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Synergetic Effects of Green Algae and Diatoms on Reproductive Hormones and Health Status of Whiteleg Shrimp, Litopenaeus vannamei Broodstock in Commercial Hatcheries

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Abstract:

This study investigated the effects of dietary addition of green algae and diatoms on reproductive hormones, redox status, and immunity response, with special emphasis to the influence of these phytobiotics on the expression of interlukine-1β (IL-1β) in Whiteleg Shrimp (Litopenaeus vannamei). The experiment was conducted during the 2022 season, during which the broodstock were fed equal proportions of different components: Spirulina platensis (SP) at 5%, T. weissflogii (TH) at 5% and a mixture of SP and TH in a 2.5:2.5 ratio as well as the control group (basal diet). The levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) and progesterone were significantly higher in the groups fed TH alone or together with SP compared to the control group ($p<0.05$). The higher concentration of estrogen hormone was observed in TH treated group compared to the control and the other treated groups ($p<0.05$). With regard to male reproductive hormone, non- significant changes were detected between the control and all examined groups for FSH ($p=0.1504$) and LH ($p=0.4550$). Meanwhile the concentration of testosterone improved significantly in the TH group compared to the control or other treated groups ($p<0.05$). Improved photomicrograph of ovarian lobules, ejaculatory and seminiferous duct through co-treatment with SP and TH supported these findings. The levels of malondialdehyde were notably shrunken in the group that received TH alone or in combination with SP compared to the control group. Conversely, the transcription of interlukine-1 β, catalase activity, and the concentrations of immunoglobulin G and M significantly induced in aforementioned two treated groups compared to the control and SP treated group ($p<0.05$). The values of glutathione, total antioxidant capacity and superoxide dismutase activity were significantly higher in TH treated group compared to the control ($p<0.05$). In conclusion, incorporating a blend of SP and TH in a 2.5:2.5 ratio into the diet of Whiteleg Shrimp could serve as an effective strategy to improve the reproductive hormones, immunity status, and redox equilibrium during spawning season .

INTRODUCTION

In The global demand for animal protein is expected to double by 2050 due to the continuous growth of the global population and an increase in protein consumption per capita (Henchion et al., 2017). The global aquaculture provides amount of protein accounted for 8% of total animal source protein consumed by humans (Boyd et al., 2022). Additionally, the consumption of aquaculture products is increasing per capita faster than meat and dairy products (Schar et al., 2020). The aquafeed industry is still relies heavily on marine ingredients excluded from wild-caught forage fish (Naylor et al., 2021). Therefore, it is crucial to explore suitable and sustainable alternatives to fish oil and fishmeal to achieve the sustainable expansion of aquaculture production.

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Spirulina platensis, is a widely utilized microalgae that offers a highly nutritional profile. It contains high crude protein content, accounting for 59%-63% of dry weight, adequate amount of n-3 polyunsaturated fatty acids (γ-linoleic acid), minerals and essential vitamins (Alagawany et al., 2021, Grosshagauer et al., 2020). Notably, Spirulina platensis also contains bioactive substances such as phenols, chlorophylls, β-carotene, and phycobiliprotein, which exhibit various biological properties, including antioxidant, antimicrobial, and anti-inflammatory activities (Mala et al., 2009; Lin et al., 2010; Carcea et al., 2015, Chentir et al., 2018). Recent research has shown that Spirulina algae can be cultivated in aquaculture effluent (Cardoso et al., 2020, Cardoso et al., 2021). This approach holds promise for sustainable aquaculture and wastewater management.

Recently, considerable studies are accumulating about the use of spirulina meal in aquatic feed. Studies have explored the beneficial effects of incorporating spirulina meal as a functional additive in diet formulations, typically using comparatively lower levels of supplementation. These studies have primarily investigated the potential effects of dietary spirulina platensis meal supplementation on reproduction (Ibrahem et al., 2013), antioxidative properties (Xia et al., 2021), pigmentation capabilities (Ren et al., 2021, Ren et al., 2022), and immunoprotective effects (Mohammadiazarm et al., 2021) which could be attributed to its aforementioned bioactive compounds. Moreover, other studies have treated spirulina meal as a protein source in aquatic animal diets, aiming to replace other high protein sources or fishmeal due to its high protein content. Higher supplemented levels of spirulina meal were examined in these studies. The nutritional properties of spirulina and the feasibility of incorporating spirulina meal into aquaculture diets have been well documented in descriptive analyses (Rosas and Poersch, 2018; Rosas et al., 2019; Ragaza et al., 2020).

Marine diatoms are known for their abundant supply of biologically substantial highly unsaturated fatty acids (HUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). These lengthy HUFA chains are recognized to be absent in terrestrial feed oil sources like soybean oil and other seed oils. Studies have showed a direct relationship between the oil produced by marine diatoms and the presence of HUFAs in marine animal flesh (Brown, 2002). Marine diatoms like Chaetoceros and Thalassiosira have been observed to possess the highest contents from EPA and DHA fatty acids, ranging from 11 to 19% of the total fatty acids (Volkman et al., 1989). Several former studies have demonstrated that incorporating Thalassiosira weissflogii as a live feed for shrimp larvae and copepods can significantly improve the nutrient profile and fatty acid composition (Arendt et al., 2005; Kiatmetha et al., 2011; Sandeep et al., 2021). The aim of the present study is to investigate the biological effects of T. weissflogii and Spirulina platensis, both individually and in combination, on reproductive hormones, redox status, and immunity response, with special emphasis to the modulation of these phytobiotics on the expression of interlukine-1β (IL-1β) in Litopenaeus vannamei shrimp.

