Research Article

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The fucose containing polymer (FCP) rich fraction of *Ascophyllum nodosum* (L.) Le Jol. protects *Caenorhabditis elegans* against *Pseudomonas aeruginosa* by triggering innate immune signaling pathways and suppression of pathogen virulence factors

Saveetha Kandasamy[1], Wajahatullah Khan[2], Garima Kulshreshtha[1], Franklin Evans[3], Alan T. Critchley[3], J. H. Fitton[4], Damien N. Stringer[4], Vicki-Anne Gardiner[4] and Balakrishnan Prithiviraj[1,*]

[1]*Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, P.O. Box 550, Truro, NS B2B 5E3, Canada*
[2]*Basic Sciences Department, College of Science and Health Professions (COSHP), King Saud Bin Abdul Aziz University for Health Sciences (KSAU-HS), P.O. Box 22490, Riyadh 11426, Saudi Arabia*
[3]*Acadian Seaplants Limited, 30 Brown Avenue, Dartmouth, NS B3B 1X8, Canada*
[4]*Marinova Pty Ltd., 249, Kennedy Drive, Cambridge, Tasmania 7170, Australia*

Brown algal extracts have long been used as feed supplements to promote health of farm animals. Here, we show new molecular insights in to the mechanism of action of a fucose containing polymer (FCP) rich fraction from the brown seaweed *Ascophyllum nodosum* using the *Caenorhabditis elegans-Pseudomonas aeruginosa* PA14 infection model. FCP enhanced survival of *C. elegans* against pathogen stress, correlated with up-regulation of key immune response genes such as: *lipases*, *lysozyme* (*lys-1*), *saponin-like protein* (*spp-1*), *thaumatin-like protein* (*tlp-1*), *matridin SK domain protein* (*msk-1*), *antibacterial protein* (*abf-1*), and *lectin family protein* (*lfp*). Further, FCP caused down regulation of *P. aeruginosa* quorum sensing genes: (*lasI*, *lasR*, *rhlI*, and *rhlR*), secreted virulence factors (lipase, proteases, and elastases) and toxic metabolites (pyocyanin, hydrogen cyanide, and siderophore). Biofilm formation and motility of pathogenic bacteria were also greatly attenuated when the culture media were treated with FCP. Interestingly, FCP failed to mitigate the pathogen stress in *skn-1*, *daf-2*, and *pmk-1* mutants of *C. elegans*. This indicated that, FCP treatment acted on the regulation of fundamental innate immune pathways, which are conserved across the majority of organisms including humans. This study suggests the possible use of FCP, a seaweed component, as a functional food source for healthy living.

Key Words: *Ascophyllum nodosum*; *Caenorhabditis elegans*; innate immunity; *Pseudomonas aeruginosa*; quorum sensing; virulence factors

INTRODUCTION

The phaeophycean, fucoid alga, *Ascophyllum nodosum* (rockweed) is an abundant intertidal brown alga of the North Atlantic on sheltered and estuarine rocky shores. It is a rich source of high-value bioactive compounds for

Received November 18, 2014, **Accepted** May 8, 2015
***Corresponding Author**
E-mail: bprithiviraj@dal.ca
Tel: +1-902-893-6643, Fax: +1-902-893-1404

biomedical applications (Turner et al. 2002, Fitton 2011). The bioactive compounds include polysaccharides (e.g., alginic acid, sulphated fucans, and laminarin), polyunsaturated fatty acids, vitamins, antioxidants, peptides, and secondary metabolites such as phlorotannins (Kim and Wijesekara 2010). The term "fucoidan" has been applied to a complex group of sulphated polymers (SPs) from marine macroalgae, the type and quantity of which differ amongst red, brown and green algae (Fitton 2011). Fucose containing polymers (FCPs) and laminarins are the major SPs of the brown algae collectively. The dry matter of *A. nodosum* contains 1-7% laminarin, 4-10% FCPs, and 22-30% alginates. The FCPs in *A. nodosum* are rich in L-fucose and sulphate ester groups (Khan et al. 2009).

