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J Pure Appl Microbiol. 2023;17(4):2140-2148. doi: 10.22207/JPAM.17.4.10
Received: 21 November 2022 | Accepted: 18 September 2023
Published Online: 23 October 2023

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Effect of Methanol Extracts of *Arthrospira platensis* on Survival and Increased Disease Resistance in *Litopenaeus vannamei* against Vibriosis

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Abstract

Vibriosis is a common bacterial infection in shrimp that causes mortality in hatcheries and farms. Various steps have been initiated to increase the resistance against bacterial pathogens and decrease the mortality rate through improved culture conditions and feed. *Arthrospira platensis* (Spirulina), a blue-green alga, is a good source of protein and other nutrients and helps to improve digestion. The effects of the methanol extract of *A. platensis* on the survival rate and resistance against vibriosis were studied. The minimum inhibitory concentration of the extract for *Vibrio* species and *in vivo* antibacterial screening were investigated using *Litopenaeus vannamei*. *Vibrio alginolyticus* was inhibited with 2000 µg mL^{-1} extract and the other two species were inhibited by 1500 µg mL^{-1} extract. Furthermore, the mortality rate and antioxidant enzyme levels of shrimps injected with pathogens reduced and increased after treatment with the methanol extract, respectively. The survival rate of *V. parahaemolyticus* and *V. harveyi*-challenged shrimps were 33.3% and 50%, respectively, after 168 h. The survival rate of *V. alginolyticus*-infected shrimp reduced (16.6%) 168 h after injection. All surviving shrimp developed resistance to *Vibrio* pathogens. This study indicated that the bioactive compounds in *A. platensis* could not only effectively prevent bacterial infection, but also serve as eco-friendly and cost-effective immune stimulants.

Keywords: *L. vannamei, Shrimp, Spirulina, V. alginolyticus, V. harveyi, V. parahaemolyticus*

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Citation: Al-Ghanayem AA. Effect of Methanol Extracts of Arthrospira platensis on Survival and Increased Disease Resistance in *Litopenaeus vannamei* against Vibriosis. *J Pure Appl Microbiol.* 2023;17(4):2140-2148. doi: 10.22207/JPAM.17.4.10

INTRODUCTION

White shrimp (*Litopenaeus vannamei*) is a major shrimp species farmed worldwide for food. These shrimps are susceptible to bacterial and viral diseases leading to huge economic loss.[1] *Vibrio* species, comprising more than 80% bacterial population in seawater, are gram-negative bacteria causing vibriosis in shrimps.[2] It is a common bacterial infection in *L. vannamei*, which causes acute hepatopancreatic necrosis syndrome (AHPNS).[3] *Vibrio harveyi*, *Vibrio fluvialis*, *Vibrio parahaemolyticus*, *Vibrio damsel*, *Vibrio vulnificus*, and *Vibrio alginolyticus* are the other major *Vibrio* species causing vibriosis.[4] They are opportunistic pathogens and infect shrimps under stressful conditions such as salinity and temperature variations. Additionally, vibriosis occurs due to the combination of physical stress and primary infection by other bacterial or viral pathogens.[1] Environmental stress triggers diseases leading to 100% shrimp mortality. Antibiotic treatment to control pathogens has developed antibiotic resistance,[5] leaving antibiotic residues such as parent drug components and metabolites.[6] Phytochemical and natural compound use has been advocated to overcome this problem, and there is great interest in the search for novel components to control pathogens. Moreover, owing to inefficient management of the virulent *Vibrio* strains through antibiotics and the consequent antibiotic residue development, farmers are forced to use probiotics and bioactive compounds.[7] Additionally, these compounds will enhance shrimp growth and stimulate the immune system. Thus, the immunostimulatory, antibacterial, and antiviral effects of several algal extracts in shrimp have been studied.

This study uses *Arthrospira platensis* (Gomont), commonly known as *Spirulina*, is a blue-green filamentous alga with numerous therapeutic and nutritional benefits. It is rich in protein, essential fatty acids such as monounsaturated and polyunsaturated fatty acids, amino acids, minerals, fibers, and vitamins and have antimicrobial and antioxidant properties.[8] Expensive fishmeal is forcing the farmers to look toward single-cell protein sources such as *A. platensis* as a promising substitute feed or feed additive for cost-effective shrimp production.[9] Several reports have focused on the effect of *A. platensis* in other shrimps and fishes in improving digestion and increasing immunity against various pathogens.[10] Hot water extracts of *A. platensis* administration in *L. vannamei* has increased immunity against vibriosis[11] and gene expression upregulation after pH stress.[12] However, no reports have focused on the effect of the methanol extract of *A. platensis* on controlling vibriosis.

MATERIALS AND METHODS

Extract preparation

Commercially available *A. platensis* (200 g, Herbolina, India) was soaked in 2 L methanol and stirred 3 times a day for 7 days. Then, the extract was filtered and the solvent was removed using a rotary evaporator under reduced pressure at 45±5°C until dry residue was obtained. The extract was transferred to airtight glass bottles and stored at 4°C until further use.