MATERIALS AND METHODS

1. Location and time:

The experiment was conducted during the 2022 season, at the Al-Ekhlas Hatchery (El Said Abo Omar) situated in the Al-Diba region, located between the Damietta and Port Said governorates in Egypt, with cooperation from the animal, poultry, and fish production department, faculty of agriculture, Damietta University, Damietta Governorate, Egypt. The hatchery provided all essential facilities required for the successful execution of the study.

2. Experimental Procedures:

2.1. Experimental design

Three different feeding regimes were implemented to prepare the female broodstock for the spawning season and reproductive traits. These regimes included Spirulina platensis (5%), Thalassiosira thira (5%), and a combination of the two (2.5:2.5 ratio) as a feed additive for L. vannamei as well as control (basal diet). The study compared these diets based on their impact on reproductive performance, redox status, and immune response, with a specific focus on how these phytobiotics influenced the expression of heat shock

protein (HSP70) and interleukin 1 β (IL-1β) in L. vannamei shrimp.

2.2. Experimental facilities:

The experiment took place in 4 rectangular tanks detailed in Table 1. Each tank had a volume of 12 m³, a width of 10 meters, a length of 3 meters, and a depth of 0.4 meters. The tanks had four drainage holes along the length of the tank, and the drainage hole was 2 inches and the tanks were black. Each tank housed 250 shrimp at a density of 9 shrimp per square meter. The water column height varied between 40 and 50 cm. The water change rate was maintained at 150-200% of the total tank volume, with 60% changed in the morning and 90% at night. The tanks operated with a lighting system for 14 hours from 6 am to 8 pm and a dark system for 10 hours from 8 pm to 6 am.

Table 1: Hatchery facilities described the experimental units used in shrimp hatchery**.**

Experimental units	Volume	Material	Shape	color	Dimensions m	Capacity
Maturation tanks	12m^3	Cement	Rectangular	Black	3*10	50%
Spawning & hatching tanks	500 L	Fiberglass	Circular conical	Black	1*0.75	85%
Basin for eyestalk ablation	500L	Plastic	Circular flat	White	1*0.6	90%
Egg& nauplii collector bucket	50 L	Plastic	Circular flat	White	0.4*0.5	50%
Precipitation Pond	6300 m^3	Earthen pond	Flat Rectangular	Brown	40*100*1.5	100%
Coloration tanks	450 m^3	Cement	Flat Rectangular	blue	30*5*3	100%

2.3.Water quality management and supply:

The hatchery features two types of saltwater sources: marine water with a salinity of 40 ppt and groundwater with a salinity of 12 ppt. The marine water is sourced through a channel linked to the Mediterranean Sea during tidal movements. This water is channeled into earthen ponds to allow for the settling of suspended plankton. Subsequently, the marine water is filtered through a ground filter composed of stones of varying diameters, acting as a mechanical filter, before being directed to a cement tank. In this tank, the salinity is adjusted by blending marine water with low-salinity groundwater to achieve a salinity level of 32 ppt. The blended water then undergoes a series of filtration processes, illustrated in Figure 1 (carbon filter + foam fractionator + ultraviolet rays + ozone gas). Following these processes, the water is transferred to another cement tank after pre-filtration steps for treatment with chlorine at a rate of 1 ppm. Additionally, EDTA (Ethylene Diamine Tetraacetic Acid) is added at 0.5 ppm to precipitate heavy metals, ensuring the elimination of any living organisms or bacteria in the water. This treatment aims to yield high-quality water suitable for hatching purposes, which is then evenly distributed throughout all sections of the hatchery. The hatchery employs various devices for monitoring water quality, including measuring oxygen levels (ppm), pH, temperature (˚C), ammonia concentration (mg/L), nitrite levels (mg/L), alkalinity (ppm), and salinity (ppt), as outlined in Table 2.

Table 2: Devices used in water quality measurements

Parameter	Device
Oxygen (ppm)	OxyGuard®
pH	HANNA (HI98107 pHep®)
Temperature °c	Thermometer
Ammonia (mg/l)	La Motte (code 3352-01)
Nitrite (mg/l)	La Motte (code 3304-02)
Nitrate (mg/l)	La Motte (code 3110-01)
Alkalinity(ppm)	HANNA (HI755 Checker® HC)
Salinity ppt	La motte (code 5-0020)

2.4.Fresh feed:

Chemicals analysis of L. vannamei feed additives (Spirulina spp, Thalthy thira, Commercial feed and Calimarae) were analyzed for crude protein, Crude ether extract, lipids, ash, moisture, and Carbohydrate according to **AOAC (2020)** methods as shown in **Table** (3)

2. **Table (3):** Proximate composition (% in dry matter basis) of *Spirulina spp*, *Thalthy thira* (Diatoms), commercial feed and calimarae.

