense. Although BASE and NKRILL were not separated in the second discriminant function, this dimension accounted for only 29.4 % of the data variability, while the first had a score of 44.2 %. Additionally, NKRILL was also significantly separated from KRILL, suggesting that the exclusion of krill meal in NKRILL may be detrimental to the whiteleg shrimp PL antioxidant mechanisms and immune response when compared to those that were fed a diet containing krill meal as the main (KRILL) or secondary marine protein source (BASE and NSOY). Similarly, in Chapter 3 a diet containing krill meal as the main ingredient (KRILL) enhanced the health status of whiteleg shrimp PL when compared to two fish meal-based diets (diet FISH and FISHHF) and a squid meal-based diet (SQUID). Although no additional studies were found supporting this hypothesis, krill meal is high in astaxanthin, which has been shown to improve whiteleg shrimp growth performances, survival, and health status. Niu et al. (2009) described increases in weight gain, final body weight, specific growth rate and survival of whiteleg shrimp PL fed diets containing graded levels of astaxanthin when compared to a non-supplemented control diet. The same authors observed an enhancement in the survival of PL fed the astaxanthin-supplemented diets in a 9 days hypoxia stress challenge. Accordingly, Chuchird et al. (2015) reported improved survival of whiteleg shrimp PL fed a diet containing 50 ppm of astaxanthin plus 0.3 % of formic acid when compared to a control diet in a 90-day feeding trial. When challenged with *Vibrio parahaemolyticus*, PL fed astaxanthin supplemented diets had significantly higher survival and improvements in immune parameters (total hemocyte count, phagocytosis activity, phenoloxidase activity, and SOD activity) when compared to those fed a control diet. The same effects on growth performances, survival in hypoxia conditions, and also increases in mRNA expression of health status related genes were observed in whiteleg shrimp juveniles (Zhang et al., 2013). Still, the growth performances of whiteleg shrimp PL fed the KRILL diet in this trial were inferior to those fed NSOY and their health status was seemingly compromised when compared to those fed the BASE diet, suggesting krill meal may not be as adequate as fish meal to be used as first ingredient but should be used as a secondary ingredient of marine origin.

Despite being fish meal-based diets, both commercial diets produced similarly health-conditioned PL status compared to KRILL (and NKRILL), and apparently compromised when compared to BASE and NSOY. ComA had significantly higher LPO levels than all the remaining diets, probably due to low CAT activity levels. These results may be related to the higher protein contents of ComA, since, as contemplated in Chapter 2, there seem to be some putative negative effects to whiteleg shrimp PL health status when using those levels of dietary protein. Furthermore, diet ComB and NKRILL had lower lipid

levels (10 and 12.3 %, respectively) than BASE, NSOY and KRILL (15.0, 13.7 and 14.6 %, respectively) which might have also contributed to the apparent deterioration of the shrimp PL health status. As previously discussed in Chapter 3, increasing lipid levels in diets for whiteleg shrimp PL seem to be related to the upregulation of immune-related genes (Xie et al., 2019). Therefore, these results suggest that krill meal inclusion in diets for whiteleg shrimp PL may be beneficial for their health status in moderate amounts and fish meal should be considered the main protein source, if appropriate dietary protein and lipid levels are maintained.

The energy reserves analysis revealed that the whiteleg shrimp PL fed ComB had significantly more carbohydrate contents than the remaining diets at the end of the feeding period. The poor growth performances and apparently compromised health status of PL fed this diet seem to indicate that they were not able to utilize this energy properly to enhance their growth and/or immune and antioxidant mechanisms. Consequently, the total EA was significantly higher in ComB (and BASE) than in NKRILL. On the other hand, whiteleg shrimp PL fed NKRILL had some of the finest growth performances in this trial but with apparent consequences to their health status, suggesting most energy was channeled to growth.

It has been shown that acute ammonia exposure can generate ROS and lead to oxidative stress or mortality in aquatic animals (Chen et al., 2019; Cheng et al., 2015; Ching et al., 2009; Hegazi et al., 2010; Yan et al., 2021; Zhang et al., 2020;) and has been used as a stressful stimulus in numerous studies with whiteleg shrimp. Several authors have reported mean lethal concentrations (LC50) of NH_3 for whiteleg shrimp juveniles to be between 2.9 to 4.1 mg L^{-1}, depending on shrimp weight, genetic lineage, or rearing conditions maintained (Alloul et al., 2021; Chen et al., 2019; Chen et al., 2020; Li et al., 2016; Lin & Chen, 2001; Zhang et al., 2012), while for younger animals (PL12), the 24 h LC50 for NH_3 has been described as around 0.75 mg L^{-1} (Frias-Espericueta et al., 2000). As no studies clearly outlining the LC50 of NH_3 for whiteleg shrimp PL of the same size of those used in this trial were available, the authors opted for an intermediate concentration (1.6 mg L^{-1}) between those reported for PL and small juveniles. However, after 24 hours of exposure to this dose of NH_3 and the following week of recovery, mortality was almost inexistent and similar between treatments, denoting that the procedures used, i.e. the NH_3 concentration and time of exposure, might have been insufficient to cause significant stress and, consequently, mortality. Hence, in future challenges, the LC50 of NH_3 should, if possible, be established previously as the results obtained in this study and the currently available literature indicate that the whiteleg shrimp tolerance to ammonia can vary with stage of development, genetic background, and culture conditions.

Nevertheless, PL fed different diets reacted distinctly to the NH_3 challenge as significant differences were found in the antioxidant parameters measured. Conversely to what was observed at the end of the feeding trial, whiteleg shrimp PL fed diet NKRILL and both commercial diets showed higher activity levels of CAT, GST and/or tGSH than those fed BASE, NSOY and KRILL, indicating a higher dependency on the activity of these antioxidant molecules to deal with the stressful stimulus. Whiteleg shrimp PL fed with ComB maintained the highest carbohydrates contents of all treatments, but the overall levels decreased with the challenge, suggesting that energy from carbohydrates was used for protection mechanisms during the NH_3 exposure. The decreased levels of protein contents observed for NKRILL and NSOY may be related to protein breakdown to increase free amino acids that can be used for energy purposes (Sokolova et al., 2012, Sokolova, 2013). Accordingly, several authors have reported decreases in energy reserves contents in response to stressful stimuli in invertebrates (Chinni and Yallapragada, 2002; Christensen et al., 2005; Rodrigues et al., 2017), including whiteleg shrimp (Mercier et al., 2006; Wang et al., 2021; Xu et al., 2022). Moreover, the energy consumption of the whiteleg shrimp PL from all dietary treatments declined after 24 hours of exposure, which may indicate a depression of their metabolism as a response to the stressful conditions and diminishing of available energy. This mechanism is known to occur in several invertebrates (Guppy, 2004) and has been recently shown in whiteleg shrimp juveniles exposed to acute ammonia-nitrogen stress (Shan et al., 2018).

After 4 hours of exposure to *V. harveyi*, mortality was minimal and survival values were similar between treatments. After 24 hours, mortality was still low indicating that the PL resistance to the pathogen was high and/or that the procedures used (i.e. challenge by immersion, concentration of the pathogen used, time of exposure) might have been inadequate for infection, as it is known that the *V. harveyi* can cause significant mortality and is frequently used to challenge whiteleg shrimp in research studies (Huang et al., 2013; Kongnum and Hongpattarakere, 2012; Ma et al., 2020; Nonwachai et al., 2010; Zokaeifar et al., 2014). Still, BASE was the diet that best conditioned the PL to resist the pathogen, having significantly higher survival values than the NKRILL, NSOY and ComB dietary treatments. These results are seemingly in agreement with those obtained in the growth trial that suggested that the exclusion of krill meal from NKRILL impaired the whiteleg shrimp PL health status. However, the multivariate analysis performed showed that the gene expression response of PL fed BASE and NKRILL was similar, which could be considered contradictory and may suggest some other protection mechanisms not analyzed might have contributed to the PL capacity to deal with the infection. Moreover, the exclusion of SPC from NSOY also seems to have been detrimental to the capacity of the PL to resist infection. The multivariate analysis of gene expression showed an apparent compromised health

status of PL fed this diet when compared to those fed the remaining dietary treatments, which was confirmed by the lower survival values when compared to BASE. It has been shown that the inclusion of soybean derived ingredients can enhance the whiteleg shrimp innate immune responses and/or antioxidant mechanisms, when fermented, hydrolyzed, or processed into a protein concentrate (Lin and Chen, 2022; Liu et al., 2019; Ray et al., 2020). Antigenic factors associated with soybean meal like trypsin inhibitors, lectins, phytic acid, soybean agglutinin, and antigen proteins are significantly reduced during processing and may increase the bioavailability of immunomodulatory active peptides (Singh et al., 2014).