Bacterial culture

V. alginolyticus, *V. harveyi*, and *V. parahaemolyticus* strains maintained in the laboratory were used for this study. Pure cultures were prepared and inoculated into trypticase soy broth for 24 h. The bacterial pellets obtained using centrifugation at 112 x*g* for 5 min were resuspended in isotonic sodium chloride solution to obtain different CFU mL^{-1} according to necessity.

Antibacterial activity

The minimum inhibitory concentration (MIC) was detected in microplates using broth dilution method.[13] Briefly, 2500, 2000, 1500, and 1000 µg mL^{-1} extract were mixed with $1x10^8$CFU mL^{-1} bacterial culture prepared using broth dilution method in trypticase soy broth using 0.5 MacFarland standard. Gentamycin (50 µg mL^{-1}) was used as a control. The lowest extract concentration that completely inhibited visible bacterial growth was considered the MIC. For determining the minimum bactericidal concentration (MBC), 20 µL test broth was re-inoculated into trypticase soy agar and incubated at 37°C for 24 h. The minimum concentration that reduced the colony count to 99.9% was considered as the MBC.

Experimental animal collection and maintenance

L. vannamei were collected from a local hatchery (Best Aqua Star Shrimp Hatcheries, Villupuram, India) and maintained in 1000 L fiberglass tanks containing seawater with airlift biological filters at 27–30°C and 20±1 ppt salinity. For each experimental trial, 12 shrimp were transferred into a 30L capacity tank (plastic) with proper aeration, and 10% water volume was exchanged daily. The animals were initially fed commercially available feeds, and the extracts were administered along with the feed during the experiments. The salinity was measured at regular intervals using a refractometer (Weswox hr-05, India). All applicable international, national, and institutional guidelines for animal care and use were followed.

***In vivo* antibacterial activity**

A. platensis extract (20 mg) was dissolved in 10 mL Tris-EDTA buffer (pH 7.4) for preliminary *in vivo* antibacterial activity assay.[14] Equal volume (10 µL) extract and *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus* suspensions were mixed separately and incubated at 37°C for 3 h. The test mixture diluted in normal saline (200 µL) was intramuscularly injected in 6–8 g healthy animals at the sixth abdominal segment. Control shrimps were administered normal saline and the general health and bacterial infection status of all animals were monitored for 168 h. The survival rates of pathogen-challenged and control shrimp were calculated using the following formula:

Challenge test

The shrimps were fed with commercially available diets (Bay White - Vannamei Shrimp Feed, India) for four weeks and further fed 2500 µg g^{-1} methanol extract of *A. platensis*. The shrimp were challenged by injecting 1x10^8 CFU ml^{-1} of the three *Vibrio* species separately into the ventral sinus of the cephalothorax. The survival rate was recorded every 24 h for168 h. The control shrimp were fed the extracts and injected with 1 mL normal saline.

Antioxidant response determination

The shrimp were dissected, and 100 mg tissue was separated and crushed using a homogenizer in tubes containing 1 mL 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was removed and used for the catalase and superoxide dismutase (SOD) activity assays. Catalase activity was calculated using hydrogen peroxide reduction kinetics with 0.04 nm cm^{-1} extinction coefficient at 240 nm using a UV–vis spectrophotometer.[13] Specific activity was calculated as unit per mg protein (CAT U $mg^{"1}$ protein). SOD activity was calculated using nitro blue tetrazolium (NBT) with riboflavin.[15] The specific activity was calculated as unit per mg protein using absorbance values.

Vibriosis detection using RT-PCR

Genomic DNA was extracted from the shrimp using a Genomic DNA Extraction Kit (Genei, Bangalore, India). Oligonucleotide primers and probes were used to amplify the *Vibrio* species DNA fragments. Briefly, 5 µL bacterial DNA samples and PCR mix in PCR buffer with 5 mM $MgCl_2$, 0.5 µL 5 U $µL^{-1}$ taq polymerase, 1 µL 20 µM dNTP, 10 µM forward and reverse primers[16-18] (1 µL each) (Table 1) and the respective probes was prepared.

RT-PCR was performed using a thermal cycler (QIAGEN Rotor-Gene Q, Software 2.3.1.49). The amplification was performed at 95°C for 3 min for initial denaturation, followed by 3s denaturation at 95°C and annealing for 30s at 60°C. Fluorescent signals based on SYBR green were sensed separately at each elongation step, and the signal was measured as positive after the threshold cycle (Ct) was set to less than 2.