Samples	Protein (%)	Crude ether extract (%)	Ash (%)	Moisture (%)	Carbohydrate (%)
Spirulina spp	65.20±0.47	0.15±2.00	16.67±0.58	8.33±0.06	7.81±0.21
Thalthy thira (Diatoms)	17.30±0.32	2.63±0.26	20.43±0.56	10.67±0.15	48.97±1.21
Commercial feed	37.00±0.38	12.70±0.26	9.13±0.15	11.37±0.32	29.80±0.78
Fresh Calamarea	13.10±0.11	2.33±0.15	1.10±0.12	77.33±0.48	6.13±0.47
Bloodworm − Glycera dibranchiata	60.06±0.04	14.29±0.02	7.64±0.01	9.70±0.01	8.31±0.03

2.5. Selection of spawners and egg collection:

Prior to the breeding season, a number of sorting and grading techniques were used in this study to determine which top broodstock prospects would be best for the spawning season. The best selection consisted of 125 female shrimp weighing about 50 g and 125 male shrimp weighing between 45 and 47 g. The shrimp were placed into four rectangular concrete tanks, one for each of the four treatments, at a density of 250 shrimp each to start the experimental period of *L. vannami* broodstock on a predetermined feeding schedule (Table, 4). At the beginning of this experiment, for each feeding regimen, four tanks were prepared using concrete tanks filled with water filtered through a 5 micron filter bag to a volume of 12 m3 per tank. The water temperature was adjusted to 26°C, with a lighting period of 14 hours and a dark period of 10 hours. Daily water changes of 100-150% were carried out, and each tank was stocked with 125 females to initiate the feeding protocols.

In preparation for eyestalk ablation, the necessary tools including surgical scissors, a gas torch, gloves, antiseptic solution (iodine), and a sedative were readied. The tanks housing the females were readied for the procedure by gradually lowering the water temperature from 22 to 26 °C over a 24-hour period. It was ensured that the females were in the post-molt phase to guarantee their health and ability to endure the eyestalk ablation process. A 600-liter plastic basin filled with water at 22°C accommodates the collected females from the original tanks. Ice is gradually introduced into the original tank to lower its temperature to 18-19°C, ensuring the prevention of any bacterial or fungal infections resulting from the eyestalk ablation process. In the small basin, ice is added to bring the temperature to 20°C, allowing the shrimp to relax before commencing the eyestalk ablation operation. One by one, females are transferred from each tank to the small basin as described. Surgical scissors are heated on both ends until fully red, following which one eye leg of each female is meticulously excised, and the female is immersed in an iodine antiseptic solution for sterilization. Subsequently, the females are returned to their original tanks, and this process is repeated for each female. After 12 hours, water is gradually reintroduced into the tank until the temperature reaches the normal level of 26°C, and the females are provided with small amounts of food until fully recovered. The eyestalk ablation procedure is performed on all broodstock in the four tanks.

Table 4: Feeding regime of male and female of L. vannamei broodstock before separating for 30 days.

Time	Control	T1 (5% *S. platensis*)	T2 (5% *T. weissflogii*)	T3 (2.5:2.5 T. *weissflogii*+*S. platensis*)
8.00am	Worm meal 400 g	Worm meal 400 g+20 g	Worm meal 400 g +20 g	Worm meal 400 g +10:10 g
12.00pm	Calamari 300 g	Calamari 300 g	Calamari 300 g	Calamari 300 g
4.00pm	Calamari 300 g	Calamari 300 g	Calamari 300 g	Calamari 300 g
8.00pm	Worm meal 400 g	Worm meal 400 g+20 g	Worm meal 400 g+20 g	Worm meal 400 g+10:10 g
12.00am	Commercial diet 100 g	Commercial diet 100 g+40 g	Commercial diet 100 g+40 g	Commercial diet 100 g +20:20 g
4.00am	Worm meal 400 g	Worm meal 400 g+20 g	Worm meal 400 g +20 g	Worm meal 400 g +10:10 g

2.6 .Biochemical parameters in hemolymph plasma:

To investigate the metabolic response of shrimp, hemolymph samples (200 µL) were individually collected from the ventral sinus at the base of the first abdominal segment. A 3 mL syringe, rinsed with a cooled 5% potassium oxalate in isotonic saline anticoagulant solution, was used for this purpose **(Mercier *et al*., 2006)**. The collected hemolymph was then centrifuged at 800 g for 10 minutes at 4°C to separate the plasma, which was preserved at -75°C for subsequent analysis.

Further analyses involved evaluating total antioxidant capacity, glutathione levels, and enzymatic antioxidant activities such as superoxide dismutase (SOD) and catalase (CAT) in muscle samples. Commercial kits from Shimadzu, Kyoto, Japan were utilized following the provided instructions. SOD activity was assessed by monitoring the auto-oxidation of pyrogallol per the method by **Marklund and Marklund (1974)**. CAT activity was determined by measuring H2O2 reduction at 240 nm, as outlined by **Claiborne (1985)**. The enzymatic activities were quantified as specific activities (IU/mg protein). Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels using the method described by Draper and Hadley (1990). This involved generating thiobarbituric acid reactive substances (TBARS) through an acid-heating reaction to evaluate lipid peroxidation, with MDA levels expressed as nmol/mg protein.

Furthermore, the levels of reproductive hormones (follicle stimulating hormone, luteinizing hormone, progesterone, estrogen, and testosterone), cortisol, immunoglobulin M (IgM), immunoglobulin G (IgG), and lysozyme activity were quantified using Elisa kits from MyBioSource, San Diego, CA, USA, following the manufacturer's instructions.