4.5 Conclusions

In conclusion, the developed experimental diets are adequate for whiteleg shrimp PL in this stage of development and for the clear water RAS husbandry conditions maintained in this study, as most of them outperformed either one or both commercial diets used for comparison in this study.

The results obtained in this study suggest SPC can be used to replace more expensive/less sustainable marine ingredients in the formulation of diets for whiteleg shrimp PL reared in clear water RAS with no adverse effects on their growth performance, survival, energy reserves, or antioxidant and immune status. Moreover, the capacity of PL to resist infection was apparently compromised, resulting in higher mortalities, when SPC was not included in the diet formulation (NSOY).

The complete removal of krill meal from the diet composition of NKRILL did not produce any changes in the whiteleg shrimp PL growth performances and survival but was detrimental to their antioxidant mechanisms and immune response when compared to those that were fed a diet containing krill meal as main (KRILL) or secondary marine protein source (BASE and NSOY). These results suggest krill meal may not be as adequate as fish meal to be used as the main ingredient but can be beneficial for the whiteleg shrimp PL health status in moderate amounts.

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Declarations

All activities were undertaken within the clear boundaries of national and EU legal frameworks directed by qualified scientists/technicians and conducted according to the European guidelines on the protection of animals used for scientific purposes (Directive 2010/63/UE of the European Parliament and on the European Union Council) and under strict monitoring and control of DGAV—(Direção Geral de Alimentação e Veterinária), Animal Welfare Division, which is the competent authority responsible for implementing the legislation on the “protection of animals used for scientific purposes”.

WP and LC worked for Sparos Lda during this study. The remaining authors declare that they have no conflicts of interest. The views expressed in this work are the sole responsibility of the authors and do not necessarily reflect the views of any third party.

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Chapter 5

Health-promoting additives supplemented in inert microdiets for whiteleg shrimp (*Penaeus vannamei*) post-larvae: effects on growth, survival and health status

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Health-promoting additives supplemented in inert microdiets for whiteleg shrimp (*Penaeus vannamei*) post-larvae: effects on growth, survival, and health status

Abstract

This study aimed to evaluate the effects of vitamins C and E, *β*-glucans, taurine and methionine supplemented in inert microdiets on the growth performance and health status of whiteleg shrimp post-larvae. Four experimental microdiets were evaluated: a positive control diet (PC); the PC with decreased levels of vitamin C and E as negative control (NC); the PC with increased taurine and methionine levels (T+M); and the PC supplemented with *β*-glucans (BG). Whiteleg shrimp post-larvae (PL16, 9 mg wet weight) were reared for 18 days in a clear water recirculating system. No significant differences in growth performance and survival were observed among dietary treatments at the end of the study. Increased vitamin C and E levels in the PC did not produce any changes in shrimp antioxidant and immune status when compared with the NC, except for lower relative expressions of *pen-3* in the NC, suggesting that lower levels of these vitamins may impact the shrimp immune status. Shrimp PL fed the T+M diet showed significantly higher relative expressions of *crus*, and significantly lower *hmc* transcripts, when compared with those fed the NC dietary treatment. Lipid peroxidation levels dropped significantly in shrimp PL fed the BG compared to those fed the PC, suggesting that *β*-glucans improved the antioxidant mechanisms of the animals. When compared with the NC diet, PL fed with BG showed significant increases in tGSH levels and in the relative expression of *crus* and *pen-3*. This suggests that the *β*-glucans supplementation coupled with higher levels of vitamin C and E can boost the antioxidant capacity and immune status of whiteleg shrimp PL. In conclusion, the results obtained in this study showed benefits to the antioxidant capacity and robustness of the shrimp PL when the vitamin C and E levels were higher than those used in the NC, like those used in the PC. Amongst the additives tested, the inclusion of *β*-glucans in the diets seems to be the most promising as it reduced lipid peroxidation in the shrimp PL, even when compared to a high-quality control diet. When compared to the NC, the interaction between the supplementation of *β*-glucans and higher levels of vitamins C and E also seems beneficial to the antioxidant capacity of whiteleg shrimp PL.

Keywords: *Penaeus vannamei*, whiteleg shrimp, post-larvae, microdiets, dietary additives, immunostimulants, antioxidants, health status

5.1 Introduction

The whiteleg shrimp (*Penaeus vannamei*) is currently the most representative animal species in aquaculture, constituting in 2020 a share of 4.7% in global production (FAO, 2022). To meet the market demands, whiteleg shrimp larvae and post-larvae (PL) yields in hatcheries has increased intensively. However, problems in larviculture can have an enormous impact on shrimp performance in the long-term, affecting the downstream production of high-quality juveniles and adults. Initial developmental stages are frequently associated with sub-optimal growth, high size dispersion, and low survival due to cannibalism and reduced disease resistance to opportunistic pathogens. The latter results from a high dependence on optimal zootechnical conditions and nutrition, as shrimp lack an adaptive immune response and depend uniquely on their innate immune system to maintain a good health status and avoid pathogenic outbreaks that can result in disastrous consequences to production and significant economic losses (de La Peña et al., 2015; Shinn et al., 2018; Song and Li, 2014). Besides the fact that shrimp cannot be vaccinated due to the lack of an adaptive immune system, the use of antibiotics in the aquaculture industry is limited due to inherent food safety concerns, environmental issues, and the increased antimicrobial resistance (Lulijwa et al., 2020). Consequently, the use of functional dietary additives to stimulate the shrimp immune system has been studied as a prophylactic alternative and is regarded as an extremely important strategy to overcome the constraints of intensive shrimp farming. Organic acids, plant/algae extracts, nucleotides, functional amino acids, vitamins, and naturally occurring immunostimulant compounds such as *β*-glucans have been studied thoroughly in diets for fish and crustaceans, as they may improve growth performance, survival, stress, and disease resistance, as recently reviewed by Dawood et al. (2018). Their potential to be included in diets for juvenile and/or adult whiteleg shrimp has also been demonstrated to some extent by several authors (Chien et al., 2020; Façanha et al., 2016; Ji et al., 2021; Liu et al., 2007; To et al., 2021; Tseng et al., 2009; Wang, 2007; Wang et al., 2021; Wu et al., 2016; Yang et al., 2010; Yue et al., 2013; Zokaeifar et al., 2012). Nevertheless, far fewer studies are available on the use of these supplements in diets for whiteleg shrimp initial developmental stages. There is evidence that vitamin C supplemented in diets for PL can be an effective antioxidant at the tissue level (Ruff et al., 2001). More recently, increases in growth performance, digestive enzymes activity, and improvement of immune condition were verified when incorporating commercial prebiotic and probiotic blends in diets for PL (Madani et al., 2018; Miandare et al., 2016, 2017).

Hence, innovative nutritional solutions that enhance development and resistance to stress and pathogenic factors during these critical stages and thus improve shrimp quality in posterior phases of production have tremendous potential to reinforce the success of

shrimp farming. Therefore, this study aimed to evaluate the effects of several health promoting nutrients/additives (i.e., vitamins C and E, *β*-glucans, taurine, and methionine) supplemented in inert microdiets on the growth performance and health status of whiteleg shrimp post larvae.

