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Development and stability analysis of a lipid-based astaxanthin nanoemulsion, its application and effect on the immune response and survival of the Pacific white shrimp

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This study aimed to develop and characterize a nanoemulsion containing lipoencapsulated astaxanthin (AST) from *Haematococcus pluvialis* and evaluate its effects on the immune performance and survival of *Penaeus vannamei* shrimp when added to feed. The nanoemulsion was prepared by high-pressure homogenization using vegetable oils, AST oleoresin, and surfactants. It was characterized for size, polydispersity, Zeta potential, pH, morphology, and physicochemical stability for 90 days at 4 °C, 25 °C, 30 °C, and 54 °C. The fatty acid content was determined by gas chromatography, while the AST content was determined by ultra-high-performance liquid chromatography (UHPLC). The *in vitro* antioxidant activity was verified by the DPPH method. The profile of vegetable oils showed significant concentrations of PUFAs (77.18% and 78.74%, respectively). The AST concentration was 1.55 mg g^{-1} of nanoemulsion. The colloidal dispersions showed an average particle size 319.6 ± 0.5 nm, a polydispersity index (PDI) of 0.290 ± 0.0 and spherical shape. The formulation showed a negative Zeta potential (–44 ± 0.40 mV). The antioxidant activity occurred for 90 days, but varied over time ($p < 0.001$). The nanoemulsion was stable during 90 days of storage at 25 °C. Six diets were tested: 10, 30, 50, 70 mg AST/kg^{-1} of feed, a negative control diet (NC) (70 mg without AST/kg^{-1}), and a control diet. After 45 days of cultivation, hemolymph samples were collected for analysis of immunological parameters [total hemocyte count (THC)], protein content (P), phenoloxidase activity (PO), and agglutination capacity (Agl). *P. vannamei* survival was evaluated after a 96 h challenge with *Vibrio parahaemolyticus* (4×10^{8} CFU mL^{-1}). There was no

significant difference for THC and Agl ($p < 0.05$). P levels were higher in the intermediate treatments (30–70 mg AST kg^{-1}) ($p = 0.0454$). PO showed higher concentrations in the treatments with 70 and 10 mg AST kg^{-1} ($p = 0.0005$). In the *V.* challenge, there was no significant difference ($p < 0.05$) however, a promising trend of protection conferred by the developed nanoemulsion was observed. The 70 mg AST kg^{-1} nanoemulsion shows strong potential for use in animal feed, offering a sustainable option for shrimp farming by enhancing serum protein levels and phenoloxidase activity in *P. vannamei*.

KEYWORDS

antioxidant, aquaculture, nanoemulsion, Penaeus vannamei, vegetable oils, *vibrio*

1 Introduction

The production of the Pacific white shrimp (*Penaeus vannamei*) has increased significantly in recent years, due to the high global demand for seafood and the development of more efficient and sustainable cultivation techniques (N'Souvi and Sun, 2025). However, shrimp farming also faces challenges, such as food safety issues related to the inappropriate use of antibiotics, which generate residues in the final product and pose a risk to public health (Jasmin et al., 2020). Additionally, the occurrence of diseases due to high stocking density in the ponds and the lack of biosecurity favoring the spread of viruses and bacteria, e.g., White Spot syndrome virus (WSSV) and *V. parahaemolyticus*, resulting in large economic losses (Shinn et al., 2018). *V. parahaemolyticus* is a pathogenic bacterium to shrimps, with *P. vannamei* being a species particularly vulnerable under captive cultivation conditions, given the high culture density (Gustilatov et al., 2022). Infection by *V. parahaemolyticus* has caused significant economic losses due to the emergence of early mortality syndrome (EMS), also known as acute hepatopancreatic necrosis disease (AHPND). Controlling pathogenic bacteria is difficult in intensive *P. vannamei* cultivation systems, with reports of antibiotic resistance development by some species (Kewcharoen and Srisapoome, 2019). Alternatively, the use of natural products has been investigated as a way to improve animal health and replace antibiotics in farming, such as the use of carotenoid pigments, due to their antioxidant and anti-inflammatory properties, improving the immune response and reducing the use of antibiotics (Li et al., 2021; Li et al., 2022a; Kumar et al., 2022). Among these pigments, astaxanthin (AST), an oxycarotenoid with anti-inflammatory and immunostimulant properties commonly found in the microalga *Haematococcus pluvialis,* stands out (Yamada et al., 1990). Crustaceans and fishes have a limited capacity to convert carotenoids and can not synthesize AST. However, through feeding, they are able to accumulate this pigment in their tissues (Miyakawa, 2021). Studies have shown that AST supplementation in feed improves the immune response of *P. vannamei*, including increased phagocytic and phenoloxidase activities, besides gene expression related to the immune response, resulting in greater resistance to *V. parahaemolyticus* infection (Wang et al., 2015; Mansour et al., 2022; Lin et al., 2023).

Nanotechnology has established itself as an innovative approach for nutritional and functional improvement in aquaculture, offering solutions capable of overcoming limitations associated with the low solubility, instability, and reduced bioavailability of bioactive compounds used in the feeding of aquatic organisms (Fath El-Bab et al., 2024). Among nanotechnology platforms, nanoemulsions have gained prominence. Nanoemulsions are colloidal particle systems with emulsions of small droplets, ranging from 10 to 200 nm, and stable against sedimentation. They are produced using oil and an aqueous phase stabilized with the use of an emulsifier. Nanoemulsion traits, e.g., high kinetic stability combined with the nanometer size of the droplets, increases the surface area and improves the efficiency of encapsulation and delivery of lipophilic compounds (Abdol Wahab et al., 2022). These properties provide protection against oxidative degradation, better dispersion in food matrices, and greater absorption in the digestive tract, making nanoemulsions superior in their performance to conventional emulsions (Jeevanandam et al., 2016). Lipid-based nanocarriers are primarily indicated for the administration of apolar compounds such as AST (Martínez-Álvarez et al., 2020). Lipid components have been used in the preparation of nanoparticles due to their biodegradability, non-toxicity, and safety (Chauhan et al., 2020). In aquaculture, such systems have shown potential to improve growth, strengthen the immune response, and enhance the antioxidant capacity of cultivated species, contributing to more functional diets aligned with the sustainability demands of the sector (Fath El-Bab et al., 2024). Studies in penaeid shrimp have shown that nutrients and phytoactives delivered via nanoemulsions can increase zootechnical performance, antioxidant enzyme activity (SOD, CAT, TAC), humoral markers (lysozyme, PO, total protein), and pathogen resistance when compared to the non-encapsulated form. Ghaffarizadeh et al. (2022) found that dietary supplementation with selenium nanoemulsion increased growth, antioxidant capacity, and transcription of immunity-related genes in juvenile Pacific white shrimps. A study with *P. vannamei* fed with curcumin-loaded olive oil nanoemulsion showed gains in growth, redox state, and immune response (Fath El-Bab et al., 2024).

In this context, AST has been widely used in aquaculture due to its positive effects on the immunity, oxidative metabolism, and zootechnical performance of crustaceans and fishes (Elbahnaswy and Elshopakey, 2024). However, its traditional application faces significant limitations due to its low water solubility, high sensitivity to oxidation, and limited bioavailability when incorporated into conventional diets. Nanoemulsification emerges as an effective strategy to overcome these barriers, increasing the physicochemical stability of AST, reducing its oxidative degradation, and enhancing its absorption in the digestive tract (Martínez-Álvarez et al., 2020).

For the assembly of AST nanoemulsion, PUFA-rich vegetable oils, e.g., flaxseed and safflower oils, are relevant, because in shrimp farming, PUFAs are vital for growth, reproduction, immunity, and the quality of the final product reaching the consumer (Li et al., 2022b). Flaxseed oil is rich in alpha-linolenic acid (C18:3 n-3, ALA), while safflower oil is mainly rich in linoleic acid (C18:2 n-6), at approximately 70%, and oleic acid (C18:1 n-9) (Amirkhiz et al., 2021; Sabaghnia et al., 2024). n-3 PUFAs play crucial roles in animal nutrition, energy metabolism, anti-inflammatory responses, and immunomodulation (Xu et al., 2020). Crustaceans require a combination of n-3 and n-6 PUFAs in their diet. However, the nutritional requirement for essential fatty acids is influenced by the biosynthesis capacity and bioconversion abilities of the crustacean species (Zheng et al., 2024). Studies have shown that crustaceans have a limited capacity to resynthesize n-6 and n-3 fatty acid families. They also have limitations in the elongation and desaturation pathways of short-chain PUFAs, which convert them into highly unsaturated fatty acids (Lim et al., 1997; González-Félix et al., 2002; Zheng et al., 2024). Indeed, the bioconversion rate appears to be insufficient to meet the metabolic needs in crustaceans, and therefore, such nutrients must be present in their diets (Li et al., 2024).

In this scenario, this study presents a novel astaxanthin-based nanoemulsion formulated with a dual functional lipid matrix composed of flaxseed and safflower oils, selected for their high contents of n-3 and n-6 PUFAs and their potential synergistic interaction with AST. Unlike previously reported astaxanthin nanoformulations, which primarily focus on physicochemical stability or bioavailability using neutral oils or polymeric carriers, the nanoemulsion developed herein was specifically designed to enhance immunological performance in *P. vannamei*. Thus, we report, for the first time, the development of an AST nanoderivative at an industrial scale for use in aquaculture.

2 Materials and methods

2.1 Astaxanthin (AST) nanoemulsion

The product developed and tested was a nanostructured formulation containing vegetable oils and AST oleoresin, derived from *H. pluvialis*, formulated in partnership with the company Nanoscoping Soluções em Nanotecnologia Ltda (Florianópolis, Brazil). This product is undergoing the patenting process.

2.2 Astaxanthin oleoresin and vegetable oils

Commercial samples of AST-containing oleoresin and vegetable oils (flaxseed and safflower) were provided by Nanoscoping Soluções em Nanotecnologia Ltda (Florianópolis, Brazil).

2.3 Preparation of AST oleoresin

The commercial AST oleoresin used in this study is a lipid extract obtained from *H. pluvialis*, in which AST is present mainly in esterified forms and solubilized in olive oil, used as a lipid carrier. Olive oil was selected due to its high carotenoid solubilization capacity, oxidative stability, and food safety, being commonly used in lipid-based carotenoid extraction systems (Li et al., 2011; Ambati et al., 2014).

For analytical purposes, 50 mg sample of AST oleoresin was transferred to a 40 mL beaker wrapped in aluminum foil and 5 mL of hexane: acetone extraction solution (1:1, v/v) was added. During the extraction time (5 min), the samples were kept under agitation (magnetic stirrer - Fisatom), protected from light, at room temperature (~23 °C). Afterwards, the sample was stored in amber vials (~10 mL) containing a N_2 atmosphere (Sühnel et al., 2009). The sample remained stored at −80 °C until further analysis of total carotenoid and AST contents.

This step was performed exclusively for carotenoid quantification and did not modify the composition of the oleoresin used in the nanoemulsion formulation. The olive oil matrix contributes to the solubility of AST and influences the physicochemical behavior and stability of the nanoemulsion (Ambati et al., 2014).

2.4 Total carotenoid analysis (TC)

The total carotenoid content in the oleoresin and nanoderived product samples was determined by UV-visible spectrophotometry (Hitachi, model U-1800), according to the protocol described by Sühnel et al. (2009). The oleoresin and nanoemulsion samples were diluted in a solution of acetonitrile and methanol (90: 10, v/v). An AST standard solution (10 mg/mL DMSO, Sigma-Aldrich, MO – USA) was used to construct a linear regression model of that oxycarotenoid (10–80 μg mL^{-1}, $y = 0.039x–r^2 = 0.999$). Three sequential readings/sample were performed, and the TC contents were expressed in mg/g.