2.7. Microscopic Examination:

Samples of the ovary, ejaculatory duct, and seminiferous duct from the examined groups were preserved in 10% formalin fixative for 24 hours. Following this, they underwent a series of processing steps including immersion in increasing alcohol concentrations, xylene treatment, and embedding in paraffin. Paraffin blocks were then used to obtain thin five-µm sections, which were subsequently rehydrated and deparaffinized. The sections were stained with eosin and hematoxylin for examination under light microscopy to study the muscle tissues. A microscope from Olympus (CX30, Tokyo, Japan) was utilized for this purpose. Images of the muscle histology were captured using a digital camera and processed with J image software (type 1, 50b. US). Measurements were taken from three shrimp specimens in each group for further analysis.

2.8. Gene expression:

Muscle samples were collected aseptically and stored in sterile Eppendorf tubes, then preserved in liquid nitrogen for RNA extraction. For the extraction process, 100mg of liver sample was used with Trizol reagent from iNtRON Biotechnology, following the manufacturer's guidelines. The quality and quantity of the isolated RNA were assessed using a Nanodrop UV-Vis spectrophotometer model Q5000 from Quawell. An OD260/OD280 ratio was calculated,

with samples ≥1.8 considered suitable for further steps. Complementary DNA (cDNA) synthesis was performed using the SensiFAST™ cDNA synthesis kit from Bioline, following the manufacturer's protocol. Only samples meeting the specified OD260/OD280 ratio were utilized in this step. Specific primers for *IL-1β* (detailed in Table 6) were employed to amplify the selected genes, with the housekeeping gene β-actin serving as a reference gene. Gene amplification was conducted using the TOP real™ preMIX SYBR Green qPCR master mix from Enzynomics on a Stratagene MX300P PCR system. The data were analyzed using the 2−ΔΔCT method, as outlined by **Livak and Schmittgen (2001)**.

Table (5): Primers used for qRT-PCR

Gene	Forward 5′- > 3′	rse 5′- > 3′	GenBank No.
β-actin	CGGACAGGTCATCACC	ATTTGCGGTGGAC	AF384096.1
IL-1β	CTGAACAACAGCACTCTC	CTCTCCACCCTCCA	115592467

Statistical analysis:

The normality and homogeneity of the data were assessed using the Levene and Shapiro–Wilk tests. Subsequently, all estimated and computed data underwent statistical analysis using the one way anova within the Statistical Analysis System (Proc ANOVA; **SAS, 2012**). Variations between means were determined using Tukey's range test, with results presented as least square mean ± pooled standard error. The level of significance for all analyses was set at $p < 0.05$.

RESULTS AND DISCUSSION

The effects of different experimental diets on females and males reproductive hormones of *Litopenaeus vannamei* shrimp have been showed in **Table** 6. The levels of follicle stimulating hormone, luteinizing hormone and progesterone were significantly higher in the groups fed T. weissflogii (TH) alone or together with Spirulina platensis (SP) compared to the control group ($p<0.05$). The higher concentration of estrogen hormone was observed in TH treated group compared to the control and the other treated groups ($p<0.05$).With regard to male reproductive hormone, non- significant changes were detected between the control and all examined groups for follicle stimulating hormone ($p=0.1504$) and luteinizing hormone ($p=0.4550$).Meanwhile the concentration of testosterone hormone improved significantly as response to the dietary treatments, maximizing in both of TH and TH-SP treated groups ($p<0.0001$).

The microalgae had fairly high antioxidant content. Additionally, the phytochemical tests of these algae have confirmed the presence of significant amount of steroid-type sterols **(Basir et al., 2017)**. Steroids in green algae can form hormones, affecting female and male reproductive processes. Therefore, it can promote balanced hormonal regulation which supports the proper functioning of the reproductive system **(Admassu *et al.*, 2015)**. The hypothalamus secretes GnRH (Gonadotropin Releasing Hormone) in reproductive system, which stimulates the anterior pituitary gland to release gonadotropin hormones, including follicle stimulating hormone (FSH) and luteinizing hormone (LH) **(Maruska and Fernald, 2010)**. During ovulation processes in females, LH can stimulate the ovary to secret progesterone and estrogen hormones. On the other hand, FSH plays a potent role in stimulating the ovarian follicles developments. Together, FSH and LH stimulate the release of estrogen hormone and facilitate ovarian secretion **(Arini, 2021)**. In this study, the levels of FSH and LH hormones in females were significantly improved as response to the dietary addition of SP alone or in combination with SP in females shrimp diets.

In entire context, Eestrogen and progesterone plays crucial roles in regulating female sex hormones. Estrogen is involved in several reproductive processes, contributing to behavioral, metabolic, and morphological changes. Moreover, it affects the proliferation of cell, differentiation, and the development and facilities the functions of reproductive tissues **(Czarny *et al.*, 2017)**. In the current study, the incorporation of SP and TH in shrimp diets significantly improved the concentration of Estrogen and progesterone compared to the control. The present results could be ascribed to the presence of bioactive compounds in marine algae, including isofucosterol and fucosterol, which belong to the sterol family are known to play an important role in oocyte maturation as demonstrated by **Corral-Rosales *et al.* (2019)**. The current results could be supported by the photomicrograph of ovarian lobules, showing a widespread presence of vitellogenic oocytes exhibiting cortical rod formation (**Figure** 1). Regarding male sex hormones, testosterone or androgens are considered the dominant sex steroid hormones in males. However, the ovaries in the female also release small amounts of these hormones and the majority of testosterone is converted into estradiol **(Rahmanisa, 2014)**. The present results clearly indicated that there was a considerable increase in the levels of testosterone in male shrimp fed TH or TH

with SP. The current findings can be substantiated by the photomicrograph ejaculatory and semineferous duct which displays duct lined spermatocyte with no to low luminal debris (**Figure** 2).