5.2 Materials and methods

5.2.1 Dietary treatments

Four experimental microdiets were evaluated in triplicates. A positive control diet (PC) was formulated to meet the nutritional requirements of whiteleg shrimp post larvae, containing 515 g kg^{-1} of SPAROS proprietary marine protein mix, 160 g kg^{-1} of SPAROS proprietary plant protein mix, 103 g kg^{-1} of fish protein hydrolysate, 19 g kg^{-1} of fish oil, 28 g kg^{-1} of marine phospholipids, and 57 g kg^{-1} of SPAROS proprietary vitamins and minerals premix. On the remaining treatments, three experimental variants based on the PC were used, differing only in the ingredient formulation by the following: (1) decreasing inclusion levels of the vitamins and minerals premix by 7 g kg^{-1} to reduce vitamin C and E contents in the negative control diet (NC); (2) supplementing 5 g kg^{-1} of taurine and 10 g kg^{-1} of methionine to increase the levels of both molecules in the T + M diet; and (3) supplementing the PC diet with 1 g kg^{-1} of *Saccharomyces cerevisiae β-(*1, 3)/(1, 6)-glucans (BG). The experimental diets formulation can be seen in Table 1. The proximate composition of the experimental diets was analyzed by Eurofins Food Testing Portugal following their standard procedures (Table 2).

All diets were produced at Sparos Lda facilities (Olhão, Portugal), using extrusion at low temperature as the main production process, as follows: powder ingredient mixing according to target formulation using a double-helix mixer; grinding in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Augsburg, Germany); addition of the oil fraction; humidification and agglomeration through low-temperature extrusion (Dominioni Group, Lurate Caccivio, Italy); drying of resultant pellets in a convection oven (OP 750-UF, LTE Scientifics, Oldham, UK) for 4 h at 60 °C; crumbling (Neuero Farm, Melle, Germany); and sieving to desired size ranges.

Table 1 Dietary formulation of the experimental diets used to culture whiteleg shrimp (*P. vannamei*) PL for 18 days.

Ingredients (g kg^{-1})	NC	PC	T + M	BG
Marine protein mix [1]	515	515	510	515
Fish protein hydrolysate [2]	103	103	103	103
Plant protein mix [3]	160	160	160	160
Cellulose [4]	17	10	0	9
Fish oil [5]	19	19	19	19
Marine phospholipids [6]	28	28	28	28
Lecithin [7]	56	56	56	56
Vitamins and minerals [8]	50	57	57	57
Cholesterol [9]	10	10	10	10
Antioxidant [10]	4	4	4	4
Monoammonium phosphate [11]	38	38	38	38
β-(1, 3)/(1, 6)-glucans [12]	0	0	0	1
DL-Methionine [13]	0	0	5	0
Taurine [14]	0	0	10	0

[1] Proprietary product for shrimp: 37% crude protein, 5% crude fat—SPAROS, Portugal

[2] Sopropêche, France

[3] Proprietary product for shrimp: 13% crude protein, 1% crude fat—SPAROS, Portugal

[4] Disproquimica, Portugal

[5] Sopropêche, France

[6] Triple nine, Denmark

[7] Lecico, Germany

[8] Proprietary premixes/products for shrimp—SPAROS, Portugal

[9] Carbogen, The Netherlands

[10] Kemin, Italy

[11] Timab Iberica, Spain

[12] MacroGard—Orffa, The Netherlands

[13] Premix—Especialidades Agricolas e Pecuárias Lda, Portugal

[14] Proprietary product for marine fish and shrimp—SPAROS, Portugal

Table 2 Proximate composition of the experimental diets used to culture whiteleg shrimp (*P. vannamei*) PL for 18 days.

	NC	PC	T + M	BG
Dry matter (DM, %)	94.0 ± 0.5	93.7 ± 0.5	94.3 ± 0.5	94.3 ± 0.5
Crude protein (% DM)	66.2 ± 1.7	66.4 ± 1.7	67.4 ± 1.7	66.1 ± 1.7
Crude fat (% DM)	15.9 ± 1.0	16.2 ± 1.0	16.3 ± 1.0	15.6 ± 1.0
Fiber (% DM)	1.4 ± 0.7	1.2 ± 0.7	1.1 ± 0.7	1.2 ± 0.7
Ash (% DM)	11.4 ± 0.4	11.7 ± 0.4	11.7 ± 0.4	11.8 ± 0.4
Phosphorous (% DM)	1.9 ± 0.4	2.0 ± 0.4	1.9 ± 0.5	2.0 ± 0.4
Energy (MJ/Kg DM)	23.0 ± 0.0	23.0 ± 0.0	23.1 ± 0.0	22.9 ± 0.0
Vitamin C (mg/kg DM)	159.6 ± 0.0	2027.7 ± 0.0	2014.8 ± 0.0	2014.8 ± 0.0
Vitamin E (mg/kg DM)	42.6 ± 0.0	1067.2 ± 0.0	1060.4 ± 0.0	1060.4 ± 0.0
Taurine (g/100 g DM)	0.31 ± 0.3	0.31 ± 0.3	0.94 ± 0.9	0.31 ± 0.3
Methionine (g/100 g DM)	1.5 ± 0.2	1.5 ± 0.2	2.0 ± 0.3	0.6 ± 0.1

Results expressed as mean ± standard deviation (*n* = 2 experimental units).

5.2.2 Shrimp rearing and sampling

Whiteleg shrimp post larvae (PL16), originated from Blue Genetics (La Paz, Mexico), were reared for 18 days at Riasearch Lda facilities (Murtosa, Portugal). Shrimp were randomly distributed to 12 tanks with approximately 50 L that were part of a clear water-recirculating system. Each tank was stocked with 200 individuals averaging 9 mg of wet weight. These were kept under a 12 h light:12 h dark photoperiod and were fed close to ad libitum with automatic feeders that supplied eight meals a day. Feeders were cleaned daily and charged with adjusted feed quantities based on the observation of the tanks and the presence/absence of remnants from the previous day. Feed size was 400–600 µm for the first week and 600–800 µm for the remaining feeding period. Water temperature was maintained at 28.8 ± 0.3 °C, dissolved oxygen concentration at 7.5 ± 0.4 mg L^{-1}, salinity at 20.3 ± 1.2, pH at 7.96 ± 0.1, NH_3 at 0.0 ± 0.0 mg L^{-1}, and NO_2 at 0.36 ± 0.3 mg L^{-1}.

At the start of the trial, a total of 60 shrimp from the initial stock were randomly selected and group weighed for initial wet weight determination. At the end of the experiment, all shrimp were weighed in groups of 20 individuals for the final wet weight determination of each tank. Additionally, 40 shrimp were randomly selected from each tank for oxidative stress and immune parameters analysis and 10 shrimp for analysis of gene expression. Shrimp were fasted for 12 h prior to samplings to ensure their guts were empty at collection. Shrimp sampled for oxidative stress and immune parameters were stored at −80 °C for subsequent analysis. Shrimp sampled for molecular biology analysis were kept in RNAlater (Sigma, St. Louis, MO, USA) at >1:5 volume ratio, at 4 °C, for 24 h prior to being stored at −20 °C. Relative growth rate (RGR), feed conversion ratio (FCR), and survival for each treatment were assessed at the end of the experiment.

5.2.3 Oxidative stress and immunity-related biomarkers

5.2.3.1 Sample preparation

A total of 40 whole whiteleg shrimp post larvae from each tank sampled at end of the trial were weighed and homogenized in quadruple groups of 10 individuals for oxidative stress and immune parameters analysis. Potassium phosphate buffer (0.1 M) was added to each group in a 1/10 (*w*/*v*) proportion followed by homogenization using a high-performance dispersing instrument (SilentCrusher M, Heidolph Instruments, Schwabach, Germany). An aliquot for lipid peroxidation (LPO) with butylated hydroxytoluene was reserved prior to centrifugation. After centrifugation (5500 rpm for 20 min), sample supernatant was collected and distributed in separate aliquots for oxidative stress parameters and immune parameters. The remaining 10 shrimp sampled for molecular biology analysis were homogenized in NZYol (Nzytech, *w*/*v* proportion according to the

manufacturer's instructions) using a Precellys 24 tissue homogenizer (Bertin instruments, Montigny-le-Bretonneux, France).