RESULTS AND DISCUSSION

The methanolic extract of *A. platensis* effectively inhibited the growth of all the three *Vibrio* species (Table 2). The MIC for *V. alginolyticus* was 2000 µg mL^{-1}, whereas 1500 µg mL^{-1} extract inhibited *V. harveyi* and *V. parahaemolyticus*. These two strains were the most susceptible to the extracts. Gentamycin was used as a positive control which inhibited all the *Vibrio* species at very low concentrations (50 µg mL^{-1}). The MBC of the extract was higher than the MIC, as the MBC for *V. alginolyticus*, *V. harveyi*,and *V. parahaemolyticus* were 2500, 2000, and 2000 µg mL^{-1}. The antimicrobial activity could be due to the presence of bioactive compounds such as alkaloids, flavonoid glycosides, phenols, and

saponins in *A. platensis.*[19] The mechanism of antimicrobial activity of these compounds is still unclear and may be genotoxic or due to the interaction with the outer membrane of the bacteria.[20]

To control acute hepato-pancreatic necrosis disease (AHPND), new approaches for using bioactive compounds from algal sources need to be considered to reduce production loss.[21] The *in vitro* antibacterial activity of various *A. platensis* extracts, particularly on *Vibrio* strains, has been reported previously.[22] The protective effect of methanol extract of *A. platensis* against vibriosis in *L. vannamei* was studied *in vivo*. The control shrimp inoculated with *Vibrio* strains were severely infected within 72 h. *Vibrio alginolyticus*- and *V. parahaemolyticus*-infected shrimp died within 120 h, whereas *V. harveyi*-infected shrimp survived slightly longer (Figure 1). *V. harveyi* is a luminescent bacterium that accounts for the total mortality of shrimp in aquaculture farms.[23] Injecting the *Vibrio* culture mixed with methanol extract into the test animals increased survival rate and reduced shrimp mortality. The survival rate of shrimp exposed to *V. alginolyticus* was 16%, whereas that of shrimp exposed to *V. parahaemolyticus* and *V. harveyi* increased (25%).

The mortality rate was higher with *V.alginolyticus* because of the virulence of the strain. The survival rate of shrimp not infected with *Vibrio*, but administered normal saline was 100%. These results clearly show that the methanol extract of *A. platensis* considerably protected *L. vannamei* against vibriosis. This result was substantiated by the observation that dry powder[24] and hot water extract[25] of *A. platensis* increased resistance against *V. alginolyticus* through increased phagocytic and lysozyme activities for pathogen clearance from shrimp. Based on the inhibitory effect of methanol extract on *Vibrio* species, the incorporation of extracts

Table 1. Primers used in the study

Bacteria	Target genes	Primer and probe sequence (52 - 32)
V. alginolyticus	*gyr B*	Forward GAGAACCCGACAGAAGCGAAG Reverse CCTAGTGCGGTGATCAGTGTTG Probe TTCTCACCCATCGCCGATTCAACCGC
V. harveyi	toxR	Forward GGAGCAGCACTCACCGAT Reverse GGTGAAGACTCATCAGCA Probe TCAAGCGATTTCTACTCTGCG
V. parahaemolyticus	*tlh*	Forward ACTCAACACAAGAAGAGATCGACAA Reverse GATGAGCGGTTGATGTCCAA Probe CGCTCGCGTTCACGAAACCGT

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration of methanol extract of Spirulina on *Vibrio* species

Bacteria	MIC90* (μgml^{-1})	MBC (μgml^{-1})
Vibrio alginolyticus	2000	2500
V. harveyi	1500	2000
V. parahaemolyticus	1500	2000

*MIC90 MIC required to inhibit the growth of 90% of organisms

Table 3. Antioxidant response of infected *L. vannamei* fed normal feed and with *Spirulina* extract incorporated feed

Parameters	Soluble protein (mg ml^{-1})	Superoxide dismutase U mg^{-1} protein	Catalase U mg^{-1} protein
Vibrio alginolyticus	14.31	1.98	8.76
V. harveyi	12.52	1.85	9.23
V. parahaemolyticus	13.57	1.78	8.23
Vibrio alginolyticus + ME	28.68	3.02	25.36
V. harveyi + ME	24.84	2.35	20.31
V. parahaemolyticus + ME	26.56	2.63	16.32
Control (Normal feed + saline)	23.59	2.05	11.35

along with feed for *Vibrio*-challenged shrimp was studied (Figure 2). The shrimp challenged with *V. alginolyticus* had a reduced mortality rate (16.7%) than the mortality rate 48 h after injecting $4x10^6$ CFU mL^{-1} *V. alginolyticus* (76.6%).[26] Further, the survival rate reduced to 16.6% after 168 h. The survival rate of *V. parahaemolyticus*- and *V. harveyi*-challenged shrimp were 33.3% and 50%, respectively, after 168 h. The survival rate of control shrimp administered saline and methanol extracts was 100%. The methanol extract of *A. platensis* considerably influences pathogen multiplication regulation. Further studies should determine the optimal dosage and administration. The effect of metabolites on inhibitory activity on pathogen depends on the dose, extract concentration, administration route, and exposure time.[27] This study clarified that extract along with feed controlled infections and increased shrimp immunity. An extract concentration below the MIC may not be effective in developing disease resistance against *Vibrio*.