Table (6): Effects of different experimental diets on reproductive hormones of Litopenaeus vannamei shrimp; Values are presented as mean ± SE.

Items	CON	SP	TH	SP+TH	*p*-value
Females					
FSH (ng/mL)	0.71±0.06^{c}	0.93±0.07^{bc}	1.18±0.19^{ab}	1.37±0.11^{a}	0.0007
LH (ng/mL)	0.94±0.04^{c}	1.13±0.06^{b}	1.39±0.12^{ab}	1.42±0.14^{a}	0.0010
P_4 (ng/mL)	1.81±0.08^{b}	2.15±0.15^{ab}	2.43±0.13^{a}	2.36±0.10^{a}	0.0017
E_2 (ng/mL)	1.44±0.08^{c}	1.62±0.07^{c}	2.33±0.11^{a}	1.90±0.08^{b}	<0.0001
Males					
FSH (ng/mL)	0.05±0.01	0.07±0.02	0.06±0.01	0.09±0.03	0.1504
LH (ng/mL)	0.21±0.05	0.27±0.04	0.26±0.06	0.25±0.03	0.4550
TEST (ng/mL)	1.7±0.07^{c}	2.1±0.11^{c}	3.2±0.24^{a}	2.9±0.20^{b}	<0.0001

FSH, follicle stimulating hormone; LH, luteinizing hormone; P_4, progesterone; E_2, estrogen. Test, testosterone. SP, *Spirulina spp*; TH, *Thalthy thira.* ^{a,b,c} Means within a row without a common superscript letter differ at p<0.05

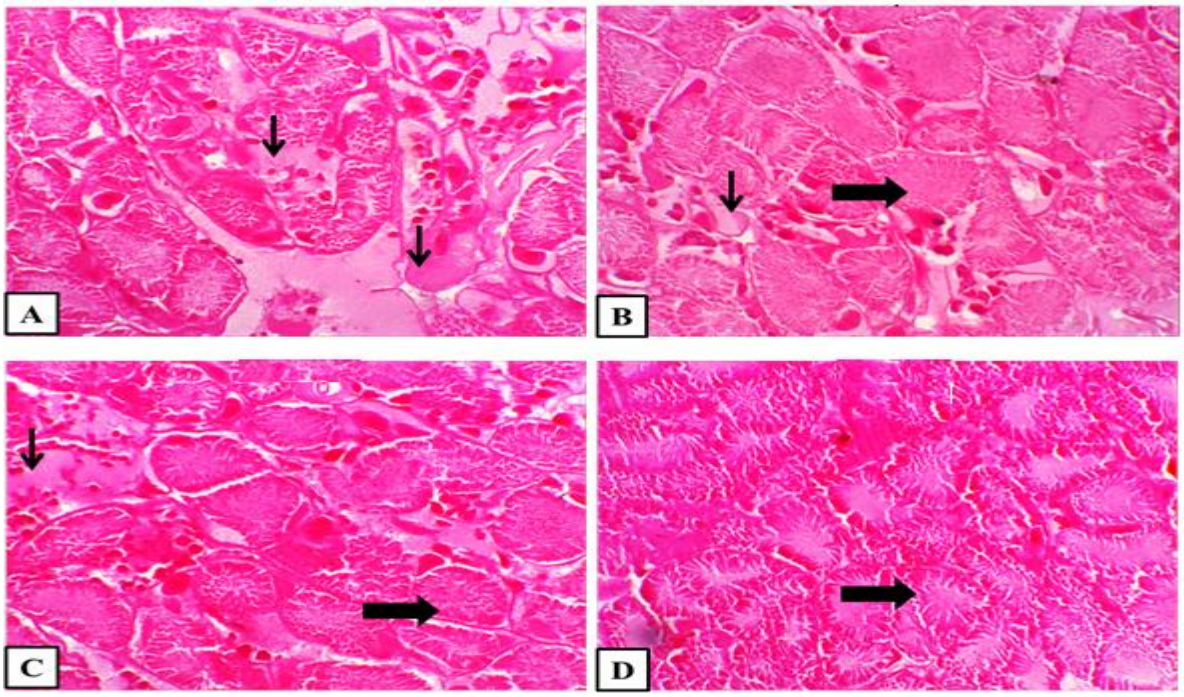

Figure 1 presents a representative photomicrograph of ovarian lobules, showing maturing and immature lobules. In panel A, scattered oocytes are showed, some of which had a loss of architectural integrity. Among these oocytes, a few are in the early stage, while others have reached maturity, characterized by the presence of vitelogenic globules and cortical rods. Panels B and C presented minimal to few oocytes, with loss of accessory cell borders. In panel D, a diffuse vitellogenic oocyte is visible with cortical rod formation. Thick arrow indicates oocyte with rod formation, while the thin arrow highlights the loss of architecture Image magnification= 400x.