5.2.3.2 Determination of oxidative stress biomarkers

Catalase (CAT), lipid peroxidation (LPO), and total glutathione (tGSH) activities as well as total proteins content were determined in the homogenized samples. Total proteins were measured by using PierceTM BCA Protein Assay Kit, as described by Costas et al. (2014). Samples were diluted in K-phosphate buffer (0.1 M; pH 7.4), and bovine serum albumin (BSA, 2 mg mL^{-1}) was used as standard. Afterwards, 25 µL of each diluted sample and standards were plated in triplicate and read at 562 nm in a Synergy HT microplate reader. Results were calculated using a standard curve and expressed as mg mL^{-1}.

CAT activity levels were determined measuring the decrease of hydrogen peroxide (H_2O_2 30%, Sigma) concentration as described by Clairborne (1985). The reaction mixture was composed of K-phosphate buffer (0.05 M pH 7.0) and H_2O_2 (30%) as substrate, and 10 µL of homogenate sample was added to the reaction mixture, effecting a total volume of 300 µL. Absorbance was read at 240 nm in UV microplates for 2 min (1 reading every 15 s) in a Synergy HT microplate reader and results expressed as enzyme units per milligram of total protein (U mg^{-1} protein). One enzyme unit is the amount of enzyme needed to catalyze one micromole of substrate per minute.

Endogenous LPO was assessed by measuring thiobarbituric acid-reactive substances (TBARS), preventing artefactual lipid oxidation by adding butylhydroxytoluene (4%; Sigma) (Torres et al., 2002). Homogenate samples incubated for 60 min at 100 °C with a 100 µL of trichloroacetic acid 100% solution and 1 mL of 2-thiobarbituric acid 0.73% (Sigma), trizma hydrochloride (Sigma), and diethylenetriaminepentaacetic acid (Fluka) solution in polystyrene microtubes. Afterwards, these were centrifuged for 5 min at 11,500 rpm, and supernatant (200 µL) was added to the microplate wells. Absorbance was read at 535 nm and results expressed as nmol g wt^{-1}.

Total glutathione content in post-larvae homogenate samples was measured based on the oxidation of glutathione by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma) as described by Rodrigues et al. (2017). Samples were diluted in K-phosphate buffer (0.1 M pH 7.4) to obtain 0.7 mg mL^{-1} of protein. Thereafter, 50 µL of each diluted sample was added to microplate wells, followed by the addition of 250 µL of a reaction solution composed by DTNB, K-phosphate buffer (0.1 M, pH 7.4), NADPH (ß-Nicotanimide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; Alpha Aesar), and glutathione reductase (Sigma). Absorbance was read at 412 nm for 3 min (1 reading every 20 s) in a Synergy HT microplate reader and results expressed as nmol mg of $protein^{-1}$.

5.2.3.3 Analysis of immune parameters

Lysozyme, pro-phenoloxidase, and bactericidal activities were determined in the homogenized samples. Lysozyme activity was measured using a turbidimetric assay as described by Costas et al. (2011). Briefly, a solution of *Micrococcus lysodeikticus* (0.25 mg mL^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was prepared and 40 µL of homogenized samples, and 130 µL of this suspension were added to a microplate, effecting a final volume of 170 µL. The reaction was carried out at 25 °C, and absorbance (450 nm) measured after 0.5 and 30 min in a Synergy HT microplate reader. Lyophilized hen egg white lysozyme (Sigma) was diluted in sodium phosphate buffer (0.05 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using a standard curve. Lysozyme was expressed as µg mg $protein^{-1}$.

Pro-phenoloxidase activity was measured spectrophotometrically using L-DOPA (L-3,4- dihydroxyphenylalanine) as substrate and trypsin (Sigma) as activator following the method described by Ji et al. (2009) with modifications. Homogenate samples of 50 µL were diluted in 100 µL of trypsin solution (0.1% in cacodylate solution) in a 96-well microplate and incubated for 30 min at room temperature. Afterwards, 100 µL L-DOPA solution (0.3% in cacodylate solution) was added. The absorbance was measured every minute during 5 min at 490 nm using a Synergy HT microplate reader. Results were calculated using the Beer–Lambert law using the molar extinction coefficient of the L-DOPA (3700). Results were expressed as units of pro-phenoloxidase mL^{-1} of sample.

Vibrio harveyi was used in the bactericidal activity assay. Exponentially growing bacteria were resuspended in sterile HBSS and adjusted to 3.1×10^9 cfu mL^{-1}. Plating serial dilutions of the suspensions onto TSA-2 plates and counting the number of cfu following incubation at 22 °C confirmed bacterial concentration of the inoculum. Homogenized samples' bactericidal activity was then determined following the method described by Machado et al. (2015). Briefly, 60 µL of homogenized samples were added to a U-shaped 96-well plate. HBSS was added to some wells instead of homogenized samples and served as positive control. To each well, 20 µL of *V. harveyi* (3.1×10^9 cfu mL^{-1}) were added, and the plate was incubated for 2.5 h at 25 °C. To each well, 25 µL of 3-(4, 5 dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide (1 mg mL^{-1}; Sigma) was added and incubated for 10 min at 25 °C to allow the formation of formazan. Plates were then centrifuged at 2000× *g* for 10 min, and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity was expressed as percentage calculated from the difference between the surviving bacteria compared to the number of bacteria from positive controls (100%).

5.2.3.4 Gene expression analysis

Extraction of RNA was performed using the NZY total RNA isolation kit (NZYTech, Lisboa, Portugal) according to the manufacturer's instructions. RNA concentration and purity was analyzed by spectrophotometry using DeNovix DS-11 FX (Wilmington, NC, USA). RNA concentration varied from 123.9 to 2180.7 ng μL^{-1} and 260:280 ratios between 1.99 and 2.17, respectively. The integrity of the RNA samples was verified through a 2% agarose gel. The cDNA was obtained using the NZY first-strand cDNA synthesis kit (NZYTech). This step was used to standardize the concentration of the samples. Reverse transcription was carried out in a Veriti DX 96-well thermal cycler (Applied Biosystems, Waltham, MA, USA), using 4.4 µL of diluted cDNA (20 ng μL^{-1}) mixed with 5 µL of NZYSpeedy qPCR Green Master Mix® (NZYTech) and 0.3 µL (10 µM) of each specific primer in a final volume of 10 µL. Real-time quantitative PCR was performed, in duplicate for each sample, using a CFX384 Touch Real-Time PCR Detection System (Biorad, Hercules, CA, USA). Nine genes were selected and analyzed due to their role in the immune response. Primer efficiency was tested for each gene (Table 3). Cycling conditions were the same between the different genes, consisting of one cycle of 95 °C for 10 min, followed by 40 cycles of 2 steps of 95 °C for 15 s and 62 °C for 1 min, with a final cycle at 95 °C for 1 min, followed by 35 s at 62 °C and ending at 95 °C for 0.5 s. The Pfaffl method (Pfaffl, 2001) was used to perform gene expression analyses, and target genes were normalized using *bactn* and *rpl-8* as housekeeping.

Table 3 Selected genes and specific primers used to evaluate the immune status of whiteleg shrimp (*P. vannamei*) PL at the end of the experimental period.