2.5 Carotenoid profile analysis of oleoresin and AST nanoemulsion by high-performance liquid chromatography (UHPLC-DAD)

The AST contents of the oleoresin and nanoemulsion samples were determined by reverse-phase ultra high-performance liquid chromatography (Thermo Scientific Ultimate 3,000 - UHPLC-DAD), according to the protocol of Sühnel et al. (2009). AST was identified based on the retention time of the analytical standard (Rt = 3.8 min - 40 μg mL^{-1}, Sigma-Aldrich–MO, USA), and its contents were determined using a linear regression model (10–70 μg mL^{-1}, $y = 0.683x–r^2 = 0.990$). The analyses were performed in triplicate, computing the average of three sequential injections (10 μL) per sample.

2.6 Fatty acid profile of vegetable oils

The fatty acid profiles in the safflower oil samples from Destilaria Bauru® and flaxseed oil from Distrol® were determined by gas chromatography with flame ionization detection (GC-FID). An aliquot (50 μL) of each oil sample was

collected and derivatized, as described by Hartman and Lago (1973). Aliquots (1 µL) of the fatty acid methyl esters in hexane were injected into a gas chromatograph (Shimadzu - 2014), equipped with a manual injector (140 °C), flame ionization detector (300 °C), and a capillary column (RTx 2,330% - 90% biscyanopropyl/10% cyanopropylphenyl polysiloxane – 105 m × 0.25 mm internal diameter × 0.20 µm). The identification of fatty acids considered the retention times of the sample peaks, according to the 37 component FAME Mix - Sigma-Aldrich standard. Table 1 presents the results of the analyses regarding the identified metabolites and the sums of PUFAs in flaxseed and safflower oils.

2.7 Nanostructured systems of AST and vegetable oils

Nanoemulsions with and without AST were prepared by the high-pressure homogenization method (Yuan et al., 2008) using a high-pressure apparatus (IBH, APL 300 model, 210–1,500 bar, 660–3000L/h, São Paulo, Brazil). The surfactants, co-surfactants, and other ingredients used to prepare the nanoemulsions were chosen based on their suitability for AST encapsulation, as well as their biocompatibility and biodegradability characteristics. The AST-based nanoderivative product is currently being patented, so its specific composition cannot be entirely disclosed. After preparation, the nanoemulsion was stored in a white plastic bottle, protected from light and at room temperature (25 °C).

2.8 Morphological analysis of nanostructured AST derivatives

The morphology of the AST nanoemulsions was investigated by scanning electron microscopy (SEM), using a Jeol JEM-1011 model (Jeol Ltd., Tokyo, Japan). Aliquots of the formulations of interest were previously diluted to 10% in Ultrapure Milli-Q® water and subsequently deposited on carbon-coated grids and stained with 2% (w/v) uranyl acetate.

2.9 Physicochemical properties and stability study of AST nanoemulsion

The determination of particle size and zeta potential of the nanoparticles used dynamic light scattering (DLS) and laser-doppler anemometry techniques, respectively, using a Zetasizer Nano ZS90 - Model ZEN3690 (Malvern Instruments, Worcestershire, UK) at 25 °C, after dilution (10×) of the samples in ultrapure water, followed by three readings/sample (n = 3). The stability of the AST nanoemulsions during storage was evaluated by subjecting the nanotechnological inputs to temperatures of 4 °C, 25 °C, 30 °C, and 54 °C for 3 months, as recommended in official guidelines (ICH, 2003; ANVISA - Agência Nacional de Vigilância Sanitária, 2012). The stability of the colloidal systems during storage was evaluated by macroscopic analysis, pH, particle size determination (nm), polydispersity index (PDI), and zeta potential (mV), followed by three readings/sample (n = 3).

TABLE 1 Fatty acid profiles (%) of flaxseed and safflower oils determined by gas chromatography with a flame ionization detector.

Fatty acids (%)	Flaxseed	Safflower
Palmitic acid (C16:0)	9.81 ± 0.6	7.07 ± 0.5
Palmitoleic acid (C16:1*n*-7)	0.11 ± 0.0	0.11 ± 0.0
Stearic acid (C18:0)	3.46 ± 0.2	3.46 ± 0.1
Oleic acid (C18:1*n*-9)	21.46 ± 0.1	21.46 ± 0.1
α-Linolenic acid (C18:3*n*-3)	17.21 ± 0.5	10.16 ± 0.3
γ-Linoleic acid (C18:2*n*-6)	40.76 ± 0.6	40.76 ± 0.5
Total W-3	17.21 ± 0.5	10.16 ± 0.3
Total W-6	40.76 ± 0.6	40.76 ± 0.5
Total W-9	21.46 ± 0.1	21.46 ± 0.1
Total polyunsaturated	79.21 ± 0.4	72.38 ± 0.3

2.10 *In vitro* antioxidant activity - DPPH method

The antioxidant activity of the AST nanostructures at 4 °C, 25 °C, 30 °C, and 54 °C was determined spectrophotometrically (l = 530 nm), as described by Mazzarino et al. (2017), using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, during 30 h of incubation at room temperature. The free radical scavenging activity (FRSA) of the nanoemulsions was calculated based on the equation:

$$FRSA\ (\%) = (Ac - As)/Ac*100,$$

Where AC represents the absorbance of the DPPH solution (control solution) and AS the absorbance in the presence of the sample. As a negative control, a DPPH solution without the addition of the AST nanoderivative was used. The results are expressed as antioxidant capacity in Trolox equivalent (TEAC, mmol g^{-1} nanostructured) and FRSA (%). All analyses were performed in triplicate (n = 3), and the results were expressed as mean ± standard deviation.

2.11 Shrimp farming

Ethical approval was not required for this study because, to date, there is no ethical procedure requirement for the crustacean category, due to the fact that they are lower invertebrates.

For the proper containment and disposal of *V. parahaemolyticus*, standard and special practices outlined in National Technical Commission on Biosafety (CTNBio - National Technical Commission on Biosafety, 1997) were followed. This included the use of safety equipment and facilities, such as appropriate personal protective equipment (PPE) like gloves, aprons, and masks, in addition to proper disinfection and waste disposal.

Marine shrimp (*P. vannamei*), acquired from Aquatec Aquacultura Ltda. (Rio Grande do Norte, Brazil), were cultivated at the Marine Shrimp Laboratory (LCM – UFSC) in a biofloc system until they reached the ideal weight for the start of the experiments (i.e., 3.0 ± 0.5 g).

The experiment was conducted in a completely randomized design and consisted of evaluating the *in vivo* effect of administering feed containing nanoemulsion with and without AST nanoemulsions to *P. vannamei* shrimp, cultivated in clear water, for 6 weeks. Six diets were tested in triplicate (n = 3), totaling eighteen experimental units (EU). For the experimental units (EU), polyethylene tanks containing 400 L of seawater were used, with a RAS system (33.3 L/min^{-1}) and diffusion aeration using MixLife® microporous tubing. Thirty animals were distributed per EU, being fed four times a day (8 a.m., 11 a.m., 2 p.m., and 5 p.m.), according to Van Wyk et al. (1999). Feces and molts were removed twice a week by siphoning.

2.12 Diet formulation and preparation

For the formulation of the diets (Table 2), Optimal Formula 2000® software (version 19102009) was used, following the recommendations and nutritional requirements of *P. vannamei* (NRC, 2011; Zhou et al., 2014). The formulated feed was stored at 4 °C until use.

After the tests with the AST nanoemulsion, a fresh solution at room temperature (25 °C) was added by spraying to the feed, followed by drying in a laminar flow hood for 60 min. Six diets were evaluated, namely: a) A - 10, B - 30, C - 50, and D - 70 mg kg^{-1} AST kg^{-1}; b) negative control (NC) containing 70 mg kg^{-1} of nanoemulsion without AST and; c) control (without nanoemulsion). The centesimal composition of the feed used to make the diets is shown in Table 3.

The water quality parameters of the shrimp cultures were determined twice a day: pH (8.10 ± 0.10), temperature (28.5 °C ± 1.0 °C), and dissolved O_2 (7.10 ± 0.42), determined with the aid of a multiparameter probe (YSI Professional Plus model); salinity (33.0 ± 1.0 mg L^{-1}, YSI EC300A digital salinometer) and alkalinity (128 ± 3.37 mg $CaCO_3$ L^{-1}) (APHA - American Public Health Association, 2005). Total ammonia (0.03 ± 0.5 mg L^{-1}), nitrate (0.06 ± 0.05 mg L^{-1}) and nitrite (0.01 ± 0.04 mg L^{-1}) levels (Boyd and Gautier, 2000) were assessed once a week.

At the end of the experiment, hemolymph was collected from the ventral sinus of the shrimp (five animals/tank) for total hemocyte count and serum collection for determination of protein concentration, phenoloxidase (PO) enzyme activity, and agglutination capacity (Agl). Hemolymph collection and serum preparation used anticoagulant solution (0.114 M sodium citrate, 0.1 M NaCl, pH 7.45) at a 1: 9 ratio (v/v; hemolymph: anticoagulant) (Cheng and Chen, 2001).

The total hemocyte count (THC) was performed as described by Cheng and Chen (2001), from a 10 μL-hemolymph sample in a Neubauer chamber. The determination of the number of hemocytes was performed in triplicate with the aid of an optical microscope.

The serum protein concentration analysis used the Bradford (1976), while the determination of phenoloxidase (PO) enzyme activity followed the Söderhäll and Häll (1984). Agglutination capacity was evaluated according to the method of Maggioni et al. (2004).

2.13 Exposure of *P. vannamei* to *V. parahaemolyticus*

For the evaluation of the challenge with *V. parahaemolyticus*, 10 shrimps per treatment were acclimated in 60 L boxes, containing seawater at 28.5 °C, for 48 h. Subsequently, the shrimps were infected with 100 μL of *V. parahaemolyticus* inoculum (viral load = 4 × 10^8 μL^{-1}, determined according to the LD_{50} test). The cumulative mortality of the animals was evaluated for 96 h after infection, according to Schleder et al. (2017).

TABLE 2 Composition of the feed administered to *P. vannamei* with or without the AST nanoemulsion.

Ingredients	g kg^{-1}
Soybean meal	348.00
Wheat flour	208.00
Chicken offal	159.57
Fish flour	131.55
Soy lecithin	25.16
Sodium chloride	10.00
Potassium chloride	17.50
Methionine	1.51
Vitamin premix[a]	15.15
Mineral premix[b]	14.84
Vitamin C	1.00
Soy oil	15.06
Fish oil	15.30
Carboxymethylcellulose	4.89
Kaolin natura	100.00
Choline	0.25

Soybean meal Adicel®; Wheat flour Moliser®; Chicken offal flour®; Fish flour ViaFlor®; Soy lecithin Adicel®; Sodium chloride Dinâmica®; Potassium chloride AurosQuímica®; Methionine Evonik®; Vitamin C Rovimix®; Soy oil Vitaliv®; Fish oil Bianquímica®; Carboxymethylcellulose Synth®; Kaolin Natura Uralis®; Choline NuSci®.

[a]Vitamin premix (*In Vivo* Mix–Paulínea, Brazil): vitamin A: 900 mg kg^{-1}; vitamin D: 25 mg kg^{-1}; vitamin E: 46,900 mg kg^{-1}; vitamin K: 14,000 mg kg^{-1}; vitamin B2: 20,000 mg kg^{-1}; Pantothenic acid: 40,000 mg kg^{-1}; Niacin: 70,000 mg kg^{-1}; vitamin B12: 50 mg kg^{-1}; Biotin: 750 mg kg^{-1}; Folic acid: 3,000 mg kg^{-1}; vitamin B1: 30,000 mg kg^{-1}; vitamin B6: 33,000 mg kg^{-1}.

[b]Mineral premix (*In Vivo* Nutrição e Saúde Animal Ltda–São Paulo, Brazil): Magnesium: 20 mg kg^{-1}; Potassium: 6.11 mg kg^{-1}; Copper: 23,330 mg kg^{-1}; Iodine: 1,000 mg kg^{-1}; Manganese: 6,500 mg kg^{-1}; Zinc: 100 mg kg^{-1}; Selenium: 125 mg kg^{-1}.

2.14 Statistical analysis

The physicochemical data of the AST nanoemulsions were subjected to one-way ANOVA variance analysis, Levene, and Shapiro-Wilk, followed by the Tukey's *post hoc* test. The results were considered significant for $p < 0.05$.