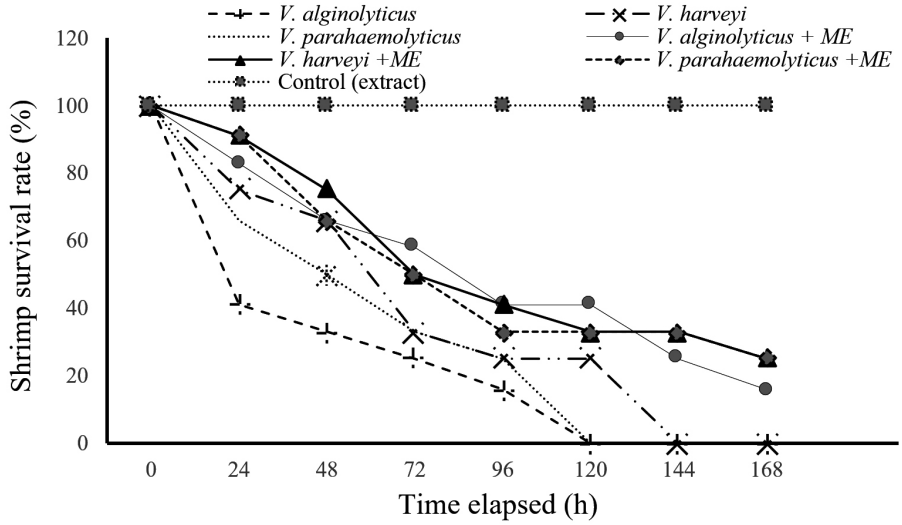

Figure 1. *In-vivo* assessment of shrimp survival rate (%) after challenging with *Vibrio* species along with *Spirulina* extract for preliminary study (each treatment is done with twelve shrimps)

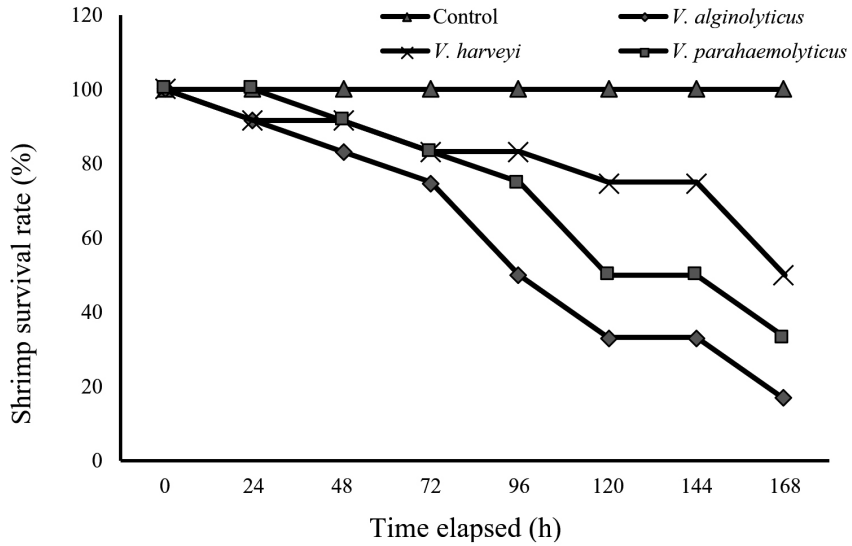

Figure 2. Survival rate of shrimps challenged with *Vibrio* species and fed with *spirulina* methanol extract incorporated in feed (each treatment is done with twelve shrimps)

To determine the efficiency of the extract in increasing immunity and disease resistance, the soluble protein content and antioxidant enzymes were measured in shrimp after the experimental period (Table 3).

The soluble protein content in *Vibrio*-infected shrimp was lower than that in control shrimp. The soluble protein content in *V. alginolyticus*-infected shrimp on normal feed and methanol extract along with feed were 14.31 and 28.68 mg mL^{-1}, respectively. Moreover, the soluble protein content in *V. harveyi*-infected shrimp on normal feed and methanol extract along with feed were 12.52 and 24.84 mg mL^{-1}, respectively. Similarly, the soluble protein content in *V. parahaemolyticus*-infected shrimp on normal feed and methanol extract along with feed were 13.57 and 26.56 mg mL^{-1}, respectively. Previous studies have reported that blue-green algae activate both humoral and cellular immune system to reduce environmental stress and infectious agents in the freshwater prawn *Macrobrachium rosenbergii.*[28] Hot water extracts of *A. platensis* induced and activated the antioxidant enzymes in *L. vannamei*.[25] SOD is an essential antioxidant enzyme that plays vital role in the defense mechanism of shrimp immune system in scavenging superoxide anion that damages the host tissues.[12] The SOD activity increases in infected shrimps as a defense against the pathogens and also shrimps fed with herbal extract-supplemented feed for resistance against the infected pathogens.[29] In this study,