Gene	Acronym	Efficiency (%)	Annealing Temperature (°C)	Accession n°	Amplicon Length (bp)	Primer Sequence (5′-3′)
Cytoplasmic-type actin 4	*bactn*	83.2	62	MF627841.1	260	F: CACGAGACCACCTACAACTCCATC R: TCCTGCTTGCTGATCCACATCTG
Ribosomal protein L8	*rpl-8*	90	62	DQ316258.1	219	F: AGCCAAGCAAGATGGGTCG R: TGTAACGATAAGGGTCACGGAAG
PvHm117 crustin P	*crus*	81	62	AY488497.1	109	F: GAAACCACCACCAACACCTACTCC R: TCTGTGCGGCCTCTTTACGG
Penaeidin-3a	*pen-3*	86.1	62	Y14926.1	137	F: ATACCCAGGCCACCACCCTT R: TGACAGCAACGCCCTAACC
Hemocyanin	*hmc*	92	62	KY695246.1	124	F: GTCTTAGTGGTTCTTGGGCTTGTC R: GGTCTCCGTCCTGAATGTCTCC
Lysozyme C-like	*lys*	73	62	XM_027352857	82	F: CGGGAAAGGCTATTCTGCCT R: CCAGCACTCTGCCATGTACT
C-type lectin 2-like	*lect*	83	62	DQ858899.2	138	F: GCTTCTGTTGGTGCTGTTGGC R: GTTCCCTTCCCGTATGTGGC
Thioredoxin 1	*trd*	85.3	62	EU499301.1	116	F: TTAACGAGGCTGGAAACA R: AACGACATCGCTCATAGA
Glutathione transferase	*gst*	99	62	AY573381	146	F: AAGATAACGCAGAGCAAGG R: TCGTAGGTGACGGTAAAGA
Glutathione peroxidase	*gpx*	86.6	62	XM_027372127.1	117	F: AGGGACTTCCACCAGATG R: CAACAACTCCCCTTCGGTA
Caspase 3	*casp-3*	93.6	62	KC660103.1	182	F: ACATTTCTGGGCGGAACACC R: GTGACACCCGTGCTTGTACA

5.2.4 Data analysis

Relative growth rate (RGR, % weight day^{-1}) was calculated as follows: RGR = (e^g − 1) × 100, where e = exponential and g = (lnWf − lnWi) × t^{-1}. Wf and Wi correspond to the final and initial weights, respectively. Feed conversion ratio (FCR) was calculated as follows: FCR = (Fi/Wg), where Fi corresponds to feed given (g) and Wg to the mean weight gain (g). Survival was expressed as percentage and calculated as follows: S = (Lf/Li) × 100, where Li and Lf correspond to the initial and final number of post larvae in the tanks, respectively. Differences in growth performance, FCR, survival, oxidative stress, immune condition, and gene expression between dietary treatments were evaluated using one-way ANOVAs, followed by Tukey multiple comparison tests. Kruskal–Wallis one way analysis of variance tests followed by Wilcoxon pairwise comparison tests were used when data did not comply with the one-way ANOVA's assumptions. Results were expressed as means ± standard deviation (SD). In results expressed as percentage, an arcsine transformation was performed prior to any statistical test: T = ASIN (SQRT (value/100)). The significance level considered was $p < 0.05$ for all tests performed.

5.3 Results

5.3.1 Growth performance and survival

No significant differences in growth performance and survival were observed among dietary treatments. Final wet weight averaged around 100 mg, RGR values 15% day^{-1}, FCR was close to 1, and survival ranged between 86 to 88% for all treatments (Table 4).

Table 4 Initial and final weight, relative growth rate (RGR), feed conversation ratio (FCR), and survival of whiteleg shrimp (*P. vannamei*) PL during the experimental period.

	NC	PC	T + M	BG
Initial weight (mg)	8.8 ± 0.0			
Final weight (mg)	110.8 ± 19.3	110.8 ± 18.4	114.0 ± 9.5	94.4 ± 9.2
RGR (% day^{-1})	15.0 ± 1.1	15.0 ± 1.1	15.3 ± 0.5	14.0 ± 0.6
FCR	0.9 ± 0.0	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
Survival (%)	87.0 ± 6.6	86.2 ± 7.6	85.5 ± 6.1	87.5 ± 5.0

Results expressed as mean ± standard deviation. For initial weight, *n* = 60 observational units; for final weight, FCR, RGR, and survival, *n* = 3 experimental units.

5.3.2 Oxidative stress and immune status-related biomarkers

Regarding the oxidative stress parameters measured, CAT levels were similar, with no significant differences being detected across treatments; LPO levels were significantly lower in shrimp PL fed the BG dietary treatment than those fed the PC diet, with no significant differences between the remaining treatments; tGSH levels were significantly higher in shrimp PL fed the BG treatment than in their counterparts fed the NC diet, with no significant differences between the remaining treatments. As for the immune condition, no significant differences between treatments were observed regarding the parameters measured (Table 5).

Table 5 Catalase (CAT), lipid peroxidation (LPO), total glutathione (tGSH), lysozyme, pro-phenoloxidase, and bactericidal activity levels in whiteleg shrimp (*P. vannamei*) PL fed the experimental diets for 18 days.

	NC	PC	T + M	BG	*p*-value
CAT (U mg^{-1} protein)	22.9 ± 8.0	22.4 ± 6.3	28.9 ± 19.3	21.4 ± 7.2	0.675
LPO (nmol g wt^{-1})	14.0 ± 2.2 ^{ab}	15.6 ± 3.1 ^{a}	14.6 ± 2.6 ^{ab}	12.7 ± 2.1 ^{b}	0.039
tGSH (nmol mg $protein^{-1}$)	4.7 ± 0.9 ^{a}	5.0 ± 0.7 ^{ab}	5.0 ± 0.8 ^{ab}	5.7 ± 1.1 ^{b}	0.018
Lysozyme (µg mg $protein^{-1}$)	1.2 ± 0.5	1.5 ± 0.6	1.1 ± 0.3	1.2 ± 0.4	0.165
Pro-phenoloxidase ($\times 10^{-3}$ U mL^{-1})	12.1 ± 6.8	14.0 ± 9.3	12.4 ± 3.8	13.0 ± 6.5	0.854
Bactericidal activity (%)	12.6 ± 6.9	12.9 ± 8.1	14.6 ± 11.9	14.5 ± 7.8	0.551

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a one-way ANOVA. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post hoc Tukey multiple comparison test.

5.3.3 Gene expression analysis

The normalized relative mRNA expression of the PvHm117 crustin P gene decreased significantly in shrimp PL fed the NC diet compared to those fed the T + M and BG dietary treatments. Similarly, the penaeidin-3a mRNA expression level decreased significantly in shrimp PL fed the NC diet compared to their counterparts fed the PC and BG dietary treatments. Hemocyanin transcripts increased significantly in shrimp PL fed the NC diet compared to PL fed the T + M dietary treatment. As for the normalized relative mRNA expression of the remaining genes, no significant differences between treatments were observed (Table 6).

Table 6 Relative expression to housekeeping (*bactn* and *rpl-8*) of target immune related genes of whiteleg shrimp (*P. vannamei*) PL fed the experimental diets for 18 days.

Gene	Acronym	Relative Expression				*p*-value
		NC	PC	T + M	BG	
PvHm117 crustin P	*crus*	0.6 ± 0.2 ^{a}	1.1 ± 0.5 ^{ab}	1.3 ± 0.4 ^{b}	1.5 ± 0.8 ^{b}	0.003
Penaeidin-3a	*pen-3*	0.4 ± 0.3 ^{a}	1.1 ± 0.4 ^{b}	0.7 ± 0.5 ^{ab}	1.2 ± 0.8 ^{b}	0.001
Hemocyanin	*hmc*	1.1 ± 0.7 ^{b}	1.9 ± 2.4 ^{ab}	0.2 ± 0.2 ^{a}	0.7 ± 0.7 ^{ab}	0.029
Lysozyme C-like	*lys*	0.7 ± 0.4	1.1 ± 0.5	1.3 ± 0.6	1.3 ± 0.9	0.212
C-type lectin 2-like	*lect*	0.7 ± 0.4	1.1 ± 0.5	1.3 ± 0.7	1.3 ± 1.0	0.236
Thioredoxin 1	*trd*	1.0 ± 0.5	1.0 ± 0.2	1.0 ± 0.3	0.90 ± 0.3	0.819
Glutathione transferase	*gst*	0.9 ± 0.5	0.9 ± 0.4	0.6 ± 0.3	0.4 ± 0.1	0.218
Glutathione peroxidase	*gpx*	1.0 ± 0.4	1.1 ± 0.4	0.9 ± 0.1	1.0 ± 0.3	0.622
Caspase 3	*casp-3*	0.6 ± 0.3	0.8 ± 0.4	0.6 ± 0.5	1.9 ± 2.5	0.410

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a one-Way ANOVA. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post hoc Tukey multiple comparison test.