In turn, the immunological parameter data were subjected to one-way analysis of variance (ANOVA) and Bonferroni's mean comparison test, when applicable, with a significance level of 5%. The survival data from the *V. parahaemolyticus* challenge assay were evaluated using the Kaplan-Meier method. Homoscedasticity and normality of sample distribution were determined using the Levene and Shapiro-Wilk tests, respectively.

All statistical tests were performed with the support of Excel Microsoft 365, Statistica 10.0 and Jamovi 2.6.25.0 software.

TABLE 3 Centesimal composition of diets containing different concentrations of nanoemulsions administered to *P. vannamei*. Control (without AST nanoemulsion), negative control (NC, 70 mg kg^{-1} nanoemulsion without AST), and diets containing 10, 30, 50, and, 70 mg kg^{-1} AST nanoemulsion.

Centesimal composition (%)	Control	NC	A	B	C	D
Dry matter	92.90	92.90	93.28	93.05	93.23	93.32
Crude protein	33.35	33.41	33.08	33.58	33.18	33.65
Ethereal extract	8.28	8.73	8.60	8.28	8.60	8.47
Mineral matter	18.65	18.59	18.59	18.57	18.66	18.47

A - 10 mg kg^{-1} AST; B - 30 mg kg^{-1} AST; C - 50 mg kg^{-1} AST; D - 70 mg kg^{-1} AST; NC – 70 mg kg^{-1} without AST and Control group.
The physicochemical characterization analyses were carried out according to the methodologies described by AOAC (1999): Dry matter, method 950.01; Mineral matter, method 942.05; Protein by LECO, method Dumas 990.03, conversion factor 6.25; Ethereal extract by Soxhlet, method 920.39C.

3 Results

3.1 Quantification of total carotenoids (TC) and astaxanthin (AST)

The contents of TC and AST in the oleoresin and AST-containing nanoemulsion samples were determined by UV-vis spectrophotometry (Hitachi, model U-1800) and high-performance liquid chromatography (UHPLC-DAD), respectively, after a saponification process. The results are shown in Table 4.

TABLE 4 Concentrations (mg g^{-1}) of total carotenoids (UV-vis, λ = 470 nm) and astaxanthin (UHPLC-DAD - λ = 476 nm) in the oleoresin and derived nanoemulsion samples.

	Oleoresin (mg g^{-1})	Nanoemulsion (mg g^{-1})
Total carotenoids	10.94 ± 0.4	2.44 ± 0.2
Astaxanthin	5.48 ± 0.4	1.55 ± 0.1

3.2 Physicochemical properties and stability study of the AST nanoemulsion

Immediately after preparation, the AST-containing nanoemulsion exhibited a dark red to brown color, low viscosity, and a characteristic odor of oleoresin. Macroscopic analysis revealed a homogeneous appearance and absence of creaming or sedimentation. The pH of the nanoemulsion was slightly acidic, i.e., 5.78 ± 0.01. The colloid presented an average particle size of 319.6 ± 0.5 nm and a polydispersity index (PDI) of 0.290 ± 0.0, revealing homogeneity in the sample. The observed zeta potential (−44 ± 0.40 mV) indicated that the particles were electrically stabilized (Table 5).

In contrast, at 54 °C, the nanoemulsion showed progressive instability, with a significant decrease in pH over time (reaching 4.04 at 90 days), likely due to thermal degradation of lipid components and hydrolysis of surfactants, leading to the formation of free fatty acids. The marked increase in average particle size (2,641.33 nm at 90 days) and the maximum PDI (1.000) indicate coalescence and flocculation processes, characterizing loss of colloidal stability. In turn, the zeta potential (mV) of the AST nanostructured derivative revealed that particles remained electrically stabilized (>|30| mV) throughout the storage period under all investigated thermal conditions (Table 5).

3.3 Morphological analysis of AST nanostructured derivatives

Transmission electron microscopy (TEM) analysis revealed spherical nanostructures, without the formation of clusters (Figure 1).

3.4 *In vitro* antioxidant activity of nanoemulsion by DPPH

The antioxidant activity values of the AST nanoemulsion, expressed as DPPH radical inhibition percentages, are presented in Table 6. Data for the 30-min reaction time were obtained and reported; however, it was not possible to calculate the IC_{50} due to limitations in the experimental design, which focused on evaluating the antioxidant activity of the formulation under different storage temperatures and durations. Future studies using a range of concentrations are recommended to allow for the determination of these parameters and to enable better comparability with the literature.

Immediately after preparation, the nanoemulsion exhibited an antioxidant activity value of 32.0% ± 0.1% at the 30-min time point. After 15 days of storage, the nanostructured product showed antioxidant activity starting at 31.9% ± 0.1% under all tested thermal conditions, with significant differences between treatments, and the highest activity detected at 30 °C (34.5% ± 0.1%) (Table 6).

At 30 and 60 days, a general fluctuation in antioxidant activity was observed. Samples stored at 54 °C showed an increase in the DPPH radical inhibitory effect, reaching 38.6% ± 0.1% at 30 days and 37.4% ± 0.1% at 60 days.

Finally, at 90 days, the nanoemulsions exhibited DPPH radical inhibition percentages starting from 31.7% ± 0.1%, with significant differences among all treatments. The highest antioxidant activity was again observed at 54 °C (41.9% ± 0.1%) (Table 6).

3.5 Immunological parameters of *P. vannamei*

The data of the immunological parameters of *P. vannamei* according to the treatments with the AST-containing

TABLE 5 Physicochemical characterization and stability of the AST nanoemulsion at different temperatures of storage.

	Temperatures	4 °C	25 °C	30 °C	54 °C
Day 15	pH	5.75 ± 0.0[a]	5.29 ± 0.0[a]	5.14 ± 0.0[a]	4.95 ± 0.0[a]
	nm	396.1 ± 11.4[a]	407.5 ± 17.4[a]	401.3 ± 10.6[a]	352.33 ± 3.2[b]
	PDI	0.260 ± 0.0[c]	0.390 ± 0.0[c]	0.580 ± 0.0[b]	0.610 ± 0.0[a]
	mV	−27.7 ± 4.8[b]	−40.7 ± 0.5[a]	−37.73 ± 1.2[a]	−40.5 ± 0.5[a]
Day 30	pH	5.36 ± 0.0[a]	5.79 ± 0.0[a]	5.80 ± 0.0[a]	4.45 ± 0.0[b]
	nm	339.97 ± 5.4[c]	339.3 ± 10.2[c]	363.6 ± 2.7[b]	486.57 ± 1.6[a]
	PDI	0.140 ± 0.0[a]	0.160 ± 0.0[a]	0.210 ± 0.0[a]	0.220 ± 0.0[a]
	mV	−35.0 ± 0.7[c]	−44.4 ± 0.5[a]	−40.7 ± 0.3[b]	−38.9 ± 0.4[b]
Day 60	pH	5.55 ± 0.0[a]	6.25 ± 0.0[a]	5.04 ± 0.0[a]	4.45 ± 0.0[b]
	nm	372.4 ± 0.9[b]	347.47 ± 6.2[b]	265.93 ± 5.5[c]	480.7 ± 26.2[a]
	PDI	0.160 ± 0.0[a]	0.170 ± 0.0[a]	0.210 ± 0.0[a]	0.666 ± 0.0[a]
	mV	−45.9 ± 0.6[a]	−37.1 ± 0.4[b]	−39.5 ± 1.6[b]	−38.1 ± 1.0[b]
Day 90	pH	5.59 ± 0.0[a]	5.66 ± 0.0[a]	4.76 ± 0.0[b]	4.04 ± 0.0[b]
	nm	329.8 ± 10.6[b]	337.0 ± 18.3[b]	259.9 ± 2.4[b]	2,641.33 ± 1,017[a]
	PDI	0.160 ± 0.0[b]	0.130 ± 0.0[b]	0.150 ± 0.0[b]	1.000 ± 0.0[a]
	mV	−41.0 ± 0.6[a]	−37.8 ± 0.5[b]	−36.7 ± 0.8[b]	−41.1 ± 1.3[a]

Characterization and physicochemical stability of the nanoemulsion at different temperatures, over the period of 15, 30, 60, and 90 days, under temperatures of 4 °C, 25 °C, 30 °C and 54 °C, until the end of the 90-day test. (nm) Particle size; (PDI) polydispersity index; (mV) Zeta potential. Distinct lowercase letters, within the same day period, represent significant differences between treatments (i.e., temperatures) ($p < 0.05$). Results are presented as mean ± standard deviation.

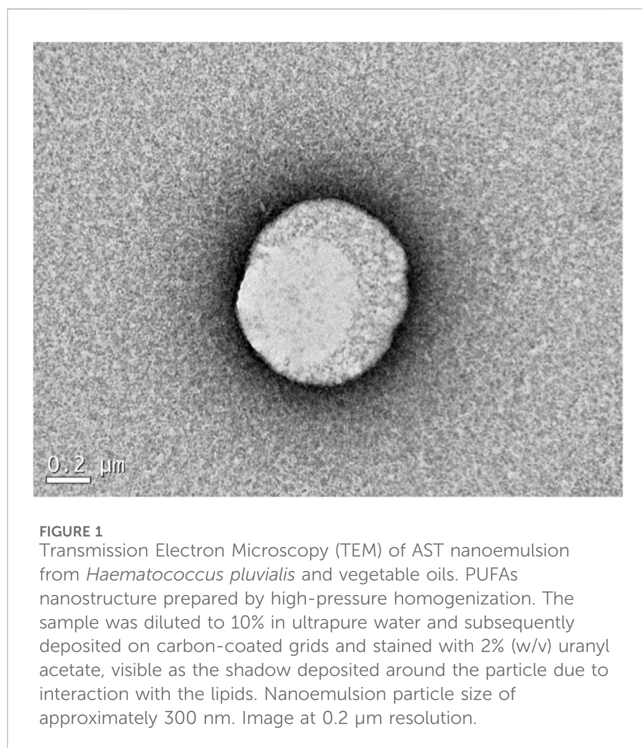

FIGURE 1
Transmission Electron Microscopy (TEM) of AST nanoemulsion from *Haematococcus pluvialis* and vegetable oils. PUFAs nanostructure prepared by high-pressure homogenization. The sample was diluted to 10% in ultrapure water and subsequently deposited on carbon-coated grids and stained with 2% (w/v) uranyl acetate, visible as the shadow deposited around the particle due to interaction with the lipids. Nanoemulsion particle size of approximately 300 nm. Image at 0.2 µm resolution.

nanoemulsion are shown in Table 7. The total hemocyte count (THC) and serum agglutination titer (Agl) of the animals treated with the nanoderived product did not differ statistically ($p > 0.05$) in relation to control. The total serum protein (P) concentration significantly differed ($p < 0.05$) according to the treatments D (3.75 ± 0.3 mg mL^{-1} P), C (3.64 ± 0.5 mg mL^{-1} P), and B (3.63 ± 0.4 mg mL^{-1} P). Similarly, the phenoloxidase (PO) activity differed ($p < 0.05$) in treatments B (84.09 ± 13.2 min^{-1} mg^{-1}), C (80.60 ± 23.1 min^{-1} mg^{-1}), and NC (89.85 ± 17.2 min^{-1} mg^{-1}) in respect to control (Table 7).

3.6 Survival of *P. vannamei* after challenge with *V. parahaemolyticus*

After 96 h exposure to *V. parahaemolyticus*, no significant difference ($p > 0.05$) in the animal survival was noted between the treatments investigated. Shrimp fed the control diet showed 40% survival, followed by treatment A (10 mg kg^{-1}) with 60%, treatments B (20 mg kg^{-1}), C (50 mg kg^{-1}), and D (70 mg kg^{-1}) with 70%, and treatment NC (70 mg kg^{-1} without AST) with 80% (Figure 2).

It was observed that the shrimp ate normally, and there was no leftover feed in the experimental units (EUs), indicating that the nanostructure is palatable. The batch of animals used in this experiment appeared to be unsuitable. The shrimp were sensitive and died after handling, even when done every 15 days. The experimental units and seawater were suitable for cultivation, with no infectious agents present.