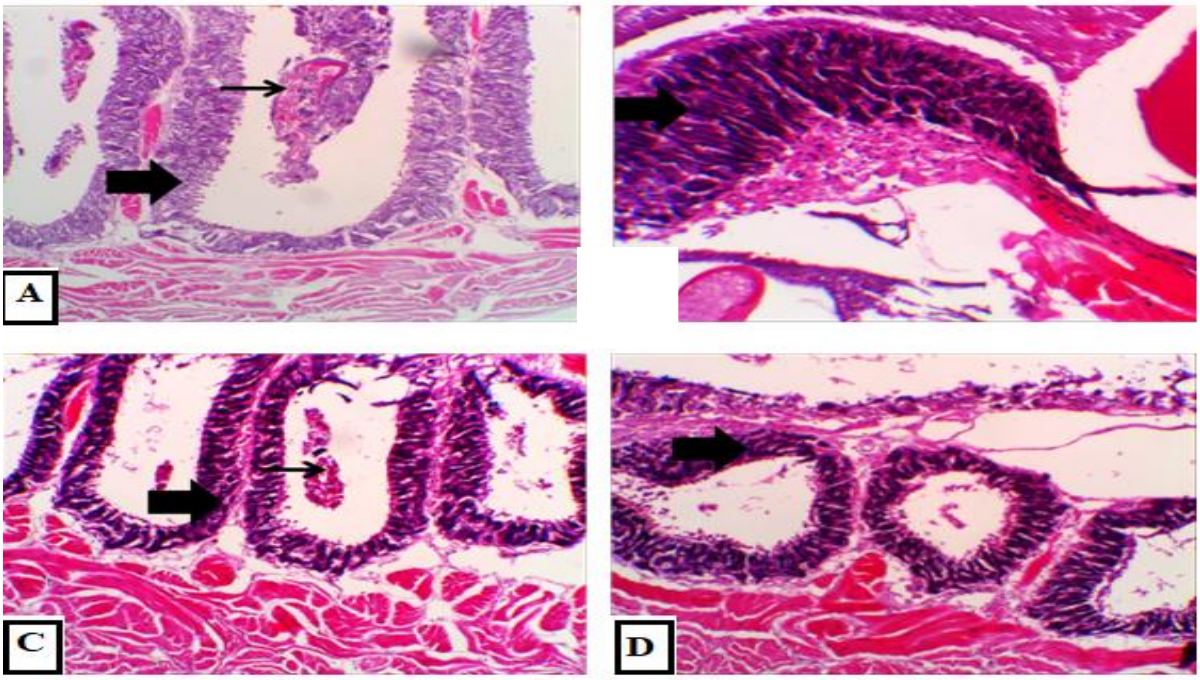

Figure 2 presents a representative photomicrograph of the ejaculatory and seminiferous duct. In panel A, the ejaculatory duct is observed, demonstrating epithelial proliferation (thick arrow) with luminal spermatophore admixed with sloughed cells (thin arrow). Panels B and C exhibit ducts lined with spermatozoa (thick arrow). The lumen contains a sperm mass with minimal to mild luminal debris or non-mature sperm cells (thin arrow). In panel D, group 4 is presented, demonstrating a duct lined with spermatocytes (thick arrow) with no to low luminal debris. Image magnification= 400x

Reactive oxygen species (ROS) are considered as byproducts of different metabolic activities in animals, and the oxidative stress occurs when their production exceeds the antioxidant capacity of body cells. Indeed, under stressful condition the cells activate many internal anti-oxidative reactions to counteract the harmful effects of ROS on cellular membranes **(Jiao *et al.*, 2019)**. These reactions involve alleviating the malondialdehyde (MDA) activation and promoting the release of antioxidant enzymes such as glutathione (GSH), catalase (CAT), and super oxide dismutase (SOD) **(Bandyopadhyay *et al.*, 1999; Naiel *et al.*, 2021)**. GSH has a dual role in cellular antioxidant defense system. It can act directly as an antioxidant, providing protection against pro-oxidants and free radicals. Moreover, it serves as a cofactor for many detoxification and antioxidant enzymes, including glyoxalases, glutathione S-transferases, and glutathione peroxidases **(Averill-Bates, 2023)**. SOD is responsible for converting superoxide into oxygen and hydrogen peroxide, and therefore represents the first line of defense against free radicals **(Li *et al.*, 2014)**. CAT facilitates the breakdown of hydrogen peroxide into oxygen and water **(Wang *et al.*, 2017)**. Focusing on antioxidant capacity of green algae and diatoms, natural products derived from different algal species, such as flavonoids, alkaloids, steroids, terpenoids, and phenols, has garnered significant interest in the scientific community over the years due to their various pharmacological properties, including their ability to act as antioxidant and exhibit anti-diabetic functions **(Paul, 2013)**. Former studies have been observed that green algae and diatoms can scavenge free radicals, inhibit DNA damage and lipid peroxidation, activate cellular antioxidant enzymes, and induce the activities of SOD, CAT and GSH as reported by **Qinghua *et al.* (2016)** and **Nowicka (2022)**.

Results in **Table 7** illustrated the effect of different experimental treatments on the redox balance of *Litopenaeus vannamei* shrimp. The levels of malondialdehyde (MDA) were significantly shrunken in TH treated group alone or in combination with SP compared to the control group. Whilest, the higher activity of CAT was showed in shrimp in aforementioned two treated groups compared to the control and SP treated group ($p<0.05$). The values of GSH, TAC and the activity of SOD were significantly higher in TH treated group compared to the control ($p<0.05$), non-significant difference were observed between SP treated group and the group received SP together with TH ($p>0.05$).