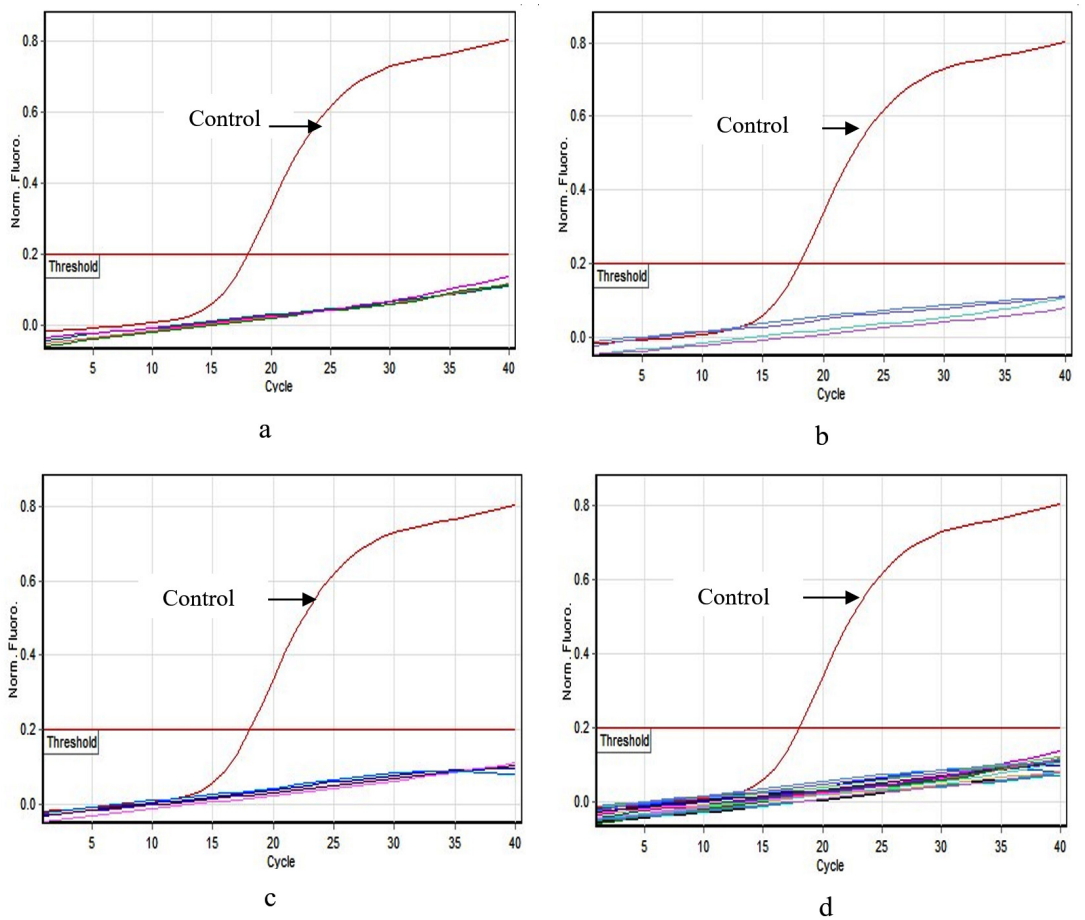

Figure 3. Real time PCR detection of pathogens in *L. vannamei* after feeding with *Spirulina* extract. (a) *L. vannamei* survived (16.6%) after challenging with *Vibrio alginolyticus* and with and fed with methanol extract along with feed (b). *L. vannamei* survived (50%) after challenging with *V. harveyi* and fed with methanol extract along with feed (c) *L. vannamei* fed with 2.5 mg concentration of extract along with feed, challenged with *Vibrio parahaemolyticus* and survived (33.3%) along with control (d) shrimps fed with methanol extract along with feed

the SOD activity in infected untreated shrimps were lower (1.78–1.98 U mg^{-1} protein) than that in the control (2.05 U mg^{-1} protein) and extract-fed shrimps. The SOD levels in *V. alginolyticus*-, *V. harveyi*-, and *V. parahaemolyticus*-infected shrimp fed with extracts were 3.02, 2.35, and 2.63 U mg^{-1} protein, respectively. SOD increased to lessen the self-damage caused by superoxide anion damage in extract-fed shrimp. It may be due to the presence of antioxidants, such as C-phycocyanin, beta carotene minerals, protein, carbohydrates vitamins and lipids, in *A. platensis*.[30] Beta carotene and C-phycocyanin scavenge free radicals such as alkoxyl, peroxyl, and hydroxyl radicals.[31] The increased SOD and other antioxidant molecules in the shrimp might have increased H_2O_2 concentration, which in turn increases catalase production to detoxify it. Catalase activity in extract-treated infected shrimp was higher than that in untreated challenged shrimp. Therefore, the immunostimulatory effects and antioxidant properties of various phytochemical components present in *A. platensis* should be evaluated. Furthermore, this study showed that the extracts stimulated SOD and catalase activities, thereby increasing the innate immunity in *L. venammei.* Similarly, *A. platensis* extracts and feeding microalgae stimulated phagocytosis in other shrimp species, such as *Penaeus merguieneis* and fish *Cyprinus carpio.*[32]