TABLE 6 *In vitro* antioxidant activity of AST nanoemulsion as determined by the DPPH assay.

Treatments	Day 15	Day 30	Day 60	Day 90
4 °C	31.9 ± 0.1^{d}	14.6 ± 0.1^{d}	27.1 ± 0.2^{d}	31.7 ± 0.1^{d}
25 °C	33.3 ± 0.1^{b}	28.6 ± 0.1^{c}	29.2 ± 0.1^{c}	34.7 ± 0.1^{c}
30 °C	34.5 ± 0.1^{a}	29.8 ± 0.2^{b}	31.1 ± 0.1^{b}	37.1 ± 0.2^{b}
54 °C	33.1 ± 0.1^{c}	38.6 ± 0.1^{a}	37.4 ± 0.1^{a}	41.9 ± 0.1^{a}
p-value	**0.001**	**0.001**	**0.001**	**0.001**

Antioxidant activity (DPPH, assay) of the AST, nanoemulsion at 20% in 80% MeOH, disrupted with acetonitrile (1: 3, v/v), stored at 4 °C, 25 °C, 30 °C, and 54 °C, after 30 min ($p < 0.05$). Measurements were performed using an UV-vis, spectrophotometer (Hitachi, model U-1800). Different lowercase letters indicate statistically significant differences between treatments. Results are expressed as mean ± standard deviation of triplicates. p-value: A statistically significant difference is considered when $p < 0.05$.

TABLE 7 Immunological parameters of *P. vannamei* treated with the AST nanoemulsions (A, B, C, and D), negative control (NC), and control.

Treatments	THC (x 10^7)	Protein concentration (mg mL^{-1})	PO activity (Unit min^{-1} mg^{-1} P)	Agglutination titer (Agl) (log^2)
Control	27.30 ± 9.6^{a}	2.41 ± 0.7^{b}	144.56 ± 29.3^{a}	10.00 ± 0.0^{a}
A	45.70 ± 6.6^{a}	2.98 ± 0.1^{a,b}	108.34 ± 18.4^{a,b,c}	10.00 ± 0.0^{a}
B	26.30 ± 35.4^{a}	3.63 ± 0.4^{a}	84.09 ± 13.2^{c}	10.00 ± 0.0^{a}
C	61.70 ± 60.6^{a}	3.64 ± 0.5^{a}	80.60 ± 23.1^{c}	10.33 ± 0.5^{a}
D	51.30 ± 9.5^{a}	3.75 ± 0.3^{a}	132.67 ± 15.8^{a,b}	10.50 ± 0.7^{a}
NC	45.3 ± 22.2^{a}	3.85 ± 0.4^{a}	89.85 ± 17.2^{c}	10.00 ± 0.0^{a}
p-value	**0.696**	**0.0454**	**0.0005**	**0.1375**

Immunological parameters analyzed from the hemolymph serum of *P. vannamei* fed with AST, nanoemulsion, negative control (NC), and control. A - 10 mg kg^{-1} AST; B - 30 mg kg^{-1} AST; C - 50 mg kg^{-1} AST; D - 70 mg kg^{-1} AST; NC, 70 mg kg^{-1} without AST. (THC): total hemocyte count; (PO): phenoloxidase; (P): protein; (Agl): Agglutination titer. Distinct lowercase letters represent significant differences between treatments. Results are presented as mean ± standard deviation of triplicates. p-value: A statistically significant difference is considered when $p < 0.05$.

4 Discussion

4.1 Physicochemical properties and nanoemulsion stability

The formulated nanoemulsion exhibited desirable characteristics achieved through a high-shear energy method. This technique effectively produces submicrometer particles, ensures better dispersion of high-lipid formulations with low polydispersion, avoids organic solvents, and is feasible for industrial scale-up (Juttulapa et al., 2017; Mistry et al., 2012).

Storing the AST nanoemulsion at higher temperatures (30 °C and 54 °C) led to significant acidification (pH 4.76 ± 0.0 and pH 4.04 ± 0.0, respectively), compromising colloidal stability compared to 4 °C and 25 °C. This pH drop often indicates chemical reactions, such as the hydrolysis of fatty acid esters in vegetable oil emulsions, generating free fatty acids that compromise product quality (Cunha et al., 2010).

Nanoemulsions stored at 4 °C, 25 °C and 30 °C showed good particle size stability, with a unimodal distribution. Since nanocarriers are thermodynamically unstable, stability depends on a robust, viscoelastic interfacial film. This study employed a synergistic mix of soy lecithin and polysorbate 80 to ensure long-term physicochemical stability, controlled release, and enhanced bioavailability (Lacatusu et al., 2014; McClements, 2015; Tadros, 2013). Nanoemulsions exposed to 54 °C maintained particle size stability only for the initial 15 days, followed by a significant increase and multimodal distribution. This size increase is primarily attributed to Ostwald ripening, where the instability of the surfactant film at high temperatures allows oil diffusion from smaller to larger droplets (Prasert and Gohtani, 2016; Taylor, 1995).

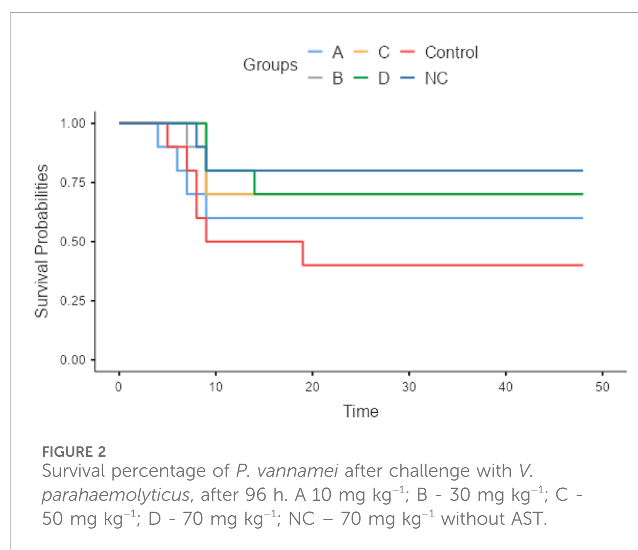

FIGURE 2
Survival percentage of *P. vannamei* after challenge with *V. parahaemolyticus*, after 96 h. A 10 mg kg^{-1}; B - 30 mg kg^{-1}; C - 50 mg kg^{-1}; D - 70 mg kg^{-1}; NC – 70 mg kg^{-1} without AST.

The formulation stored at 30 °C surprisingly showed a gradual reduction in average particle size over time. This apparent contradiction results from the inherent limitation of the Dynamic Light Scattering (DLS) technique: large, degraded droplets sediment or precipitate and escape detection, leading to an underestimation of the true size distribution (Beliciu and Moraru, 2009; Marucco et al., 2019). The observed reduction in particle diameter likely does not signify enhanced stability, but rather a structurally compromised system. Thermal degradation of the surfactant layer can cause larger droplets to break down into smaller, measured micellar structures, skewing the DLS signal toward smaller values (Beliciu and Moraru, 2009; Farkas and Kramar, 2021).

High PDI values indicate a wide particle size distribution, which negatively impacts nanoemulsion stability (Gaumet et al., 2008). The combination of phospholipids and polysorbates aids stability by reducing internal phase droplet size, which, in turn, favors cellular uptake and active ingredient delivery (McClements et al., 2017; Lin et al., 2014).

Although zeta potential values remained in the electrostatically stable range (>|30| mV), a minor magnitude reduction occurred at higher temperatures. This suggests a weakening of the repulsive barrier due to temperature-induced disruptions in the surfactant interface, lower continuous phase viscosity, and accelerated particle collisions, leading to destabilization (Rudin and Choi, 2013; Montoya-Anaya et al., 2023).

In conclusion, the nanoemulsion exhibited good physicochemical stability when stored under refrigeration (4 °C) and at room temperature (25 °C), but showed pronounced thermal sensitivity at elevated temperatures (30 °C and 54 °C), which is critical for defining application and storage strategies.

4.2 Morphology of the nanostructured system containing AST

Conventional negative staining using uranyl acetate allowed for the identification of the lipid droplets present in the nanoemulsion, which exhibited a spherical shape and homogeneous distribution in their morphology (Figure 1). The results of the present study are similar to those previously observed (Araújo et al., 2011; Schuh et al., 2014; Mattos et al., 2015; Abdou et al., 2018) for the morphology of particles in nanoemulsion systems developed as nanocarriers for bioactive compounds.

The use and choice of vegetable oil is a crucial step in the design and development of nanoemulsions. It not only determines the loading capacity and bioavailability of the active compound, but also influences the physical stability, shelf life, and additional functional properties of the final product (Rostamabadi et al., 2020). TEM is also used at this stage to determine the most appropriate stabilizer and carrier oil (Banasaz et al., 2020). Abdou et al. (2018) used TEM to characterize the morphology and size of nanoemulsion particles prepared with garlic oil (CGN), cinnamon oil (CCN), and sunflower oil (CSN). As a result, both CGN and CCN provided spherical nanoemulsion droplets with higher homogeneity and uniform distribution, which was not the case for CSN, due to the discrepancy in the viscosity of the carrier oils.

4.3 Antioxidant activity – DPPH assay

The temporal pattern observed in the antioxidant activity of the nanoemulsion (Table 6), i.e., relatively high values in the first 15 days, a significant drop at 30 days, and a subsequent increase up to 90 days, especially under storage at 54 °C) is consistent with the dynamic interaction between degradation, isomerization, and physicochemical changes in the nanostructured matrix. In the initial stages, the homogeneous distribution of the droplets and the protection conferred by nanoencapsulation preserve the antioxidant activity, resulting in a high radical chelating capacity. The reduction at 30 days likely reflects primary degradation and a decrease in the fraction of intact AST available to react with the DPPH radical, as described in kinetic studies of the thermal stability of nanoencapsulated carotenoids. However, the subsequent increase in antioxidant activity between 60 and 90 days can be explained by the combination of progressive thermal release of the compound from the nanoemulsion matrix and conversion of all-*trans* AST into *cis* isomers, especially 9-*cis*, which exhibit greater radical-scavenging activity *in vitro* (Liu and Osawa, 2007). Thermal conditions and solvents promote *trans*→*cis* isomerization and structural modifications capable of increasing antioxidant reactivity (Yuan and Chen, 1999). Furthermore, elevated temperatures can generate degradation products with residual radical neutralization capacity, contributing to the observed increase (Rao and McClements, 2012; Ahmad et al., 2019; Jia et al., 2021). Finally, fluctuations in activity during storage can also reflect changes in the physical stability of the nanoemulsion, such as partial coalescence, Ostwald ripening, or alterations in PDI, which modify the release and extractivity of AST during sample preparation for chemical analysis (Liu et al., 2019).

4.4 Immunological parameters

Although the present experiment did not reveal a clear and uniform linear effect of AST-based nanoproduct on hemolymph agglutination activity (Agl) or total hemocyte count (THC) in *P. vannamei*, the serum protein (P) and phenoloxidase (PO) results strongly suggest the occurrence of a non-linear dose-dependent phenomenon (Costantini, 2019), possibly with an optimal window of immune response (Table 7).

The immunoregulatory effects in *P. vannamei*, mediated by AST, have been reported in previous studies. Flores et al. (2007) observed significantly higher values of hemocyanin concentrations and total hemocyte count (THC) in the hemolymph of shrimp supplemented with 80 mg kg^{-1} AST compared to shrimp fed other diets. Chuchird et al. (2015) found that the THC and PO of shrimp fed AST showed significant improvement compared to the other groups. Experimental studies in *P. vannamei* have demonstrated that dietary supplementation with AST increased important enzymatic activities related to immunity, such as acid phosphatase (ACP) and alkaline phosphatase (AKP), by approximately 20%–45%, as well as increasing total hemocyte count (THC) and antioxidant enzyme activities by 30%–60% (Niu et al., 2009; Zhang et al., 2025).