5.4 Discussion

This study aimed at evaluating the potential health-promoting effects of including several dietary supplements in inert microdiets for whiteleg shrimp PL. Vitamin C and E, methionine, taurine, and *β*-glucans were selected for this purpose since their potential ability to enhance the health status of whiteleg shrimp in the initial stages of development is still promising but yet to be experimentally validated. A control diet formulated to fulfil the nutritional requirements of whiteleg shrimp PL was used as positive control, and the remaining experimental diets were based on it, differing only in the reduction or addition of the previously mentioned nutrients. In overall, the formulation changes in the diets did not compromise their adequacy, as good growth performances and survival results were obtained in all experimental treatments, also revealing that good zootechnical conditions were maintained during the trial. Growth results were similar to those reported by Wang et al. when using graded levels of *Schizochytrium* meal (2017a) and as a replacement of fish oil (2017b) in practical diets for whiteleg shrimp PL, but survival results were considerably inferior in those studies (40.3–44.5% and 42.7–45.6%, respectively) than in the current trial.

Increasing the vitamin C and E supplementation levels in the PC dietary treatment did not produce any changes in growth performance and survival when compared with the NC, suggesting that the levels of these vitamins present in the NC diet still allowed the shrimp post larvae to maintain an adequate development. Like other vitamins, vitamin C and E are essential nutrients, as animals are unable to synthesize sufficient amounts to meet their physiological needs, and a deficient supply in the diet often results in poor growth, possibly leading to severe health issues and even compromising survival (Dawood et al., 2018; NRC, 2011). Additionally, no changes were verified in the activity levels among most of the immune and antioxidant parameters measured in this study when increasing the levels of these vitamins in the diets. Accordingly, other studies reported thresholds in inclusion levels for these vitamins in diets for whiteleg shrimp juveniles at which their beneficial effects did not increase after a certain incorporation percentage (Liu et al., 2007; Wu et al., 2016). The only significant dissimilarity detected was the lower relative expression of *pen-3* in shrimp fed the NC diet compared to those fed the PC diet, suggesting that lower levels of these vitamins may impact the shrimp immune status. Penaeidins, a key group of antimicrobial peptides in penaeid shrimp, have antibacterial and antifungal activities, which are particularly effective against Gram+ bacteria and filamentous fungi (Destoumieux et al., 2000; Muñoz et al., 2003). These findings indicate that higher supplementation levels of vitamins C and E in microdiets for whiteleg shrimp PL did not directly enhance growth and survival in the current study but may have improved their robustness. Although not confirmed in the current experiment, this may be an indication that shrimp may have a higher survival capacity in the long term and particularly in a potentially challenging

husbandry situation. Therefore, inert diets with adequate levels of vitamins C and E can be vital during critical stages of production, particularly in farms where a nursery system is employed (intermediate step between the early PL stage and the grow out phase), in which PL are kept at extremely high stocking densities that can induce stress and vulnerability to opportunistic pathogens (Mishra et al., 2008).

The supplementation of methionine to balance the nutritional profiles of aquafeeds rich in plant-based proteins has become a common practice (Ji et al., 2021; Qiu et al., 2018). Traditionally, methionine supply was ensured by fish meal, but continuous efforts are underway to reduce the industry dependence on this ingredient and replace it by plant-based proteins, where methionine and lysine are generally low in the amino acid profile. It has been shown by several authors that when dietary requirements are not met, generally, when low-fish-meal diets are concomitantly used, growth performances and survival of whiteleg shrimp can be affected (Façanha et al., 2016; Guo et al., 2020; Ji et al., 2021; Wang et al., 2019; Xie et al., 2018). Additionally, methionine has a recognized role in the immune system and has recently been used to improve the antioxidant capacity, innate immune response, and/or disease resistance of whiteleg shrimp juveniles (Ji et al., 2021; Machado et al. 2022; Wang et al., 2021). Besides that, methionine is also a precursor for taurine. The supplementation of this nutrient in diets for whiteleg shrimp is also recommended since it can provide beneficial effects on their growth and immune response (To et al., 2021; Yue et al., 2013). Yet, the evaluation of the supplementation of both molecules in diets for the initial developmental stages of shrimp is still necessary. In this study, whiteleg shrimp PL fed with the T + M diet showed similar growth performances, survival, oxidative status, and immune condition to those fed the PC dietary treatment, suggesting that the ingredient formulation of the control diet was capable of covering the shrimp PL requirements for taurine and methionine, and no extra benefits were obtained through the supplementation of these amino acids. These results can probably be explained by the fact that the PC was a high-quality diet with considerable levels of protein of marine origin. However, shrimp PL fed the T + M diet showed significantly higher relative expressions of *crus* and significantly lower *hmc* transcripts when compared with those fed the NC dietary treatment. Both PvHm117 crustin P and hemocyanin are associated with important broad-spectrum antimicrobial peptides involved in the first line of the shrimp defense (Fajardo et al., 2021; Vargas-Albores et al., 2004; Yang et al., 2018). These results could be considered contradictory, as it would be expected that variations in the expressions of both genes would follow the same trend. Still, it is important to bear in mind that hemocyanin is a multifunctional protein involved in several physiological processes beyond innate immunity, such as oxygen transport, protein storage, molt cycle, exoskeleton formation, and osmoregulation (Zhang et al., 2009; Zheng et al., 2016). Concomitantly,

taurine is also one of the main organic osmolytes in osmoregulation for decapods (Chen et al., 1994; Li et al., 2015), and it has been shown that increases in dietary taurine inclusion levels increases the molecule contents in different tissues and hemolymph of whiteleg shrimp (To et al., 2021). Therefore, the hemocyanin levels needed to maintain osmolality were probably lower in shrimp fed the T + M diet, which may have caused a downregulation of the *hmc* gene. Still, it should be noted that the analysis only focused on measuring mRNA transcripts of *hmc* and not the taurine molecule levels. Therefore, to better understand these interactions and clarify if the supplementation of taurine and methionine in diets for whiteleg shrimp PL is beneficial when lower vitamin C and E inclusion levels are used, further studies should be conducted.

The Inclusion of *β*-glucans in the diets did not significantly affect the shrimp PL growth performance and survival. Still, shrimp PL fed the BG diet tended to grow less and achieved final weights around 15% lower than those fed the PC, although it was not supported by the statistical analysis. Nonetheless, lipid peroxidation levels dropped significantly in shrimp PL fed the BG dietary treatment compared to those fed the PC diet, suggesting that *β*-glucans improved the antioxidant mechanisms of the animals. In fact, the immunostimulatory and antioxidant-boosting properties of *β*-glucans as aquafeed additives have been reported for several species, as recently reviewed by Pogue et al. (2021). These can be tremendously valuable in the larval/PL stages, where shrimp undergo extremely fast development, as accelerated growth is likely to produce excess reactive oxygen species that can result in oxidative stress, damaging key physiological structures (Monaghan et al., 2009). Although there are reports of the enhancement of whiteleg shrimp disease resistance through the dietary supplementation of *β*-glucans (Bai et al., 2014; Burgents et al., 2004; Li et al., 2019), in the present study, no significant improvements in immune condition were observed in the shrimp PL fed the BG diet compared with those fed the PC diet. Bai et al. (2010) suggested that discontinuous feeding, changing between a basal diet and one with the inclusion of *β*-glucans, is the most suitable strategy to enhance the immunity of whiteleg shrimp, as continuous feeding for long periods of time with the supplemented diet can cause immune fatigue, mitigating the beneficial effects provided in the short term. Considering this hypothesis, the BG diet potential to improve the whiteleg shrimp PL immunity could have been clearer if a different feeding strategy had been employed. When compared with the NC diet, PL fed with BG showed significant increases in tGSH levels as well as in the relative expression of *crus* and *pen-3*. This suggests that the *β*-glucans supplementation coupled with higher levels of vitamin C and E can boost the antioxidant capacity and immune status of whiteleg shrimp PL. In fact, Wu et al. (2016) proposed that there is an interaction between *β*-glucans and vitamin C that is capable of increasing the nonspecific immune response of the whiteleg shrimp. The results obtained in that study corroborate this hypothesis, as the

addition of *β*-glucans to the PC diet amplified the differences in the shrimp's immune condition and antioxidant capacity relative to the NC diet.