FCPs have been reported to exhibit a wide variety of bioactivities such as antioxidant, anti-bacterial, anti-coagulant, anti-inflammatory, and anti-proliferative activities (Fitton 2011). Fucoidan from the brown seaweed *Cladosiphon* sp. has been shown to inhibit *Helicobacter pylori* attachment to porcine gastric mucin *in vitro* and also reduced the prevalence of *H. pylori* infected gerbils *in vivo* (Shibata et al. 2003). Recently, *in-vitro* studies on low-molecular-weight fragments of fucoidan isolated from the brown alga, *Fucus evanescens* showed anti-cancer activity in human malignant melanoma cell lines (Anastyuk et al. 2012). A cyto-protective effect of fucoidan on 5-fluorouracil-treated dendritic cells has also been shown (Jeong et al. 2012). The antibacterial activities of *A. nodosum* extracts against several pathogenic bacteria were demonstrated by Vacca and Walsh (1954). Besides fucoidans, the carbohydrate-binding proteins (i.e., lectins and lectin-like molecules) present in *A. nodosum*, selectively bind to specific carbohydrates on the surface of bacterial cells, inhibiting the attachment of microbes to the gut wall (Fabregas et al. 1989).

Pseudomonas aeruginosa is an opportunistic human pathogen that causes serious infections in immune-compromised, human immunodeficieny virus, and cancer patients and also widespread lung infection in cystic fibrosis patients. Pathogenesis of *P. aeruginosa* is largely mediated by secretory proteins (i.e., elastase, alkaline protease, and lipases) and secondary metabolites (e.g., pyoverdine, siderophores, and hydrogen cyanide). The bacterium produces a redox-active phenazine called pyocyanin, which is a characteristic chloroform-soluble, blue-green pigment, which kills higher animal cells through the generation of reactive oxygen species and the arrest of cellular respiration (Stewart-Tull and Armstrong 1972). Biosynthesis of hydrogen cyanide (HCN) is one of the primary components in microbial secondary metabolism, which occurs during the late log and early stationary phases of bacterial growth; it has been well characterized in *P. aeruginosa* pathogenesis (Castric 1975).

Bacteria communicate through an extensive array of extracellular signal molecules. Production and secretion of these extracellular signal molecules mediate cell-to-cell communication which coordinates the expression of various genes within the bacterial population and aids in the formation of biofilms in response to specific environmental or physiological conditions, which, in turn, enhances successful infection in humans and in animals (Davies et al. 1998, Wiener-Kronish et al. 2001). The process of sensing individual cells by the accumulation of diffusible, low-molecular-weight signal molecules is known as "quorum sensing" (QS) (Williams et al. 2000). There are two well-defined QS systems identified in *P. aeruginosa*, namely, *las* (mediated by transcriptional activators *lasR* and *lasI*) and *rhl* (mediated by transcriptional activators *rhlR* with *rhlI*). An understanding of QS has opened a new avenue for treating bacterial infections. The importance of bacterial motility in pathogenesis was well explained by Burrows (2012).

In this study, we used the *C. elegans–P. aeruginosa* (PA14) patho-system to test the effects of commercially extracted *A. nodosum* FCPs against infection, secreted virulence factors, biofilm formation, motility and QS properties of *P. aeruginosa*. Since, PA14 is a clinical isolate of *P. aeruginosa,* which is known to infect the model nematode *C. elegans*, and this patho-system has been studied extensively, it was chosen as the most appropriate for this study (Tan et al. 1999). It has been used by Sifri et al. (2005) to screen anti-infective and anti-microbial agents and also by other researchers (Aballay and Ausubel 2002). An advantage of this model system is that it is genetically traceable because of the availability of genome maps for both model organisms, and the results can be readily used as a reference for mammalian-bacterial pathogenesis.

MATERIALS AND METHODS

Chemicals and *Caenorhabditis elegans* strains

All chemicals were purchased from Sigma-Aldrich, Oakville, Ontario, Canada, unless otherwise stated. The wild type N2 (var. Bristol), mutant (*skn-1*, *daf-2*, *pmk-1*) *C. elegans* stains and *Escherichia coli* strain OP-50 were obtained from the *C. elegans* were obtained from the Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN, USA. Cultures of OP-50 *E. coli*

were grown over-night in Luria-Bertani (LB) broth and concentrated 10 times by centrifugation at 3,500 ×g for 10 min. The *C. elegans* strains were maintained at 20°C on 1.2% solid nematode growth medium (NGM), seeded with 50 µL of live OP-50 *E. coli* as a food source.