Nutritional studies demonstrate that AST supplementation elevates humoral and cellular markers relevant to agglutination (Agl), THC, PO, and phagocytic capacity in *P. vannamei* and

other crustaceans, suggesting a functional improvement in hemolymph that may be reflected in greater agglutinating activity (Chuchird et al., 2015; Mansour et al., 2022). Transcriptomic and gene expression studies indicate that AST regulates redox pathways and immunological genes (proPO, lectins, AMPs, caspases), which provides a plausible mechanism for preserving and increasing the activity of plasma proteins involved in agglutination (Pan et al., 2008; Zhang et al., 2009; Tan et al., 2020; Li et al., 2022a).

In particular, intermediate doses (30–70 mg kg^{-1}) resulted in significant increases in P, indicating that AST nanoemulsion may stimulate the synthesis or release of plasma proteins, such as lectins, hemocyanins, or acute phase factors, while PO was elevated at both the low dose (10 mg kg^{-1}) and the highest dose (70 mg kg^{-1}), suggesting an inverted U-shaped or hormetic response curve (Calabrese and Baldwin, 2002). This pattern is consistent with previous data on conventional AST diets, where Mansour et al. (2022) observed a dose-dependent upregulation of immune genes (proPO, lysozyme, crustin) and SOD in *P. vannamei* with 2–6 g AST kg^{-1}, in parallel with improved overall immunocompetence. Chuchird et al. (2015) also reported that 50 ppm of AST increased THC, PO activity, phagocytosis, and SOD in shrimp, although without affecting growth, defining the dose- and treatment duration-sensitive nature of the response. In turn, the study by Pan et al. (2008) demonstrates that agglutination activity in shrimp depends on the oligomeric conformation of hemocyanin, suggesting that AST could modulate the structural stability of these plasma proteins, but this modulation requires specific conditions (concentration, redox, and time) to translate into increased AgI (Beltramini et al., 2005). Finally, Lin et al. (2023) found that different sources and doses of AST (100–200 mg kg^{-1}) increase PO, phagocytosis, superoxide production, and expression of antioxidant and antimicrobial genes in *P. vannamei*, demonstrating that the immune response to AST is sensitive to the dose and source of the carotenoid. Therefore, the results of the present study are consistent with a non-linear response pattern. AST at moderate doses stimulates the production of plasma proteins and the redox pathway, while the activation of functional defense pathways, such as PO, depends on the dose and the route of administration, which in this study was via rationing of a nanoemulsion containing that oxycarotenoid. This justifies the need to expand the range of doses tested, explore the temporal kinetics of the response, and directly quantify lectins and hemocyanins to correlate with Agl. Based on the results obtained (Table 7), the most promising concentration is 70 mg kg^{-1}.

Recent studies demonstrate that dietary supplementation with AST elevates immunocompetence and antioxidant markers in *P. vannamei*, including promoting increases in plasma total protein (P) content. In an assay with natural AST extracted from *Arthrospira platensis*, the authors reported a dose-dependent regulation of immune genes (prophenoloxidase, lysozyme, crustin, β-GBP, transglutaminase) and antioxidants (SOD), associated with improved growth and feed utilization (Mansour et al., 2022). Another study that supplemented AST derived from *Adonis amurensis* observed increased antioxidant capacity and various immunological responses at 60–120 mg kg^{-1} diet, suggesting that the carotenoid may favor the overall health and protein homeostasis of *P. vannamei* (Zhang et al., 2025). Eldessouki et al. (2022) administered AST at 100–200 mg kg^{-1} and observed increases in immunological parameters and total serum protein, concomitantly with a reduction in lipid peroxidation and modulation of the expression of antioxidant enzymes in shrimp, supporting the hypothesis that AST contributes to the preservation or synthesis of plasma proteins under oxidative stress conditions.

PO is a key enzyme in the innate immune system of crustaceans, involved in the melanization and encapsulation of pathogens (Söderhäll and Cerenius, 1998). The elevated PO activity in the control group may reflect a compensatory immune response triggered by environmental stressors during cultivation. In contrast, in the groups treated with the nanoemulsion, especially those receiving AST, the reduced PO activity may be attributed to the antioxidant and anti-inflammatory properties of AST and the vegetable oils, which mitigate oxidative stress and consequently modulate the activation of the proPO system (Guerin et al., 2003; Chen et al., 2017). This regulation of PO activity, accompanied by the increase in serum proteins, suggests an immunomodulatory effect, indicating a balanced immune state that prevents excessive inflammatory hyperactivation without compromising defense against pathogens. These findings are consistent with previous reports showing that dietary antioxidants reduce stress-induced immune hyperactivation in crustaceans (Zhou et al., 2016; Ahmad et al., 2019).

Studies have shown that dietary inclusion of AST improves the expression of antioxidant and immune-related genes, such as beta-glucan binding protein (Bgp) and phenoloxidase (PO) in *P. vannamei* muscle tissue (Mansour et al., 2022). Chuchird et al. (2015) observed that *P. vannamei* fed diet containing 50 ppm AST for 90 days showed increased PO activity compared to control. Similarly, Wang et al. (2015) demonstrated that supplementation of Pacific white shrimp with 80 mg kg^{-1} of AST for 4 weeks affected ($p < 0.05$) serum PO activity compared to control. Additionally, a significant increase in PO activity was detected in *P. vannamei* fed diets containing 25, 50, 100, and 200 mg kg^{-1} AST for 8 weeks (Eldessouki et al., 2022).

PO enzyme activity is an immunoparameter commonly used to assess the health status of shrimp. When an organism is infected by pathogens, these microorganisms bind either directly or indirectly (i.e., via plasma pattern recognition proteins (PRPs)) to granular hemocytes, inducing degranulation or regulated exocytosis and releasing effector molecules, such as the proPO system (Barracco et al., 2014). The proPO system is known to play an important defensive role against pathogens. The Bgp gene functions as a vital factor for proPO system activation, coagulation progression, and the expression of antimicrobial peptides after the recognition of microbial components (Gonçalves et al., 2012). It is worth noting the existence of positive regulation of the Bgp gene in shrimp fed diets supplemented with AST (Mansour et al., 2022). Consequently, serum PO significantly improves when AST is added to *P. vannamei* feed (Wang et al., 2015).

Within the scope of nanotechnology, the use of nanoemulsions allows for the formation and stability of small-sized particles with improved bioavailability and absorption, increased solubility of lipophilic compounds, and rapid and efficient penetration of the compound (Soukoulis and Bohn, 2018; Dos Santos et al., 2018). Currently, studies using nanotechnology in crustacean diets are

scarce (Santos et al., 2024). Due to this, the bioavailability of nanoemulsified compounds and their absorption by the crustacean intestine still require further investigation for a better understanding of their mode of action.

The antioxidant properties of AST may directly participate in enhancing immunity in *P. vannamei, eventually by counteracting the effect of reactive oxygen species*. However, the immunostimulatory activity of astaxanthin in crustaceans still requires further investigation to better understand its mode of action (Mansour et al., 2022). Importantly, this study provides the first *in vivo* evidence that such a formulation significantly increases serum protein levels and modulates phenoloxidase activity in *P. vannamei*, key indicators of crustacean immune function. These findings highlight a non-obvious integration of lipid composition, processing technology, and biological efficacy, establishing the nanoemulsion as a promising functional feed additive and advancing the application of nanotechnology in sustainable shrimp aquaculture.

4.5 Survival of *P. vannamei* after challenge with *V. parahaemolyticus*

Despite the positive effects observed in baseline immunological parameters, such as increased serum protein (P) concentration and modulation of phenoloxidase (PO) activity, no significant differences were observed in the survival rate of *P. vannamei* after challenge with *V. parahaemolyticus* between the experimental groups, including those supplemented with the AST-containing nanoemulsion (Figure 2). However, the results demonstrated a promising protective trend conferred by the nanoemulsion developed in this study. Although the statistical analysis was inconclusive due to the low sample size (n = 10 per treatment) and the known sensitivity of the experimental batch, the observed difference in final mortality, reducing from 60% in the control group to 30% in the optimized treatments (B – 30 mg kg^{-1}, C – 50 mg kg^{-1} and D – 70 mg kg^{-1}), is biologically significant and has been observed in other studies (Chen et al., 2017; Zhou et al., 2016; Wei et al., 2018). Indeed, studies have shown positive effects of AST on the immune response, anti-stress capacity, and disease resistance in shrimp (Lim et al., 2018; Wang et al., 2018). Chuchird et al. (2015) found a higher survival rate in *P. vannamei* fed 50 ppm AST after infection with *V. parahaemolyticus*. Similar results were reported by Lin et al. (2023) in *P. vannamei* shrimp fed 100 mg kg^{-1} AST.

This result can also be attributed to interrelated factors. First, a potentially high bacterial load used in the challenge protocol (4×10^8 CFU mL^{-1} for 96 h) may have overwhelmed the shrimp's immune system, even with prior tests to determine the ideal concentration of *V. parahaemolyticus*, making it difficult to detect differences between groups, regardless of the treatment. Additionally, although the 45-day supplementation period was sufficient to alter physiological parameters, it may not have been enough to promote a robust protective response against a highly virulent acute infection (Eldessouki et al., 2022). Compared to other studies, the culture period was longer, lasting from 60 to 90 days (Zhou et al., 2007; Wei et al., 2018; Kumar et al., 2018; Eldessouki et al., 2022). Previous studies have also presented mixed results. For example, Ahmad et al. (2019) reported improved survival of *P. vannamei* supplemented with AST nanoemulsion under pathogenic challenge. However, those authors used higher doses and a longer supplementation period comparatively to this study. Similarly, Zhou et al. (2016) observed that the protective effect of AST depends on the stress level and the intensity of the challenge.

PUFAs also appear to participate in the expression of antimicrobial peptides that act against Gram-negative bacteria (Zuo et al., 2017). Furthermore, studies have demonstrated that essential fatty acids improve the immune response of shrimps and their resistance to *Vibrio* spp. infection (Lim et al., 1997; González-Félix et al., 2002; Nonwachai et al., 2010). Pacific white shrimp treated with exogenous alpha-linolenic acid (C18: 3 n-3, ALA) showed increased levels of EPA and DHA in the hepatopancreas, with or without challenge with *V. parahaemolyticus*, greater stimulation of the shrimp's antimicrobial immune response, and increased survival against pathogenic bacteria (Chen et al., 2024). The authors also observed that the increase in DHA and EPA levels and the induction of immune system gene expression were mediated by delta (Δ) desaturases, especially Δ desaturase, showing that *P. vannamei* has the ability to synthesize DHA and EPA from ALA, mainly during challenge with pathogens, but the details of the molecular mechanisms remain unknown.

Commonly, the composition of the gut microbiota exerts a considerable influence on the health of aquatic organisms, encompassing processes such as digestion, nutrient absorption, immune responses, and biological antibiosis (Sharawy et al., 2020; Li et al., 2018). The bacteria present in the gut demonstrate sensitivity to changes in food intake, diet composition, and its components (Ringø et al., 2016). Consistently, Mansour et al. (2022) observed a significant reduction ($p < 0.05$) in total heterotrophic bacteria (THB) and *Vibrio* spp. (TVC) counts in *P. vannamei* as AST inclusion levels increased, in respect to control group. In parallel, the quantity of bacteria of the genus *Vibrio* showed a decline in all diets enriched with AST compared to control group. Corroborating these findings, Chuchird et al. (2015) reported an increase in survival, growth, and resistance to *V. parahaemolyticus* in *P. vannamei* fed a diet supplemented with AST. Additionally, shrimp diets containing this oxycarotenoid resulted in significantly lower counts of total gut bacteria and *Vibrio* spp. (Chuchird et al., 2015). According to the aforementioned authors, the precise mechanism by which AST modulates the bacterial population remains uncertain and requires further investigation. In this regard, Mansour et al. (2022) concluded that AST presents itself as a promising compound in controlling the load of pathogenic bacteria throughout the shrimp culture cycle. These results were attributed to the significant biological activities of the acetone extract of *A. platensis* NIOF17/003, whose main component is astaxanthin, giving it the potential to be a remarkable, sustainable, and ecologically beneficial feed additive for aquaculture applications.