5.5 Conclusions

In conclusion, the results obtained in this study suggest that although no improvements in growth performances and survival were observed at the end of the experimental period, all dietary additives tested have the potential to add value to inert microdiets for whiteleg shrimp PL. Benefits to the antioxidant capacity and robustness of the shrimp PL were clearer when the vitamin C and E levels were higher than those used in the NC, similar to those used in the PC. However, the control diet can be considered a premium option, and it should be expected that the positive effects provided by these supplements are augmented when incorporated into more economical alternatives. Amongst the additives tested, the inclusion of *β*-glucans in the diets seems to be the most promising, as it reduced lipid peroxidation in the shrimp PL even when compared to a high-quality control diet. When compared to the NC, the interaction between the supplementation of *β*-glucans and higher levels of vitamins C and E also seems beneficial to the antioxidant capacity of whiteleg shrimp PL.

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Declarations

All activities were undertaken within the clear boundaries of national and EU legal frameworks directed by qualified scientists/technicians and conducted according to the European guidelines on the protection of animals used for scientific purposes (Directive

2010/63/UE of the European Parliament and on the European Union Council) and under strict monitoring and control of DGAV—(Direção Geral de Alimentação e Veterinária), Animal Welfare Division, which is the competent authority responsible for implementing the legislation on the "protection of animals used for scientific purposes".

WP and LC worked for Sparos Lda during this study. The remaining authors declare that they have no conflicts of interest. The views expressed in this work are the sole responsibility of the authors and do not necessarily reflect the views of any third party.

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Chapter 6

General Discussion

6.1 Dietary protein requirements of whiteleg shrimp PL in clear water recirculating aquaculture systems are higher than in traditional production methods

The results obtained in **Chapter 2** of this Thesis indicate that protein levels around 47 % are suitable for growth and survival of whiteleg shrimp PL reared in clear water RAS. Moreover, increasing protein inclusion levels up to 54 % seems to enhance the PL antioxidant status, which can be particularly advantageous in this stage of production, as the accelerated development whiteleg shrimp undergo in the larval/PL stages may produce excess ROS and result in oxidative damage (Monaghan et al., 2009). These high protein requirements can probably be explained by the fact that RAS water is devoid of natural productivity, known to be a particularly important supplemental feed source for the shrimp in traditional production methods. In this study, when shrimp PL were fed a diet containing 34 % of protein, which is equal or above of what has been suggested by several authors as the optimal level when PL are reared in pond or bioflocs systems (Jatobá et al., 2014; Martinez-Cordova et al., 2002; Olier et al., 2020; Pinho and Emerenciano, 2021; Yun et al., 2016), their growth performance, survival and/or antioxidant status was impaired when compared to higher protein diets. On the other hand, a low protein diet seems to have stimulated the shrimp PL immune system when compared to diets containing 44 %, 49 % and 54 % protein levels, which may be related to the increased levels of wheat meal (and possibly antinutritional factors that may have caused an innate immune response in the PL) when compared to higher protein diets. Parallelly, fish and algae oil quantities were also increased in the 34 % protein diet, which may have positively influenced the shrimp PL immune mechanisms, as both ingredients have been shown to enhance the health condition of whiteleg shrimp PL (Nonwachai et al., 2010; Xie et al., 2019). A diet containing 63 % of protein also seemed to equally modulate the shrimp PL health condition, yet it is unclear what were the mechanisms underlying this response.

Therefore, results obtained in **Chapter 2** indicate that a minimum of 47 % protein should be included in microdiets for whiteleg shrimp PL produced in clear water RAS but levels up to 54 % may be beneficial for the PL antioxidant status. Microdiets developed in **Chapter 3, Chapter 4** and **Chapter 5** were formulated in consonance with these premises.

6.2 Marine ingredients and fat levels used in inert microdiets for whiteleg shrimp PL impact survival, health status and feed conversion

In **Chapter 3**, shrimp PL were fed five experimental microdiets: a control diet (CTRL) formulated to contain 54 % of protein and 14 % lipids utilizing a blend of fish and squid meals as main ingredients; three diets (FISH, SQUID and KRILL) formulated to contain the same levels of protein and lipids but with fish, squid and krill meals, respectively, as main ingredients; and an additional fish meal based diet (FISHHF) formulated to contain a higher

lipid content (16 %) through higher inclusion levels of fish oil and reduced wheat meal. Growth performance was similar between dietary treatments, but results obtained showed that diets utilizing a blend of fish and squid meals, or krill meal as main ingredients improved PL survival and health status when compared to the remaining diets. Shrimp PL fed a krill meal-based diet showed higher lipid peroxidation (LPO) levels than those fed a diet containing a mixture of fish and squid meals, probably due to the lower activity levels of the antioxidant enzyme CAT observed for this dietary treatment. Nevertheless, this was not reflected in the survival values, similar in both treatments at the end of the trial.

Furthermore, the results obtained demonstrate that a lipid level of around 14% seems to be preferable over 16 % when tailoring diets for whiteleg shrimp PL reared in clear water RAS and at this stage of development. When whiteleg shrimp PL were fed the higher lipid diet, an apparent activation of the antioxidant defenses and immune mechanisms occurred. This may signal a more stressful condition of PL, what seems corroborated by the lower survival values observed for this treatment. Additionally, FCR values were higher in this dietary treatment than in those fed a krill meal-based diet. Increasing dietary lipid levels did not produce any changes in the PL whole body lipid total contents but slightly higher n-3 PUFA and lower n-6 PUFA levels were measured, probably reflecting the higher fish oil contents.

Thus, results obtained in **Chapter 3** suggest that a blend of fish and squid meals or krill meal as main ingredients and a lipid level of around 14% should be considered when tailoring diets for whiteleg shrimp PL reared in clear water RAS and at this stage of development.

6.3 Krill meal enhances whiteleg shrimp PL health status but is preferable as a secondary marine ingredient

Given the good results obtained in **Chapter 3** with a krill meal-based diet, the potential of this ingredient to be included in aquafeeds for whiteleg shrimp PL produced in clear water RAS was further evaluated in **Chapter 4**. In this study, the complete exclusion of krill meal from the formulation did not produce any change in the whiteleg shrimp PL growth performance or survival when compared to the remaining experimental diets tested which had krill meal as first or secondary marine ingredient. However, shrimp PL fed a diet with no krill meal apparently had their health status hindered when compared to the remaining experimental diets. In fact, when PL were challenged with *Vibrio harveyi*, those fed a diet where krill meal was excluded, experienced significantly higher mortalities than those fed a diet containing krill meal as secondary ingredient. Similarly, in **Chapter 3** a diet containing krill meal as main ingredient seemingly enhanced the health status of whiteleg shrimp PL when compared to two fish meal-based diets and a squid meal-based diet. Although no

studies were found supporting this hypothesis, krill meal is high in astaxanthin, which has been shown to improve whiteleg shrimp growth performances, survival, and health status (Chuchird et al., 2015; Niu et al., 2009; Zhang et al., 2013), and might have been the intrinsic cause for the modulation of the antioxidant and immune mechanisms of whiteleg shrimp PL fed diets containing krill meal in this Thesis.

Consequently, results obtained in **Chapter 4** suggest that using fish meal as first ingredient may be preferable over krill meal as the latter is usually more expensive and does not seem to enhance growth performances. However, its inclusion in moderate amounts is advisable, as it enhances whiteleg shrimp PL health status.