In the present study, TAC, SOD, GSH, and CAT activities were significantly induced and MDA levels were significantly decreased in the *Litopenaeus vannamei* received diets containing a combination of TH and SP, demonstrating the ability of green algae and diatoms to enhance redox homeostasis, which could be ascribed to the presence of bioactive antioxidants components in microalgae along with the anti-inflammatory constituents **(Yu *et al*., 2016)**. The phenolic compounds and the antioxidant activity of SP were determined to be 28.76 and 6.30 ppm, respectively **(Elsalhin *et al*., 2016)**. Additionally, Asha Shalini et al. (2019) found a higher amount of Total phenolic compounds in the methanolic extract of *T. weissflogii*, measuring 11.34 mg GAE g-1 DW. In accordance with the present study, **Sayed *et al*. (2018)** reported that the dietary supplementation of aquatic and terrestrial animals with SP inhibited oxidative DNA damage, diminished lipid peroxidation (MDA), and restored antioxidant levels. Also, **AlMulhim *et al*. (2023)** demonstrated that the replacing of fish mail by 10 % SP significantly improved the activities of antioxidant enzymes, including SOD, CAT by 142.81 and 212.87%, respectively.

Table (7): Effects of different experimental diets on redox balance of Litopenaeus vannamei shrimp; Values are presented as mean ± SE.

Items	Control	SP	TH	SP+TH	*p*-value
MDA (nmol/mg)	57.31±1.38^{a}	40.56±2.25^{b}	29.67±4.21^{c}	33.27±3.96^{c}	<0.0001
TAC (µmol/g)	26.82±1.50^{c}	37.44±3.81^{b}	44.45±1.65^{a}	37.35±0.55^{b}	<0.0001
SOD (U/mg)	12.85±1.01^{c}	22.84±1.55^{b}	28.43±1.51^{a}	20.20±1.60^{b}	0.0013
CAT (U/mg)	1.45±0.04^{b}	1.6±0.03^{b}	2.15±0.05^{a}	2.05±0.06^{a}	0.0008
GSH (U/mg)	31.25±2.35^{c}	37.85±0.75^{b}	38.11±2.51^{a}	41.37±1.45^{ab}	0.0012

MDA, malondialdehyde; TAC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione. SP, *Spirulina spp; TH, Thalthy thira*.; ^{a,b,c} Means within a row without a common superscript letter differ at p<.05

In **Table** 8, the effect of different experimental diets on immunological response of *Litopenaeus vannamei* shrimp have been presented. Both of immunoglobulin G and M as well as the transcription of *IL-1β* were significantly higher in the TH and TH+SP treated groups compared to the control and SP treated group (p<0.05). Lysozyme activity was significantly higher in the TH treated group compared to the control (p<0.05), non-significant differences were observed between the groups fed SP or SP+TH (p>0.05). Diatoms and filamentous blue-green algae such as SP show promising potential as immune stimulants and growth promoters in aqua feed applications **(Ayoub *et al*., 2019; Sheikhzadeh *et al*., 2019)**. Dried SP is known for its High-protein content (up to 57% of its dry weight). Additionally, SP and TH have several beneficial compounds, including polysaccharides, carotenoids, gamma-linolenic acid (GLA), vitamins (particularly B12), phycobiliproteins, and minerals **(Zhang *et al*., 2022; Sattanathan *et al*., 2023)**. These microalgae also possess immune-boosting effects, as documented in research performed on several fish species **(Al-Deriny *et al*., 2020; Huervana *et al*., 2022)**. Scientific researchers have demonstrated the importance of activating non-specific immune mechanisms and the advantages of using both microalgae and macroalgae in enhancing fish immune status. Moreover, Changes in the expression of immune genes have been identified as reliable indicators of improved immune states in aquatic animals **(Teles *et al*., 2013)**.The significant improvements in cellular immunity in shrimp fed diets enriched with SP alone or in combination with and TH were on line with the findings of **Egamberdieva *et al*. (2017)**, who highlighted the widespread use of algae and other medical plants as alternatives to expensive antibiotics for enhancing fish health due to their cost-effectiveness, reduced adverse effects, and environmental friendliness.

Lysozyme activity, pro-inflammatory cytokines, and immunoglobulin are examples of humeral substances that play crucial roles in both non-specific and specific immunity in aquatic animals **(Zhao *et al*., 2009)**. The increased lysozyme activities were observed in shrimp received TH and /or SP compared to the control, what in general agreement with the results of **Wan *et al*. (2016)** who found that supplementing 10% of the algae enhanced serum lysozyme and complement activity in Atlantic salmon. Similarly, **Gabrielsen *et al*. (1998)** reported that the incorporating of *A. platensis* in the diets of juvenile great sturgeon can boost the serum lysozyme and complement activity. These results support the outcomes of the present study, highlighting the positive effects of green algae and diatoms supplementation on humeral immune responses in *Litopenaeus vannamei* shrimp. Several studies demonstrated that the inclusion of SP increased immunological parameters in coral trout *Plectropomus leopardus* **(Yua *et al*., 2018)**, *Ctenopharyngodon*

idella **(Chen *et al.*, 2019)**, *Padina gymnospora* **(Rajendran *et al.*, 2016)**. The *IL-1β* also plays an important role in the host's immune response to microbial invasion, impaired immune function, and tissue damage **(Corripio-Miyar *et al.*, 2007)**. It is up regulated in several species, including mollusks, invertebrates, birds, mammals, plants, reptiles, fish, and others, with some exhibiting unique forms or tissue-specific expression patterns **(Jimenez-Cantizano *et al.*, 2008)**. In this study, the group of shrimp fed a mixture of TH and SP algae showed an increase in the expression of the IL-1β gene, suggesting an enhanced immune response in the shrimp.