The high biological efficacy observed in this study, even with the limited sample size, can be attributed to nanoemulsion. Nanoencapsulation, using vegetable oils, increases the bioavailability and absorption of AST (Castro et al., 2020),

ensuring that the shrimp metabolize the compound efficiently, which is crucial for a rapid and robust immune response against an aggressive pathogen such as *V. parahaemolyticus*.

5 Conclusion

This study provides the first *in vivo* evidence that the developed AST nanoemulsion significantly increases serum protein levels and modulates phenoloxidase activity in *P. vannamei* treated with 70 mg kg^{-1} AST, and its potential application in animal feed due to its physicochemical characteristics represents a promising and sustainable alternative for shrimp farming. Future studies should focus on unraveling the molecular mechanisms underlying the observed effects, improving treatment strategies, and examining long-term effects.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The requirement of ethical approval was waived by Ethics Committee on Animal Use of the Federal University of Santa Catarina - CEUA/UFSC for the studies involving animals because In view of the request from the researchers responsible for the project entitled “Development and stability analysis of a lipid-based astaxanthin nanoemusion, its application and effect on the immune response and survival of the Pacific white shrimp,” we declare, for all due purposes and in accordance with the provisions of Law no 11,794/2008, that the Animal Use Ethics Committee (AEC) is only responsible for the analysis of projects involving animals of the phylum Chordata, subphylum Vertebrata (except humans). Therefore, the research project conducted with invertebrate animals is not within the scope of AEC’s activities and does not require analysis and approval of the research project by this instance. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

CPB: Formal Analysis, Resources, Visualization, Writing – original draft, Project administration, Data curation, Writing – review and editing, Conceptualization, Validation, Investigation, Supervision, Methodology, Software. CMB: Methodology, Data curation, Investigation, Software, Writing – original draft. CS: Investigation, Methodology, Data curation, Software, Writing – original draft. TG: Writing – original draft, Investigation, Software, Data curation, Methodology. ERdO: Software, Data curation, Writing – original draft, Investigation, Methodology. CZ: Methodology, Software, Writing – original draft, Data curation. NCBR: Validation, Project administration, Writing – review and editing, Formal Analysis, Supervision, Methodology, Data curation, Software. CSM: Methodology, Data curation, Software, Writing – review and editing, Project administration. MHdAM: Methodology, Project administration, Supervision, Writing – review and editing. FCdA: Project administration, Supervision, Methodology, Writing – review and editing. JVRH: Project administration, Writing – review and editing, Supervision, Methodology, Formal Analysis. FFR: Methodology, Project administration, Writing – review and editing, Supervision. IdAG: Supervision, Formal Analysis, Writing – review and editing, Project administration, Methodology. MdSG: Methodology, Data curation, Supervision, Writing – review and editing, Formal Analysis. CCC: Supervision, Writing – review and editing, Formal Analysis, Project administration. SAPD: Visualization, Methodology, Data curation, Investigation, Validation, Conceptualization, Project administration, Writing – review and editing, Supervision, Software, Formal Analysis. FBV: Resources, Writing – review and editing, Methodology, Data curation, Formal Analysis, Supervision, Conceptualization, Visualization, Validation. LM: Supervision, Methodology, Conceptualization, Software, Writing – original draft, Investigation, Funding acquisition, Writing – review and editing, Visualization, Formal Analysis, Validation, Resources, Data curation. MBdRV: Data curation, Funding acquisition, Writing – original draft, Conceptualization, Visualization, Supervision, Software, Writing – review and editing, Validation, Investigation, Resources, Formal Analysis, Methodology. MM: Visualization, Data curation, Methodology, Validation, Project administration, Conceptualization, Writing – original draft, Software, Funding acquisition, Supervision, Writing – review and editing, Resources, Formal Analysis, Investigation.

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Conflict of interest

Nanoscoping Soluções em Nanotecnologia Ltd., represented by LM and MBdRV, was involved in planning of the study.

Remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that generative AI was not used in the creation of this manuscript.

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References

Abdol Wahab, N. R., Meor Mohd Affandi, M. M. R., Fakurazi, S., Alias, E., and Hassan, H. (2022). Nanocarrier system: state-of-the-art in oral delivery of astaxanthin. *Antioxidants* 11 (9), 1676. doi:10.3390/antiox11091676

Abdou, E. S., Galhoum, G. F., and Mohamed, E. M. (2018). Curcumin loaded nanoemulsions/pectin coatings for refrigerated chicken fillets. *Food Hydrocoll.* 83, 445–453. doi:10.1016/j.foodhyd.2018.05.026

Ahmad, M., An, S., and Zhang, X. (2019). Astaxanthin nanoemulsion improves growth and immunity in shrimp. P. Vannamei. *Aquac. Nutr.* 25 (6), 1284–1293. doi:10.1111/anu.12867

Ambati, R. R., Phang, S. M., Ravi, S., and Aswathanarayana, R. G. (2014). Astaxanthin: sources, extraction, stability, biological activities and its commercial applications - a review. *Mar. Drugs* 12, 128–152. doi:10.3390/md12010128

Amirkhiz, K. F., Dehaghi, M. A., Sanavy, S. A. M. M., and Rezazadeh, A. (2021). Evaluation of changes in fatty acid profile, grain, and oil yield of *Carthamus tinctorius* L. in response to foliar application of polyamine compounds under deficit irrigation conditions. *Industrial Crops Prod.* 161, 113231. doi:10.1016/j.indcrop.2020.113231

ANVISA - Agência Nacional de Vigilância Sanitária. (2012). *RESOLUÇÃO - RDC No 45, DE 9 DE AGOSTO DE 2012* 45. Available online at: https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2012/rdc0045_09_08_2012.html. (Accessed August 10, 2021).

AOAC (1999). *Official methods of analysis of the association of official analytical chemists*. 17th ed. Washington, D.C., USA: Association of Official Analytical Chemists.

APHA - American Public Health Association (2005). *Standard methods for the examination of the water and wastewater*. 21st ed.

Araújo, F. A., Kelmann, R. G., Araújo, B. V., Finatto, R. B., Teixeira, H. F., and Koester, L. S. (2011). Development and characterization of parenteral nanoemulsions containing thalidomide. *Eur. J. Pharm. Sci.* 42 (3), 238–245. doi:10.1016/j.ejps.2010.11.014

Banasaz, S., Morozova, K., Ferrentino, G., and Scampicchio, M. (2020). Encapsulation of lipid-soluble bioactives by nanoemulsions. *Molecules* 25 (17), 3966. doi:10.3390/molecules25173966

Barracco, M. A., Perazzolo, L. M., and Rosa, R. D. (2014). "Avances em la Inmunología del Camarón," in *Guía Técnica de Patología y Inmunología de Camarones Penaeidos*. Editors M. Q. Vielka and J. Cuéllar-Anjel (CYTED), 237–288.

Beliciu, C. M., and Moraru, C. I. (2009). Effect of solvent and temperature on the size distribution of casein micelles measured by dynamic light scattering. *J. Dairy Sci.* 92, 1829–1839. doi:10.3168/jds.2008-1467

Beltramini, M., Colangelo, N., Giomi, F., Bubacco, L., Di Muro, P., Hellmann, N., et al. (2005). Quaternary structure and functional properties of Penaeus monodon hemocyanin. *FEBS J* 272:2060–2075. doi:10.1111/j.1742-4658.2005.04634.x

Boyd, C. E., and Gautier, D. (2000). Effluent composition and water quality standards. *Glob. Aquac. Advocate* 3 (5), 61–66. Available online at: https://www.globalseafood.org/advocate/effluent-composition-and-water-quality-standards/ (Accessed May 5, 2021).

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72 (1–2), 248–254. doi:10.1006/abio.1976.9999

Calabrese, E. J., and Baldwin, L. A. (2002). Defining hormesis. *Hum. and Exp. Toxicol.* 21 (2), 91–97. doi:10.1191/0960327102ht217oa

Castro, G. M. M. A., Passos, T. S., Nascimento, S. S. da C., Medeiros, I., Araújo, N. K., Maciel, B. L. L., et al. (2020). Gelatin nanoparticles enable water dispersibility and potentialize the antimicrobial activity of Buriti (*Mauritia flexuosa*) oil. *Bmc. Biotechnoly.* 20 (1), 55. doi:10.1186/s12896-020-00649-4

Chauhan, I., Yasir, M., Verma, M., and Singh, A. P. (2020). Nanostructured lipid carriers: a groundbreaking approach for transdermal drug delivery. *Adv. Pharmaceutical Bulletin* 10 (2), 150–165. doi:10.34172/apb.2020.021

Chen, F., Sun, X., and Zheng, D. (2017). Effects of astaxanthin on oxidative stress and immune responses in crustaceans. *Fish and Shellfish Immunol.* 60, 337–344. doi:10.1016/j.fsi.2016.11.053

Chen, Z., Kong, Y., Huang, Z., Zheng, X., Zheng, Z., Yao, D., et al. (2024). Exogenous alpha-linolenic acid and *Vibrio parahaemolyticus* induce EPA and DHA levels mediated by delta-6 desaturase to enhance shrimp immunity. *Int. J. Biol. Macromol.* 257 (2), 128583. doi:10.1016/j.ijbiomac.2023.128583

Cheng, W., and Chen, J. C. (2001). Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. *Fish Shellfish Immunol.* 11 (1), 53–63. doi:10.1006/fsim.2000.0293

Chuchird, N., Rorkwiree, P., and Rairat, T. (2015). Effect of dietary formic acid and astaxanthin on the survival and growth of Pacific white shrimp (*Litopenaeus vannamei*) and their resistance to *Vibrio parahaemolyticus*. *SpringerPlus* 4 (1), 440–512. doi:10.1186/s40064-015-1234-x

Costantini, D. (2019). Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. *J. Exp. Biol.* 222 (13). doi:10.1242/jeb.194688

CTNBio - National Technical Commission on Biosafety (1997). Normative instruction N° 7, of 06.06. Available online at: https://antigo.mctic.gov.br/mctic/opencms/legislacao/outros_atos/instrucoes_normativas/Instrucao_Normativa_CTNBio_n_7_de_06061997.html#:~:text=EQUIPAMENTOS%20DE%20CONTEN%C3%87%C3%83O%20PARA%20O,da%20cabine%20de%20seguran%C3%A7a%20biol%C3%B3gica (Accessed May 21, 2025).