The shrimp survived for 168 h and were subjected to RT-PCR to detect *Vibrio* infection. Real-time PCR is used for the molecular detection of target DNA under automated conditions by fluorescence changes at low cost and in modest steps, avoiding difficult electrophoretic methods. Currently, it is widely used to detect pathogens at earlier stages. *V. alginolyticus* was not detected in the tissue samples of challenged shrimp that survived after 168 h (Figure 3a). The gene encoding the beta subunit of DNA gyrase is *gyr*B, which has a unique sequence for *V. alginolyticus* that is recognized as a molecular marker.[33] The survival rate of *V. alginolyticus*-infected shrimp was minimal than that of other strain-infected shrimp. The *V. alginolyticus*-infected shrimp are more susceptible to other pathogens and prone to decreased immunity, ultimately leading to death. However, the survival time increased, and the surviving shrimp were not infected when compared with that in the control. The survival rate of *L. vannamei* on methanol extract-incorporated feed after challenge with V. *harveyi* was 50%. None of the surviving shrimp showed signs of vibriosis in RT-PCR analysis (Figure 3b-d).

V. harveyi is considered one of the most common opportunistic pathogens in *L. vannamei* and other shrimp farms and hatcheries and infects shrimp under environmental stress, thus increasing the mortality rate.[34] The secretion of extracellular products such as enzymes (proteases and lipases), toxins (hemolysins), lipopolysaccharides, and bacteriocin helps in the survival of these bacteria in the host.[35] *V. harveyi* causes luminous vibriosis and a 382 bp *tox*R gene fragment is widely used for the rapid and specific identification of this pathogen. The survival rate of *V. harveyi*-infected shrimp increased after treatment.*V. parahaemolyticus* produces a thermolabile hemolysin responsible for its pathogenicity via *tlh* gene, which is a basic molecular marker for the species. The survival rate of *L. vannamei* fed with 2500 µg extract along with feed and challenged with *V. parahaemolyticus* was 33.3% and the shrimps were subjected to RT-PCR analysis for pathogen infection. All surviving shrimps were negative for *Vibrio* infection. *V. parahaemolyticus* is a major pathogenic strain for *L. vannamei* and causes Early Mortality Syndrome (EMS), with 100% mortality within 20 days of stocking. It is present in the water and digestive tract of shrimp and enters the host through gills, feed, and injuries. Shrimps are susceptible to infection due to stress or less immune response.[36] Previous invivo studies have showed that ethanol extracts of seaweed *Gracilaria fisheri* decreased *V. harveyi* and *V. parahaemolyticus* colonization and protected against mortality.[37] This study shows that, to some extent, *A. platensis* extract helps to overcome stress and increase immunity against the pathogens.

CONCLUSION

Vibriosis, the most common infection caused by *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus*, is a serious threat to the aquaculture industry. Introducing novel bioactive compounds against bacterial pathogens through feed will diminish the bacterial community and improve shrimp immunity. Incorporating *A.*

platensis in feed stimulates disease resistance by acting as an immunoprophylaxis against vibriosis in *L.vannamei*. It also increases antioxidant enzyme production, thereby acting as an immune stimulant in shrimps. Therefore, the methanol extract of *A. platensis*is an eco-friendly alternative to chemical agents for controlling vibriosis.

ACKNOWLEDGMENTS

The author would like to thank the Deanship of Scientific Research at Shaqra University for supporting this work.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