6.4 Soy protein concentrate can reduce costs of microdiets for whiteleg shrimp PL produced in clear water recirculating aquaculture systems

In **Chapter 4**, the potential of using soy protein concentrate (SPC) to reduce the preponderance of marine ingredients in the experimental microdiets' formulations was evaluated, as it has been shown in several studies that it can partially/fully replace fish meal in aquafeeds for whiteleg shrimp juveniles (Bauer et al., 2012; Chen et al., 2017; Guo et al., 2020; Ray et al., 2020; Sá et al., 2013; Xie et al., 2016; Zhu et al., 2021). Even so, knowledge on the potential of using SPC in microdiets for whiteleg shrimp PL is limited. An experimental diet based of CTRL used in **Chapter 3**, was formulated keeping the same nutritional premises but employing a more economical approach. In CTRL, the main protein sources were a blend of fish meal and squid meal, while in this study, fish meal and SPC were used, which allowed for a significant reduction in the overall cost of the diet. The inclusion of this ingredient was not detrimental to the PL development, survival and health status, as similar results were obtained with a diet where SPC was excluded. Moreover, the exclusion of SPC from the formulation also seems to have been detrimental to the capacity of the PL to resist infection by *V. harveyi*, as significantly higher mortalities were observed for whiteleg shrimp PL fed this diet. It has been shown that the inclusion of soybean derived ingredients can enhance the whiteleg shrimp innate immune responses and/or antioxidant mechanisms, when fermented, hydrolyzed, or processed into a protein concentrate (Lin and Chen, 2022; Liu et al., 2019; Ray et al., 2020), as antigenic factors associated with soybean meal like trypsin inhibitors, lectins, phytic acid, soybean agglutinin and antigen proteins are significantly reduced in the degradation process and may increase the bioavailability of immunomodulatory active peptides (Singh et al., 2014).

Hence, results obtained in this trial corroborate that SPC can be used to replace more expensive marine ingredients in the formulation of diets for whiteleg shrimp PL reared in clear water RAS with no adverse effects on growth performances or survival, while possibly improving their health status and increasing their resistance to pathogens.

6.5 *β*-glucans improve antioxidant mechanisms of whiteleg shrimp PL while Vitamins C and E, methionine and taurine have modulatory effects on their health status

In **Chapter 5**, the potential health-promoting effects of several dietary supplements in inert microdiets for whiteleg shrimp PL were evaluated. Vitamin C and E, methionine, taurine, and *β*-glucans were selected for this purpose since their potential ability to enhance the health status of whiteleg shrimp in the initial stages of development is promising but yet to be experimentally validated. These additives had no effect on the shrimp PL growth performances or survival, under the optimal clear-water conditions used in this Thesis, but modulations on their antioxidant and immune mechanisms were observed. When lowering the inclusion levels of vitamins C and E, a downregulation of the gene *pen-3* occurred, suggesting these vitamins can impact the shrimp PL immune status, as Penaeidins are a key group of antimicrobial peptides in penaeid shrimp that have antibacterial and antifungal activities (Destoumieux et al., 2000; Muñoz et al., 2003). These results confirm the importance of maintaining adequate levels of vitamins C and E in microdiets for whiteleg shrimp PL.

The inclusion of taurine and methionine in the diets did not produce any change in the parameters analyzed if adequate levels of vitamins C and E were supplemented. However, when the levels of vitamins C and E were lowered, the supplementation with taurine and methionine resulted in an upregulation of gene *crus* and a downregulation of the hemocyanin *hmc* gene, both associated with the shrimp innate immune response. Still, hemocyanin is multifunctional protein involved in several physiological processes beyond innate immunity, including osmoregulation (Zhang et al., 2009; Zheng et al., 2016). Concomitantly, taurine is also one of the main organic osmolytes in osmoregulation for decapods (Chen et al., 1994; Li et al., 2015), which suggests that increasing levels of this amino acid in the diets may have caused a downregulation of the *hmc* gene in the shrimp PL, as hemocyanin levels needed to maintain osmolality were probably lower.

Lipid peroxidation levels dropped significantly with the inclusion of *β*-glucans in the diets, when compared to the Control, suggesting this supplement improved the antioxidant mechanisms of the animals. In fact, the immunostimulatory and antioxidant-boosting properties of *β*-glucans as aquafeed additives have been reported for several species (Pogue et al., 2021), and can be tremendously valuable in the shrimp larval/PL stages, where they undergo an extremely fast development that can result in oxidative stress. Still, no changes in the immune condition were observed in this study. It has been suggested that discontinuous feeding, changing between a basal diet and one with the inclusion of *β*-glucans, is the most suitable strategy to enhance the immunity of whiteleg shrimp, as continuous feeding for long periods of time with the supplemented diet can cause immune fatigue, mitigating the beneficial effects provided in the short term (Bai et al., 2010). When

the levels of vitamins C and E were reduced, *β*-glucans supplementation resulted in the upregulation of genes *crus* and *pen-3*, as well as increases in tGSH activity in the PL. These results suggest that the *β*-glucans supplementation when coupled with higher levels of vitamin C and E can boost the antioxidant capacity and immune status of whiteleg shrimp PL.

In conclusion, the results obtained in **Chapter 5** indicate all dietary additives tested have the potential to add value to inert microdiets for whiteleg shrimp PL produced in clear water RAS. The inclusion of *β*-glucans in the diets seems to be the most promising, as it reduced lipid peroxidation in the shrimp PL even when compared to a high-quality Control diet.

6.6 Final conclusions

This Thesis highlights the following conclusions:

- A minimum of 47 % protein should be considered when tailoring microdiets for whiteleg shrimp PL produced in clear water RAS to ensure optimal growth performances and survival, while increasing levels up to 54 % may be beneficial for their antioxidant status (**Chapter 2**).
- Dietary lipid levels of around 14 % are more suitable than 16 % for whiteleg shrimp PL reared in RAS (**Chapter 3**). Increasing dietary lipid levels may result in lower shrimp PL survival and a tendency for higher FCR values.
- A blend of fish and squid meals seems to be preferable over using uniquely fish or squid meals as main marine ingredient in microdiets for whiteleg shrimp PL (**Chapter 3**). Utilizing a mixture of these ingredients seems to positively modulate the shrimp PL health status and enhance their survival.
- Krill meal enhances the whiteleg shrimp PL health status (**Chapter 3** and **4**). However, it should be used as a secondary ingredient of marine origin as a krill meal based diet can produce inferior growth performances than a fish meal based diet (**Chapter 4**).
- Soy protein concentrate can be used to replace more expensive marine ingredients in microdiets for whiteleg shrimp PL (**Chapter 4**). The inclusion of this ingredient has no adverse effects on PL growth performances and survival, while it may improve their health status and increase their resistance to pathogens.
- Vitamins C and E, methionine and taurine have modulatory effects on the PL health status (**Chapter 5**). Changes in the relative expression of antioxidant and immunity related genes may be observed when levels of these additives are adjusted in the microdiets.
- β-glucans boost the antioxidant mechanisms of whiteleg shrimp PL (**Chapter 5**). The inclusion of this supplement decreases lipid peroxidation in the PL.

6.7 Future research

The present Thesis aimed to contribute to the development of a microdiet for whiteleg shrimp PL, specially tailored for production in clear water RAS, but simultaneously with potential to be used in all production methods as a functional diet capable of improving the health status of shrimp PL. Although the performance of the developed microdiets was very satisfactory, they can be considered premium options compared to some of the commercially available diets for whiteleg shrimp PL. Further studies should prioritize evaluating the effectiveness of including alternatives to some of the more expensive or less sustainable ingredients. As demonstrated in this Thesis, it is possible to replace some ingredients of marine origin while maintaining the adequacy of the diets and reducing its overall costs. Doing so, may decrease the preponderance of feeds in the overall expenditures of production and contribute to the financial success and sustainability of the whiteleg shrimp aquaculture industry.

Assessing the health modulatory effects of additives, like the ones tested in this Thesis, is only the first step to understanding their potential to be included in a functional microdiet. Although results obtained in this Thesis are promising, future experiments should also address on clarifying the benefits of the tested, and other, additives for whiteleg shrimp PL in challenging environmental conditions and/or when subject to pathogenic infection.

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