Cunha, J. P. A. R., Alves, G. S., and Reis, E. F. (2010). Temperature effect on the physical-chemical characteristics of aqueous solutions with spray adjuvants. *Planta Daninha* 28 (3), 665–672. doi:10.1590/S0100-83582010000300024

Dos Santos, P. P., Andrade, L. d.A., Flôres, S. H., and Rios, A. D. O. (2018). Nanoencapsulation of carotenoids: a focus on different delivery systems and evaluation parameters. *J. Food Sci. Technol.* 55, 3851–3860. doi:10.1007/s13197-018-3316-6

Elbahnaswy, S., and Elshopakey, G. E. (2024). Recent progress in practical applications of a potential carotenoid astaxanthin in aquaculture industry: a review. *Fish Physiology Biochem.* 50 (1), 97–126. doi:10.1007/s10695-022-01167-0

Eldessouki, E. A. A., Diab, A. M., Selema, T. A. M. A., Sabry, N. M., Abotaleb, M. M., Khalil, R. H., et al. (2022). Dietary astaxanthin modulated the performance, gastrointestinal histology, and antioxidant and immune responses and enhanced the resistance of *Litopenaeus vannamei* against *Vibrio harveyi* infection. *Aquac. Int.* 30, 1869–1887. doi:10.1007/s10499-022-00876-w

Farkas, N., and Kramar, J. A. (2021). Dynamic light scattering distributions by any means. *J. Nanoparticle Res.* 23, 120. doi:10.1007/s11051-021-05220-6

Fath El-Bab, A. F., El-Ratel, I. T., Abdel-Warith, A. A., Younis, E. M., Davies, S. J., and El-Raghi, A. A. (2024). Investigating the impact of nanoemulsion of curcumin-loaded olive oil on growth performance, feed utilization, immunological responses, and redox status of *Litopenaeus vannamei* shrimp with emphasis on economic efficiency of supplementation. *J. Animal Physiology Animal Nutr.* 108 (6), 1877–1889. doi:10.1111/jpn.14027

Flores, M., Díaz, F., Medina, R., Re, A. D., and Licea, A. (2007). Physiological, metabolic and haematological responses in white shrimp *Litopenaeus vannamei* (Boone) juveniles fed diets supplemented with astaxanthin acclimated to low-salinity water. *Aquac. Res.* 38 (7), 740–747. doi:10.1111/j.1365-2109.2007.01720.x

Gaumet, M., Vargas, A., Gurny, R., and Delie, F. (2008). Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *Eur. Journal Pharmaceutics Biopharmaceutics* 69 (1), 1–9. doi:10.1016/j.ejpb.2007.08.001

Ghaffarizadeh, A., Sotoudeh, E., Mozanzadeh, M. T., Sanati, A. M., and Ghasemi, A. (2022). Supplementing dietary selenium nano-particles increased growth, antioxidant capacity and immune-related genes transcription in Pacific white leg shrimp (*Penaeus vannamei*) juveniles. *Aquac. Rep.* 25, 101215. doi:10.1016/j.aqrep.2022.101215

Gonçalves, P., Vernal, J., Rosa, R. D., Yepiz-Plascencia, G., de Souza, C. R. B., Barracco, M. A., et al. (2012). Evidence for a novel biological role for the multifunctional β-1,3-glucan binding protein in shrimp. *Mol. Immunol.* 51 (3-4), 363–367. doi:10.1016/j.molimm.2012.03.032

González-Félix, M. L., Gatlin, D. M., Lawrence, A. L., and Perez-Velazquez, M. (2002). Effect of various dietary lipid levels on quantitative essential fatty acid requirements of

juvenile Pacific white shrimp *Litopenaeus vannamei*. *J. World Aquac. Soc.* 33 (3), 330–340. doi:10.1111/j.1749-7345.2002.tb00509.x

Guerin, M., Huntley, M. E., and Olaizola, M. (2003). *Haematococcus* astaxanthin: applications for human health and nutrition. *Trends Biotechnol.* 21 (5), 210–216. doi:10.1016/S0167-7799(03)00078-7

Gustilatov, M., Ekasari, J., and Pande, G. S. J. (2022). Protective effects of the biofloc system in Pacific white shrimp (*Penaeus vannamei*) culture against pathogenic *Vibrio parahaemolyticus* infection. *Fish and Shellfish Immunol.* 124, 66–73. doi:10.1016/j.fsi.2022.03.037

Hartman, L., and Lago, R. C. A. (1973). Rapid preparation of fatty acid methyl esters. *Laboratory Practice*, 22 (6), 475–494. Available online at: https://www.researchgate.net/profile/Regina-Lago-3/publication/18441624_Rapid_preparation_of_fatty_acid_methyl_esters/links/0c960539edbca2127f000000/Rapid-preparation-of-fatty-acid-methyl-esters.pdf (Accessed May 8, 2021).

ICH (2003). Stability testing of new drug substances and products Q1A(R2). Available online at: https://www.ema.europa.eu/en/ich-q1a-r2-stability-testing-new-drug-substances-drug-products-scientific-guideline (Accessed August 10, 2021).

Jasmin, M. Y., Syukri, F., Kamarudin, M. S., and Karim, M. (2020). Potential of bioremediation in treating aquaculture sludge: review article. *Aquaculture* 519, 734905. doi:10.1016/j.aquaculture.2019.734905

Jeevanandam, J., Chan, Y. S., and Danquah, M. K. (2016). Nano-formulations of drugs: recent developments, impact and challenges. *Biochimie* 128, 99–112. doi:10.1016/j.biochi.2016.07.008

Jia, Z., Xu, Y., Wang, J., and Song, R. (2021). Antioxidant activity and degradation kinetics of astaxanthin extracted from Penaeus sinensis (*Solenocera crassicornis*) byproducts under pasteurization treatment. *LWT – Food Sci. Technol.* 152, 112336. doi:10.1016/j.lwt.2021.112336

Juttulapa, M., Piriyaprasarth, S., Takeuchi, H., and Sriamornsak, P. (2017). Effect of high-pressure homogenization on stability of emulsions containing zein and pectin. *Asian J. Pharm. Sci.* 12 (1), 21–27. doi:10.1016/j.ajps.2016.09.004

Kewcharoen, W., and Srisapoome, P. (2019). Probiotic effects of *Bacillus* spp. from Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). *Fish and Shellfish Immunol.* 94, 175–189. doi:10.1016/j.fsi.2019.09.013

Kumar, V., Habte-Tsion, H.-M., Allen, K. M., Bowman, B. A., Thompson, K. R., El-Haroun, E., et al. (2018). Replacement of fish oil with *Schizochytrium* meal and its impacts on the growth and lipid metabolism of Pacific white shrimp (*Litopenaeus vannamei*). *Aquac. Nutr.* doi:10.1111/anu.12816

Kumar, S., Verma, A. K., Singh, S. P., and Awasthi, A. (2022). Immunostimulants for shrimp aquaculture: paving pathway towards shrimp sustainability. *Environ. Sci. Pollut. Res.* 30, 25325–25343. doi:10.1007/s11356-021-18433-y

Lacatusu, I., Badea, N., Niculae, G., Bordei, N., Stan, R., and Meguea, A. (2014). Lipid nanocarriers based on natural compounds: an evolving role in plant extract delivery. *Eur. J. Lipid Sci. Technol.* 116, 1708–1717. doi:10.1002/ejlt.201300488

Li, Y., Sommerfeld, M., Chen, F., and Hu, Q. (2011). Consumption of oxygen by astaxanthin biosynthesis: a protective mechanism against oxidative stress in *Haematococcus pluvialis* (chlorophyceae). *Bioresour. Technol.* 102, 860–864. doi:10.1016/j.jplph.2007.12.007

Li, E., Xu, C., Wang, X., Wang, S., Zhao, Q., Zhang, M., et al. (2018). Gut microbiota and its modulation for healthy farming of Pacific white shrimp *Litopenaeus vannamei*. *Rev. Rev. Fish. Sci. and Aquac.* 26 (3), 381–399. doi:10.1080/23308249.2018.1440530

Li, F., Huang, J., Wang, M., Chen, L., and Xiao, Y. (2021). Sources, distribution and dynamics of antibiotics in *Litopenaeus vannamei* farming environment. *Aquaculture* 545, 737200. doi:10.1016/j.aquicultura.2021.737200

Li, W., Li, Y., Zheng, N., Ge, C., and Yao, H. (2022a). Occurrence and distribution of antibiotics and antibiotic resistance genes in the guts of shrimp from different coastal areas of China. *Sci. Total Environ.* 815, 152756. doi:10.1016/j.scitotenv.2021.152756

Li, W., Wang, J., Li, J., Liu, P., and Li, J. (2022b). Transcriptomics revealed the effect of astaxanthin on apoptosis and immunity of the adult prawn of *Exopalaemon carinicauda*. *Fish and Shellfish Immunol.* 131, 480–486. doi:10.1016/j.fsi.2022.09.065

Li, M., Li, X., Yao, W., Wang, Y., Li, L., and Leng, X. (2024). Effects of substituting fish meal by chlorella meal in practical diet on growth, feed utilization, and flesh quality of Pacific white shrimp (*Penaeus vannamei*). *Aquac. Nutr.* 19, 9969518. doi:10.1155/2024/9969518

Lim, C., Ako, H., Brown, C. L., and Hahn, K. (1997). Growth response and fatty acid composition of juvenile *Penaeus vannamei* fed different sources of dietary lipid. *Aquaculture* 151, 143–153. doi:10.1016/S0044-8486(96)01500-1

Lim, K. C., Yusoff, F., Shariff, M., and Kamarudin, M. S. (2018). Astaxanthin as a feed supplement in aquatic animals. *Rev. Aquac.* 1, 1–36. doi:10.1111/raq.12200

Lin, C. C., Lin, H. Y., Chi, M. H., Shen, C. M., Chen, H. W., Yang, W. J., et al. (2014). Preparation of curcumin microemulsions with food-grade soybean oil/lecithin and their cytotoxicity on the HepG2 cell line. *Food Chem.* 154, 282–290. doi:10.1016/j.foodchem.2014.01.012

Lin, Y. J., Chang, J. J., Huang, H. T., Lee, C. P., Hu, Y. R., Wu, M. L., et al. (2023). Improving red-color performance, immune response and resistance to *Vibrio parahaemolyticus* on white shrimp *Penaeus vannamei* by an engineered astaxanthin yeast. *Sci. Reports* 13, 2248. doi:10.1038/s41598-023-29225-4

Liu, X., and Osawa, T. (2007). Cis astaxanthin and especially 9-cis astaxanthin exhibits a higher antioxidant activity *in vitro* compared to the all-trans isomeres. *Bioquimical Biophysical Res. Commun.* 357 (1), 187–193. doi:10.1016/j.bbrc.2007.03.120

Liu, Q., Huang, H., Chen, H., Lin, J., and Wang, Q. (2019). Food-grade nanoemulsions: preparation, stability and application in encapsulation of bioactive compounds. *Molecules* 24. doi:10.3390/molecules24234242

Maggioni, E. S., Andreatta, E. R., Hermes, E. M., and Barracco, M. A. (2004). Evaluation of some hemato-immunological parameters in female shrimp *Litopenaeus vannamei* submitted to unilateral eyestalk ablation in association with a diet supplemented with superdoses of ascorbic acid as a form of immunostimulation. *Aquaculture* 241, 501–515. doi:10.1016/S0044-8486(03)00530-1

Mansour, A. T., Ashour, M., Abbas, E. M., Alsaquf, A. S., Kelany, M. S., El-Sawy, M. A., et al. (2022). Growth performance, immune-related and antioxidant genes expression, and gut bacterial abundance of Pacific white leg shrimp, *Litopenaeus vannamei*, dietary supplemented with natural astaxanthin. *Front. Physiology* 13, 874172. doi:10.3389/fphys.2022.874172

Martínez-Álvarez, Ó., Calvo, M. M., and Gómez-Estaca, J. (2020). Recent advances in astaxanthin micro/nanoencapsulation to improve its stability and functionality as a food ingredient. *Mar. Drugs* 18 (8), 406. doi:10.3390/md18080406

Marucco, A., Aldieri, E., Leinardi, R., Bergamaschi, E., Riganti, C., and Fenoglio, I. (2019). Applicability and limitations in the characterization of poly-dispersed engineered nanomaterials in cell media by dynamic light scattering (DLS). *Materials* 12 (23), 3833. doi:10.3390/ma12233833

Mattos, C. B., Argenta, D. F., Melchiades, G. L., Cordeiro, M., Tonini, M. L., Moraes, M. H., et al. (2015). Nanoemulsions containing a synthetic chalcone as an alternative for treating cutaneous leshmaniasis: optimization using a full factorial design. *Int. J. Nanomedicine* 5529, 5529. doi:10.2147/ijn.s83929

Mazzarino, L., Pitz, H. S., Voytena, A. P. L., Trevisan, A. C. D., Ribeiro-Do-Valle, R. M., and Maraschin, M. (2017). Jaboticaba (*Plinia peruviana*) extract nanoemulsions: development, stability, and *in vitro* antioxidant activity. *Drug Dev. Industrial Pharm.* 44 (4), 643–651. doi:10.1080/03639045.2017.1405976

McClements, D. J. (2015). *Food Emulsions: Principles, Practices, and Techniques*. 3rd Edn. CRC. doi:10.1201/b18868

McClements, D. J., Bai, L., and Chung, C. (2017). Recent advances in the utilization of natural emulsifiers to form and stabilize emulsions. *Annu. Rev. Food Sci. Technol.* 8, 205–236. doi:10.1146/annurev-food-030216-030154

Mistry, P. H., Mohapatra, S. K., and Dash, A. K. (2012). Effect of high-pressure homogenizationand stabilizers on the physicochemical properties of curcumin-loaded glycerol monooleate/chitosan nanostructures. *Nanomedicine* 7 (12), 1863–1876. doi:10.2217/nnm.12.49

Miyakawa, K. (2021). Commercial production of astaxanthin from the green alga *Haematococcus pluvialis*. *Adv. Exp. Med. Biology* 1261, 3–10. doi:10.1007/978-981-15-7360-6_1

Montoya-Anaya, D. G., Madera-Santana, T. J., Aguirre-Mancilla, C. L., Grijalva-Verdugo, C. P., Gonzáles-García, G., Núñez-Colín, C. A., et al. (2023). Caracterización fisicoquímica de almidón recuperado de papa (*Solanum tuberosum*) residual de la industria de papas fritas en México: Caracterización fisicoquímica de almidón recuperado de papa. *Biotecnia* 25 (2), 60–72. doi:10.18633/biotecnia.v25i2.1880

Niu, J., Tian, L. X., Liu, Y. J., Yang, H. J., Ye, C. X., Gao, W., et al. (2009). Effect of dietary astaxanthin on growth, survival, and stress tolerance of postlarval shrimp, *Litopenaeus vannamei*. *J. World Aquac. Soc.* 40 (6), 795–802. doi:10.1111/j.1749-7345.2009.00300.x

Nonwachai, T., Purivirojkul, W., Limsuwan, C., Chuchird, M. V., and Dhar, A. K. (2010). Growth, nonspecific immune characteristics, and survival upon challenge with *Vibrio harveyi* in Pacific white shrimp (*Litopenaeus vannamei*) raised on diets containing algal meal. *Fish. &Shellfish Immunol.* 29, 298–304. doi:10.1016/j.fsi.2010.04.009

NRC (2011). *Nutrient requirements of fish and shrimp*. 3 ed. Washington: The National Academies Press, 376.