REFERENCES

1. Patil PK, Geetha R, Ravisankar T, et al. Economic loss due to diseases in Indian shrimp farming with special reference to *Enterocytozoon hepatopenaei* (EHP) and white spot syndrome virus (WSSV). *Aquaculture.* 2021;533:736231. doi: 10.1016/j.aquaculture.2020.736231
2. Chellapandian H, Sivakamavalli J, Anand AV, Balasubramanian B. Challenges in controlling vibriosis in shrimp farms. *Infections and Sepsis Development.* 2021;195. doi: 10.5772/intechopen.97018
3. Kumar V, Roy S, Behera BK, et al. Acute hepatopancreatic necrosis disease (AHPND): virulence, pathogenesis and mitigation strategies in shrimp aquaculture. *Toxins.* 2021;13(8):524. doi: 10.3390/toxins13080524
4. de Souza Valente C, Wan AH. *Vibrio* and major commercially important vibriosis diseases in decapod crustaceans. *J Invertebr Pathol.* 2021;181:107527.
5. Kim DH, Rajapaksha LGTG, Gunasekara CWR, et al. Phylogenetic relationships and antibiotic resistance of *Vibrio parahaemolyticus* isolates related to acute hepatopancreatic necrosis disease in Korea. *Aquaculture.* 2021;545:737253. doi: 10.1016/j.aquaculture.2021.737253
6. Hu Y, Lei D, Wu D, Xia J, Zhou W, Cui C. Residual β-lactam antibiotics and ecotoxicity to Vibrio fischeri, Daphnia magna of pharmaceutical wastewater in the treatment process. *J Hazard Mat.* 2022;425:127840. doi: 10.1016/j.jhazmat.2021.127840
7. Dewi NR, Huang HT, Wu YS, et al. Guava (*Psidium guajava*) leaf extract enhances immunity, growth, and resistance against *Vibrio parahaemolyticus* in white shrimp *Penaeus vannamei. Fish Shellfish Immun.* 2021;118:1-10. doi: 10.1016/j.fsi.2021.08.017
8. Kavisri M, Abraham M, Prabakaran G, Elangovan M, Moovendhan M. Phytochemistry, bioactive potential and chemical characterization of metabolites from marine microalgae (*Spirulina platensis*) biomass. *Biomass Conversion and Biorefinery.* 2023;13:10147-10154. doi: 10.1007/s13399-021-01689-2
9. Awad LZ, El-Mahallawy HS, Abdelnaeim NS, Mahmoud MMA, Dessouki AA, Elbanna NI. Role of dietary *Spirulina platensis* and betaine supplementation on growth, hematological, serum biochemical parameters, antioxidant status, immune responses, and disease resistance in Nile tilapia. *Fish Shellfish Immun*. 2022;126:122-130. doi: 10.1016/j.fsi.2022.05.040
10. Sharawy ZZ, Ashour M, Labena A, Alsaqufi AS, Mansour AT, Abbas EM. Effects of dietary *Arthrospira platensis* nanoparticles on growth performance, feed utilization, and growth-related gene expression of Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture*. 2022;551:737905. doi: 10.1016/j.aquaculture.2022.737905
11. Tayag CM, Lin YC, Li CC, Liou CH, Chen JC. Administration of the hot-water extract of *Spirulina platensis* enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus. Fish Shellfish Immun.* 2010;28(5-6):764-773. doi: 10.1016/j.fsi.2010.01.023
12. Lin YC, Tayag CM, Huang CL, Tsui WC, Chen JC. White shrimp Litopenaeus vannamei that had received the hot-water extract of *Spirulina platensis* showed earlier recovery in immunity and up-regulation of gene expressions after pH stress. *Fish Shellfish Immun.* 2010;29(6):1092-1098. doi: 10.1016/j.fsi.2010.09.002
13. Abdel-Moneim AME, El-Saadony MT, Shehata AM, et al. Antioxidant and antimicrobial activities of *Spirulina platensis* extracts and biogenic selenium nanoparticles against selected pathogenic bacteria and fungi. *Saudi J Biol Sci.* 2022;29(2):1197-1209. doi: 10.1016/j.sjbs.2021.09.046
14. Velmurugan S, Jerin N, Babu MM, Bindhu F, Dhas SA, Citarasu T. Screening and characterization of antiviral compounds from *Enteromorpha flexuosa* against white spot syndrome virus (WSSV) and its *in-vivo* influence on Indian white shrimp *Fenneropenaeus indicus*. *Aquacult Int.* 2010;23:65-80. doi: 10.1007/s10499-014-9798-y
15. Liu Y, Zhang Y, Liu Q, et al. *In vitro* measurement of superoxide dismutase-like nanozyme activity:a comparative study. *Analyst*. 2021;146(6):1872-1879. doi: 10.1039/D0AN02164D
16. Zhou S, Hou Z, Li N, Qin Q. Development of a SYBR Green I real-time PCR for quantitative detection of *Vibrio alginolyticus* in seawater and seafood. *J ApplMicrobiol.* 2007;103(5):1897-1906. doi: 10.1111/j.1365-2672.2007.03420.x
17. Pang L, Zhang XH, Zhong Y, Chen J, Li Y, Austin B. Identification of *Vibrio harveyi* using PCR amplification of the toxR gene. *Lett Appl Microbiol.* 2006;43(3):249-