Source of FCP

A powder form of commercially extracted FCP was a kind gift, prepared by Marinova (http://www.marinova.com.au/). The material was derived from *Ascophyllum nodosum*, which was sustainably harvested in Nova Scotia, Canada by Acadian Seaplants Limited (http://www.acadianseaplants.com/marine-plant-seaweed-manufacturers/resource-management). A stock solution of FCP (40 mg mL^{-1}) was made by dissolving the powder in distilled water and diluted to required concentrations, as required by each experiment. The compositional analysis of FCP is given in Appendix 1, the molecular weight of the fucoidan was 633 kDa.

Bacterial strains and growth conditions

The clinical, pathogenic isolate of *P. aeruginosa* PA14 was a kind gift from Dr. Eric Déziel (INRS-Institute Armand-Frappier-Microbiologieet Biotechnologie, Laval, Québec, Canada). Kings B, complete medium (with peptone) was used for bacterial culture and maintenance. The following media were used, as required in different assays: 1) modified nematode growth agar (MNGA) / slow killing (SK) agar medium (with 0.35% peptone instead of 0.25%), 2) LB broth (tryptone 10 g, yeast extract 5 g, sodium chloride 10 g, final pH 7.0 ± 0.2), 3) glycerol alanine minimal medium (10 mL glycerol, 6 g L-alanine, 2 g $MgSO_4$, 0.1 g K_2HPO_4, 0.018 g $FeSO_4$ per liter of medium. All assays and experiments were repeated with three biological and three technical replicates.

Testing of antimicrobial susceptibility

The disk diffusion method of the Clinical and Laboratory Standards Institute (CLSI) was used to determine the antimicrobial susceptibility of the *P. aeruginosa* strains.

Motility assays

Bacterial culture was streaked on the control and the different concentrations of FCP-treated, LB agar. Single colonies of bacteria were subjected to different motility assays. All the assays were repeated three times, each with three replicates.

Swarming assay

A single colony of PA14 bacteria was inoculated into 5 mL of LB media and grown for 15 h at 37°C with aeration. Two microliters of each overnight culture was spotted onto the surface of LB 0.5% agar and SK 0.5% agar plates and then incubated at 37°C overnight.

Swimming motility

A single bacterial colony was picked with a straight end loop and inoculated into LB swim agar (0.35% agar). Plates were incubated 8-12 h at 37°C. The diameter of the flagellum-mediated motility generated turbid zone was measured.

Twitching motility assay

A portion of a single bacterial colony was picked with a straight end inoculation loop and stabbed to the bottom of a LB agar plate (1.5% agar). Plates were incubated overnight at 37°C and then for 2 days at room temperature. The growth, at the interface between the agar and the polystyrene plate (radius from the inoculation point), was measured.

The effect of FCP on *Pseudomonas aeruginosa* protease

Protease activity was determined by measuring the ability of culture supernatants to lyse skimmed milk powder. Supernatants from the 18-hour-old culture, grown at 37°C, with constant shaking, were used for the assay. A 100 µL aliquot of *P. aeruginosa* LB culture supernatant, with or without 150 µg mL^{-1} FCP was added to 900 µL of 0.5% (w/v) skimmed milk in 50 mM Tris-HCl (pH 8.0). Absorbance at OD_{600} was measured at 24 and 48 h. Enzyme activity was expressed as A600 per microgram of protein.

Effect of FCP on *Pseudomonas aeruginosa* alkaline protease

Alkaline protease activity of the supernatant from an overnight bacterial culture in LB broth was determined by adding 0.5 mL of supernatant to 1.5 mL of assay buffer (20 mM Tris-HCl, 1 mM $CaCl_2$ buffer, pH 8.0) which contained 50 mg of hide remazol blue powder (Sigma-Aldrich). Tubes were incubated at 37°C for 1 h with constant

shaking; the reaction was stopped by placing the tube on ice. After centrifuging 5 min at 4,000 ×g, the absorbance of the supernatant was measured at 590 nm after 24 and 48 h. The enzyme activity was expressed as units where an increase of 1.0 in the OD_{590} per milliliter per hour was defined as 1 unit.