N'Souvi, K., and Sun, C. (2025). Do exports and domestic prices influence aquaculture shrimp production: evidence from the key South and Southeast Asian countries. *Aquac. Int.* 33 (1), 42. doi:10.1007/s10499-024-01693-z

Pan, J. Y., Zhang, Y. L., Wang, S. Y., and Peng, X. X. (2008). Dodecamer is required for agglutination of *Litopenaeus vannamei* hemocyanin with bacterial cells and red blood cells. *Mar. Biotechnol.* 10, 645–652. doi:10.1007/s10126-008-9115-8

Prasert, W., and Gohtani, S. (2016). Effect of temperature on low-energy nanoemulsification and phase behavior in water/polyoxyethylene sorbitan fatty acid ester (Tweens)/vegetable oil systems. *J. Food Eng.* 180, 101–109. doi:10.1016/j.jfoodeng.2016.02.014

Rao, J., and McClements, D. J. (2012). Stability of antioxidant-loaded nanoemulsions to simulated environmental stress conditions. *Food Hydrocoll.* 26 (2), 350–356. doi:10.1016/j.foodhyd.2011.02.003

Ringø, E., Zhou, Z., Vecino, J. L. G., Vencino, S., Wadsworth, J., Romero, A., et al. (2016). Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquac. Nutr.* 22 (2), 219–282. doi:10.1111/anu.12346

Rostamabadi, H., Falsafi, S. R., and Jafari, S. M. (2020). Transmission electron microscopy (TEM) of nanoencapsulated food ingredients. *Charact. Nanoencapsulated Food Ingredients* 4, 53–82. doi:10.1016/B978-0-12-815667-4.00002-X

Rudin, A., and Choi, P. (2013). Dispersion and emulsion polymerizations. *Elem. Polym. Sci. and Eng.*, 427–447. doi:10.1016/B978-0-12-382178-2.00010-9

Sabaghnia, N., Fattahi, M., Janmohammadi, M., and Abbasi, A. (2024). Graphic analysis of various sulfur applications on safflower fatty acids profile. *J. Biotechnol. Comput. Biol. Bionanotechnol.* 105 (1), 33–39. doi:10.5114/bta.2024.135640

Santos, R. N. F. D., Passos, T. S., Fernandes, R. S., Matsui, K. N., de Sousa Júnior, F. C., Damasceno, K. S. F. S. C., et al. (2024). Effect of nanoencapsulation on the solubility and antioxidant activity of astaxanthin pigmented oil extracted from shrimp waste (*Litopeneaus vannamei*). *PLoS ONE* 19 (11), e0313059. doi:10.1371/journal.pone.0313059

Schleder, D. D., Rosa, J. R., Guimarães, A. M., Ramlov, F., Maraschin, M., Seiffert, W. Q., et al. (2017). Synergistic effect of two brown seaweeds as feed additive fot white-leg shrimp: effects on thermal stress resistance, midgut microbiology, and immunology. *J. Appl. Phycol.* 29, 2471–2477. doi:10.1007/s10811-017-1129-z

Schuh, R. S., Bruxel, F., and Teixeira, H. F. (2014). Physicochemical properties of lecithin-based nanoemulsions obtained by spontaneous emulsification or high-pressure homogenization. *Quimica Nova* 37 (7), 1193–1198. doi:10.5935/0100-4042.20140186

Sharawy, Z. Z., Ashour, M., Abbas, E., Ashry, O., Helal, M., Nazmi, H., et al. (2020). Effects of dietary marine microalgae, *Tetraselmis suecica*, on production, gene expression, protein markers and bacterial count of pacific white shrimp *Litopenaeus vannamei*. *Aquac. Res.* 51 (6), 2216–2228. doi:10.1111/are.14566

Shinn, A. P., Pratoomyot, J., Griffiths, D., Trong, T. Q., Vu, N. T., Jiravanichpaisal, P., et al. (2018). Asian shrimp production and the economic costs of disease. *Asian Fischeries Sci.* 31S, 29–58. doi:10.33997/j.afs.2018.31.S1.003

Söderhäll, K., and Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* 10 (1), 23–28. doi:10.1016/s0952-7915(98)80026-5

Söderhäll, K., and Häll, L. (1984). Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. *Biochem. Biophys. Acta* 797, 99–104. doi:10.1016/0304-4165(84)90387-8

Soukoulis, C., and Bohn, T. (2018). A comprehensive overview on the micro- and nano-technological encapsulation advances for enhancing the chemical stability and bioavailability of carotenoids. *Crit. Rev. Food Sci. Nutr.* 58, 1–36. doi:10.1080/10408398.2014.971353

Sühnel, S., Lagreze, F., Ferreira, J. F., Campestrini, L. H., and Maraschin, M. (2009). Carotenoid extraction from the gonad of the scallop *Nodipecten nodosus* (Linnaeus, 1758) (Bivalvia: pectinidae). *Braz. J. Biol.* 69 (1), 209–215. doi:10.1590/S1519-69842009000100028

Tadros, T. F. (2013). *Emulsion formation and stability*. Wiley-VCH.

Tan, K., Zhang, H., Leong-Seng, L., Ma, H., Li, S., and Zheng, H. (2020). Roles of carotenoids in invertebrate immunology. *J. Front. Immunol.* 10. doi:10.3389/fimmu.2019.03041

Taylor, P. (1995). Ostwald ripening in emulsions. *Colloids Surfaces A Physicochem. Eng. Aspects* 99 (2-3), 175–185. doi:10.1016/0927-7757(95)03161-6

Van Wyk, P. M., Davis-Hodgkins, M., Laramore, R., Main, K. L., and Scarpa, J. (1999). Farming marine shrimp in recirculating fresh water systems. *Flórida Harb. Branch Oceanogr. Inst.* Available online at: https://www.researchgate.net/profile/John-Scarpa/publication/242621708_Farming_Marine_Shrimp_in_Recirculating_Fresh_Water_Systems/links/574c4f0508ae8d6e6a7b678c/Farming-Marine-Shrimp-in-Recirculating-Fresh-Water-Systems.pdf (Accessed September 30, 2023).

Wang, H., Dai, A., Liu, F., and Guan, Y. (2015). Effects of dietary astaxanthin on the immune response, resistance to white spot syndrome virus and transcription of antioxidante enzyme genes in Pacific white shrimp *Litopenaeus vannamei*. *Iran. J. Fish. Sci.* 14 (3), 699–718. doi:10.22092/IJFS.2018.114476

Wang, W., Ishikawa, M., Koshio, S., Yokoyama, S., Sakhawat Hossain, M., and Moss, A. S. (2018). Effects of dietary astaxanthin supplementation on juvenile kuruma shrimp, *Marsupenaeus japonicus*. *Aquaculture* 491, 197–204. doi:10.1016/j.aquaculture.2018.03.025

Wei, B., Yang, Z., Cheng, Y., Wang, J., and Zhou, J. (2018). Label-free quantification proteomics reveals the effects of dietary fish oil and soybean oil on the immune response of Chinese mitten crab, *Eriocheir sinensis*. *Aquac. Res.* 49 (9), 2927–2937. doi:10.1111/are.13787

Xu, Z., Liu, A., Li, S., Wang, G., and Ye, H. (2020). Hepatopancreas immune response during molt cycle in the mud crab, *Scylla paramamosain*. *Sci. Rep.* 10 (1), 13102. doi:10.1038/s41598-020-70139-2

Yamada, S., Tanaka, Y., Sameshima, M., and Ito, Y. (1990). Pigmentation of prawn (*Penaeus japonicus*) with carotenoids: I. Effect of dietary astaxanthin, β-carotene and canthaxanthin on pigmentation. *Aquiculture* 87 (3–4), 323–330. doi:10.1016/0044-8486(90)90069-y

Yuan, J. P., and Chen, F. (1999). Isomerization of trans-astaxanthin to cis-Isomers in organic solvents. *J. Agric. Food Chem.* 47 (9), 3656–3660. doi:10.1021/jf981319u

Yuan, Y., Gao, Y., Zhao, J., and Mao, L. (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Res. Int.* 41, 61–68. doi:10.1016/j.foodres.2007.09.006

Zhang, Y., Yan, F., Hu, Z., Zhao, X., Min, S., Du, Z., et al. (2009). Hemocyanin from shrimp *Litopenaeus vannamei* shows hemolytic activity. *Fish and Shellfish Immunol.* 27 (2), 330–335. doi:10.1016/j.fsi.2009.05.017

Zhang, T., Wen, B., Huo, X., Ren, J., and Chen, X. (2025). Dietary supplementation of Adonis-derived astaxanthin enhances antioxidant capacity and immune response in *Litopenaeus vannamei*. *Comp. Immunol. Rep.* 7, 200233. doi:10.1016/j.cirep.2025.200233

Zheng, J. Y., Gan, Z. R., Huang, T. Y., Xiao, Y., Xu, W. Y., Li, X. Q., et al. (2024). Replacement of fish meal with defatted black soldier fly (*Hermetia illucens*) in diet of Pacific white shrimp (*Litopenaeus vannamei*): growth, flesh quality and transcriptome. *J. Insects As Food Feed* 11 (5), 873–893. doi:10.1163/23524588-00001235

Zhou, Q.-C., Li, C.-C., Liu, C.-W., Chi, S.-Y., and Yang, Q.-H. (2007). Effects of dietary lipid sources on growth and fatty acid composition of juvenile shrimp, *Litopenaeus vannamei*. *Aquac. Nutr.* 13 (3), 222–229. doi:10.1111/j.1365-2095.2007.00470.x

Zhou, Y. G., Davis, D., and Buentello, A. (2014). Use of new soybean varieties in practical diets for the Pacific white shrimp. *Litopenaeus Vannamei. Aquac. Nutr.* 21 (5), 635–643. doi:10.1111/anu.12181

Zhou, X., Wang, Y., and Wang, Y. (2016). Dietary astaxanthin modulates immune response and antioxidant capacity of shrimp under stress. *Fish and Shellfish Immunol.* 58, 159–165. doi:10.1016/j.fsi.2016.09.018

Zuo, H., Gao, J., Yuan, J., Deng, H., Yang, L., Weng, S., et al. (2017). Fatty acid synthase plays a positive role in shrimp immune responses against *Vibrio parahaemolyticus* infection. *Fish and Shellfish Immunol.* 60, 282–288. doi:10.1016/j.fsi.2016.11.054