255. doi: 10.1111/j.1472-765X.2006.01962.x
18. Nordstrom JL, Vickery MCL, Blackstone GM, Murray SL, DePaola A. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl Environ Microbiol.* 2007;73(19):5840-5847. doi: 10.1128/AEM.00460-07
19. Hardiningtyas SD, Putri FA, Setyaningsih I. Antibacterial activity of ethanolic *Spirulina platensis* extract-water soluble chitosan nanoparticles. *IOP Conference Series:Earth and Environmental Science* 2022;1033(1):012053. doi: 10.1088/1755-1315/1033/1/012053
20. Esquer-Miranda E, Nieves-Soto M, Rivas-Vega A, Miranda -Baeza A, Pina-Valdez P. Effects of methanolicmacroalgae extracts from *Caulerpa sertularioides* and *Ulva lactuca* on *Litopenaeus vannamei* in the presence of Vibrio bacteria. *Fish Shellfish Immun.* 2016;51:346-350. doi: 10.1016/j.fsi.2016.02.028
21. El-Saadony MT, Swelum AA, Ghanima MMA, et al. Shrimp production, the most important diseases that threaten it, and the role of probiotics in confronting these diseases:A review. *Res Vet Sci.* 2022;144:126-140. doi: 10.1016/j.rvsc.2022.01.009
22. Gunasundari E, Kumar PS, Christopher FC, Arumugam T, Saravanan A. Green synthesis of metal nanoparticles loaded ultrasonic assisted *Spirulina platensis* using algal extract and their antimicrobial activity. *IET Nanobiotechnol.* 2017;11(6):754-758. doi: 10.1049/iet-nbt.2016.0223
23. Wang Y, Liu X, Su C, Ding Y, Pan L. Process optimization for fermented siwu decoction by multi-index-response surface method and exploration of the effects of fermented siwu decoction on the growth, immune response and resistance to *Vibrio harveyi* of Pacific white shrimp (*Litopenaeus vannamei*). *Fish Shellfish Immun.* 2022;120:633-647. doi: 10.1016/j.fsi.2021.11.029
24. Chen YY, Chen JC, Tayag CM, et al. Spirulina elicits the activation of innate immunity and increases resistance against *Vibrio alginolyticus* in shrim. *Fish Shellfish Immun.* 2016;55:690-698. doi: 10.1016/j.fsi.2016.06.042
25. Tayag CM, Lin YC, Li CC, Liou CH, Chen JC. Administration of the hot-water extract of *Spirulina platensis* enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against Vibrio alginolyticus. *Fish Shellfish Immun.* 2010;28(5-6):764-773. doi: 10.1016/j.fsi.2010.01.023
26. Liu CH, Cheng W, Hsu JP, Chen JC. *Vibrio alginolyticus* in the white shrimp *Litopenaeus vannamei* confirmed by polymerase chain reaction and 16S rDNA sequencing. *Dis Aquat Organ.* 2004;61(1-2):169-174. doi: 10.3354/dao061169
27. Santra HK, Maity S, Banerjee D. Production of bioactive compounds with broad spectrum bactericidal action, bio-film inhibition and antilarval potential by the secondary metabolites of the endophytic fungus Cochliobolus s APS1 Isolated from the Indian Medicinal Herb *Andrographis paniculata*. *Molecules.* 2022;27(5):1459. doi: 10.3390/molecules27051459
28. Radhakrishnan S, Belal IE, Seenivasan C, Muralisankar T, Bhavan PS. Impact of fishmeal replacement with *Arthrospira platensis* on growth performance, body composition and digestive enzyme activities of the freshwater prawn, *Macrobrachium rosenbergii*. *Aquacult Rep.* 2016;3:35-44
29. Bussabong P, Rairat T, Chuchird N, et al. Effects of isoquinoline alkaloids from *Macleaya cordata* on growth performance, survival, immune response, and resistance to *Vibrio parahaemolyticus* infection of Pacific white shrimp (*Litopenaeus vannamei*). *Plos one.* 2021;16(5):0251343. doi: 10.1371/journal.pone.0251343
30. Gogna S, Kaur J, Sharma K. Spirulina-An edible cyanobacterium with potential therapeutic health benefits and toxicological consequences. *J Am Nutri Assoc.* 2023;42(6):559-572. doi: 10.1080/27697061.2022.2103852
31. Abdel-Moneim AME, Shehata AM, Mohamed NG, Elbaz AM, Ibrahim NS. Synergistic effect of *Spirulina platensis* and selenium nanoparticles on growth performance, serum metabolites, immune responses, and antioxidant capacity of heat-stressed broiler chickens. *Biol Trace Elem Res.* 2022;200(2):768-779. doi: 10.1007/s12011-021-02662-w
32. Watanuki H, Ota K, Tassakka AC MAR, Kato T, Sakai M. Immunostimulant effects of dietary *Spirulina platensis* on carp, *Cyprinus carpio*. *Aquaculture.* 2006;258(1-4):157-163. doi: 10.1016/j.aquaculture.2006.05.003
33. Luo P, Hu C. Vibrio alginolyticus *gyrB* sequence analysis and *gyrB*-targeted PCR identification in environmental isolates. *Dis Aquat Organ.* 2008;82(3):209-216. doi: 10.3354/dao01984
34. Chrisolite B, Thiyagarajan S, Alavandi SV, et al. Distribution of luminescent *Vibrio harveyi* and their bacteriophages in a commercial shrimp hatchery in South India. *Aquaculture.* 2008;275(1-4):13-19. doi: 10.1016/j.aquaculture.2007.12.016
35. Prasad S, Morris PC, Hansen R, Meaden PG, Austin B. A novel bacteriocin like substance (BLIS) from a pathogenic strain of Vibrio harveyi. *Microbiol.* 2005;151:3051-3058. doi: 10.1099/mic.0.28011-0
36. Khimmakthong U, Sukkarun P. The spread of *Vibrio parahaemolyticus* in tissues of the Pacific white shrimp *Litopenaeus vannamei* analyzed by PCR and histopathology. *Microb Pathogenesis* 2017;113:107-112. doi: 10.1016/j.micpath.2017.10.028
37. Karnjana K, Soowannayan C, Wongprasert K. Ethanolic extract of red seaweed *Gracilaria fisheri* and furanone eradicate *Vibrio harveyi* and *Vibrio parahaemolyticus* biofilms and ameliorate the bacterial infection in shrim *Fish Shellfish Immun.* 2019;88:91-101. doi: 10.1016/j.fsi.2019.01.058