Effect of FCP on *Pseudomonas aeruginosa* elastin

The secreted elastase in the supernatant of PA14 was measured using Congo Red as the substrate. The bacterium was grown in LB broth at 37°C for 16 h, centrifuged at 15,000 ×g, at 4°C, for 10 min; 0.5 mL of the supernatant was added to 1 mL of the assay buffer (30 mM Tris buffer, pH 7.2) containing 10 mg of Congo Red (Sigma-Aldrich). The mixture was incubated at 37°C for 6 h with constant shaking. The insoluble substrate was removed by centrifugation at 1,200 ×g for 10 min and the absorbance of the supernatant was measured at 495 nm. Elastase activity was defined as an increase in the OD_{495} per milliliter of PA14 culture filtrate.

Effect of FCP on *Pseudomonas aeruginosa* pyocyanin

Pyocyanin was extracted from a 24-hour-old *P. aeruginosa* culture, grown in glycerol alanine, minimal medium. The cells were removed by centrifugation and the pyocyanin in the supernatant was extracted in chloroform, by mixing 5 mL of supernatant with 3 mL of chloroform. Pyocyanin was then re-extracted into 1 mL of acidified water (0.2 N HCl), as a pink-red solution. The pyocyanin concentration was determined from the absorbance measured at 520 nm.

Effect of FCP on the production of hydrogen cyanide in *Pseudomonas aeruginosa*

A bacterial culture was streaked onto tryptic, soya agar medium. Filter paper discs (1.5 cm diameter) were soaked in picric acid solution (2.5 g picric acid, 12.5 g Na_2CO_3, and 1 L distilled water) and placed on the upper lids of Petri dishes. The dishes were sealed with parafilm and incubated for four days. HCN production was assessed by the presence of a coloured zone around the bacterial lawn and the colour change of the filter paper from yellow to a brown to reddish-brown. Reaction colours were scored as "weak" (i.e., yellow to light brown), "moderate" (i.e., brown) and "strong" (i.e., reddish brown).

Quantification of hydrogen cyanide

Bacterial isolates were grown in tryptic soya broth with picric acid solution saturated filter paper strips (10 cm long and 0.5 cm wide) in a hanging position, inside the flask at 28 ± 2°C for 48 h. The sodium picrate in the filter paper was reduced to a reddish compound in proportion to the amount of hydrocyanic acid evolved. The coloured pigment was eluted by placing the filter paper in a clean test tube containing 10 mL of distilled water and measuring absorbance at 625 nm.

Quantitation of siderophores

The bacterium was grown in KB broth for 3 days and centrifuged at 2,000 rpm for 10 min. The pH of the supernatant was adjusted to 2.0 with HCl and an equal volume of ethyl acetate was added in a separating funnel, mixed well and the ethyl acetate fraction was collected. This process was repeated three times to recover most of the siderophores from the supernatant. The ethyl acetate fractions were pooled, air-dried and dissolved in 5 mL of ethanol (50%). Five milliliters of ethyl acetate fraction was mixed with 5 mL of Hathway's reagent (1.0 mL of 0.1 M $FeCl_3$ in 0.1 N HCl to 100 mL of distilled water containing 1.0 mL of potassium ferricyanide). The absorbance for dihydroxyphenol was measured at 700 nm using dihydroxy benzoic acid as a standard. The synthesis of siderophores was expressed as μM mL^{-1} of culture filtrate.

Real time-polymearse chain reaction (RT-PCR) analysis of quorum sensing and virulence genes of *Pseudomonas aeruginosa*

The relative expressions of quorum sensing genes (i.e., *lasI*, *lasR*, *rhlI*, and *rhlR*) and other virulence related genes (i.e., *hcnC*, *aroE*, *rpoN*, *sbe*, and *sodB*) were analyzed by isolating RNA from the FCP-treated and control *P. aeruginosa* (PA14) samples. The primer sequences used are listed in Appendix 1.