Table (8): Effects of different experimental diets on immunological response of *Litopenaeus vannamei* shrimp; Values are presented as mean ± SE.

Items	CON	SP	TH	SP+TH	*p*-value
IgG (ng/mL)	35.4±1.02^{c}	50.15±1.45^{b}	64.05±2.25^{a}	65.10±1.55^{a}	<0.0001
IgM (ng/mL)	32.61±0.90^{c}	48.20±2.32^{b}	61.75±1.95^{a}	58.8±2.51^{a}	<0.0001
Lysozyme (µg/dL)	6.07±0.44^{c}	7.275±0.62^{b}	11.51±0.39^{a}	7.35±0.16^{b}	<0.0001
IL-1β (Fold change)	1.00±0.00^{c}	1.65±0.05^{b}	2.5±0.11^{a}	2.35±0.04^{a}	<0.0001

IgG, immunoglobulin G; IgM, immunoglobulin M; IL-1β, mRNA gene expression of interlukine-1*β*. SP, *Spirulina spp; TH, Thalthy thira*. ^{a,b,c} Means within a row without a common superscript letter differ at p<.05.

CONCLUSION:

According to the findings outlined, a dietary addition of a mixture of SP and TH in a 2.5:2.5 ratio to *Litopenaeus vannamei* shrimp brood stock enhanced reproductive hormones, immune response, and redox equilibrium. Future research efforts should focus on utilizing these green algae and diatoms at molecular levels to explore their proprieties as environmentally friendly feed additives specifically during the spawning season.

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الملخص العربي

التأثيرات التآزرية للطحالب الخضراء والدياتومات على الهرمونات التناسلية والحالة الصحية للجمبري ابيض الساق (الفانمى) في المفرخات التجارية

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تناولت هذه الدراسة تأثير الإضافة الغذائية للطحالب الخضراء والدياتومات على الهرمونات التناسلية وحالة الأكسدة والاختزال والاستجابة المناعية، مع التركيز بشكل خاص على تأثير هذه المواد الحيوية النباتية على التعبير عن إنترلوكين-1 بيتا في الجمبري ذو الساق البيضاء (الفانمى). أجريت التجربة خلال موسم 2022، حيث تم تغذية الأسماك الأمهات بنسب متساوية من مكونات مختلفة: طحلب سبيرولينا بنسبة 5٪، وطحلب ثلاسوسيرا بنسبة 5٪ ومزيج من سبيرولينا وثلاسوسيرا بنسبة 2.5: 2.5 بالإضافة إلى مجموعة التحكم (النظام الغذائي الأساسي). كانت مستويات الهرمون المنشط للحويصلات والهرمون المنشط للجسم الاصفر والبروجسترون أعلى بشكل ملحوظ في المجموعات التي تغذت على ثلاسوسيرا بمفرده أو مع سبيرولينا مقارنة بمجموعة التحكم ($p < 0.05$). لوحظ ارتفاع تركيز هرمون الاستروجين في المجموعة المعالجة بـثلاسوسيرا مقارنة بالمجموعة الضابطة والمجموعات المعالجة الأخرى ($p<0.05$). وفيما يتعلق بالهرمون التناسلي الذكري، تم الكشف عن تغييرات غير مهمة بين المجموعة الضابطة وجميع المجموعات المفحوصة للهرمون المنشط للحويصلات و هرمون المنشط للجسم الاصفر ($p=0.4550$). وفي الوقت نفسه، تحسن تركيز هرمون التستوستيرون بشكل ملحوظ في المجموعة ثلاسوسيرا مقارنة بالمجموعة الضابطة أو المجموعات المعالجة الأخرى ($p<0.05$). وقد دعمت هذه النتائج الصورة المجهرية المحسنة لفصيصات المبيض والقناة القذفية والمنوية من خلال العلاج المشترك بـسبيرولينا وثلاسوسيرا. وانكمشت مستويات مالونديالدهيد بشكل ملحوظ في المجموعة التي تلقت ثلاسوسيرا بمفردها أو بالاشتراك مع سبيرولينا ب مقارنة بمجموعة التحكم. وعلى العكس من ذلك، تم تحريض نسخ إنترلوكين-1 بيتا ونشاط الكاتالاز وتركيزات الجلوبيولين المناعي ج و م بشكل كبير في المجموعتين المعالجتين المذكورتين أعلاه مقارنة بالمجموعة الضابطة والمجموعة المعالجة بـبـسبيرولينا ($p<0.05$). كانت قيم الجلوتاثيون، والقدرة الكلية المضادة للأكسدة ونشاط أكسيد الفائق ديسميوتاز أعلى بشكل ملحوظ في المجموعة المعالجة بـثلاسوسيرا مقارنة بالمجموعة الضابطة ($p<0.05$). وعليه، يوصى بدمج مزيج من طحلبى سبيرولينا وثلاسوسيرا بنسبة 2.5:2.5 في النظام الغذائي لجمبري ذو الساق البيضاء حيث أنه يمكن أن يكون بمثابة استراتيجية فعالة لتحسين الهرمونات التناسلية وحالة المناعة وتوازن الأكسدة والاختزال خلال موسم التبويض