Biofilm formation

The biofilm forming ability of the isolates was observed using polystyrene microtitre plates. Overnight cultures in LB broth were diluted 1 : 100 into fresh LB broth and then 0.1 mL of the freshly inoculated medium was dispensed into a 96-well polystyrene microtitre plate. The plates were incubated at 37°C for 8 h without agitation. Biofilm formation was observed by staining the wells

with 10 µL of crystal violet [0.1% (w/v) in water]. After the stain was added, the plates were incubated for a further 15 min at room temperature and then washed thoroughly with distilled water to remove cells and residual dye. Ethanol (95%) was used to elute any crystal violet from the biofilms and the absorbance of the solubilized dye was measured at 590 nm using a microtitre plate reader (BioTek, Winooski, VT, USA).

Slow-killing assay

MNGA medium was used in slow-killing assays to test the efficacy of FCP on PA14-induced killing. Treatment plates were prepared by spreading 10 µL of saturated PA14 culture on the center of the plate. The plates were incubated at 37°C, for 12 h to establish the PA14 lawn. *E. coli* (OP-50) plates were used as the control. Production of progenies was stopped by treating the worms with 50 µM fluorodeoxyuridine before their transfer to assay plates. About 30-40 synchronized L4 worms (Kandasamy et al. 2014) were transferred to each treatment plate; the number of dead was recorded at 12-h intervals by microscopic observations. The experiment was performed as three combinations of treatments with three different concentrations of FCP (50, 150, and 250 µg mL^{-1}): 1) synchronized worms were maintained on treatment plates, from their egg stage and exposed to bacterial infection, 2) the worms, synchronized on plain MNGA were infected with a bacterial lawn grown in FCP-treated culture plates, and 3) synchronized worms on their treatment plates were transferred to a bacterial lawn which was grown on FCP-treated MNGA plates.

Killing assays with mutant *Caenorhabditis elegans* worms

Three different immune responsive, functional mutant strains (i.e., *skn-1*, *daf-2*, and *pmk-1*) were used in these killing experiments. Synchronized, pre-treated worms were transferred to a bacterial lawn, which was grown on FCP-treated, MNGA plates, and the killing effects were monitored at regular interval. The experimental conditions were maintained as mentioned above; just one concentration of FCP (150 µg mL^{-1}) was used.

Effect of FCP on immune-response gene expression of *Caenorhabditis elegans*

About 500 synchronized, young adult worms from each treatment were used in this study. The treatment details are as follows: 1) control (worms cultured on *E. coli* OP-50 lawn), 2) worms cultured on *E. coli* OP-50 lawn, treated with FCP, 3) and worms cultured on a pathogen lawn without FCP treatment, 4) worms cultured on FCP-treated NGM plates were exposed to a pathogen lawn for 18 h. The worms were then collected from the treatment plates at their young adult stage. The total RNA was isolated and converted in to cDNA. The differential expression of immune-response genes, as affected by FCP, was studied by quantitative, RT-PCR using an ABI 7900HT Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA) with appropriate primers (Appendix 2). The expression of the following genes was studied: *zk6.7* (*lipases*), *lys-1* (*lysozyme*), *spp-1* (*saponin-like protein*), *f28d1.3* (*thaumatin-like protein*), *t20g5.7* (*matridin SK domain protein*), *abf-1* (*anti-bacterial protein*), and *f38a1.5* (*lectin family protein*). The primers were designed using the online Universal Probe Library Array Design Center. Reverse transcription was performed with 2 µg total RNA for 2 h at 37°C, using the ABI-RT system.

Statistical analysis

Significance of the data was analyzed using COSTAT; $p < 0.05$ was considered to be statistically significant. Data were analyzed using Fisher's least significant difference test with $p \leq 0.05$ using COSTAT statistical software.

RESULTS

FCP prevented *Pseudomonas aeruginosa* biofilm formation, without showing direct anti-microbial effect

Biofilms act as environmental reservoirs of pathogens; the film provides the organisms with survival advantages in natural environments and increases their virulence. The FCP-treatment reduced the biofilm formation by a factor of 4, as compared to the control ($p < 0.0001$) (Fig. 1). The direct antimicrobial activity of FCP was tested by placing paper discs with different concentrations of FCP on petri-plates cultured with *P. aeruginosa*. The plates were examined at regular intervals for 3 days. It was established that FCP-treatment did not exhibit a direct antimicrobial effect on *P. aeruginosa* (data not shown).

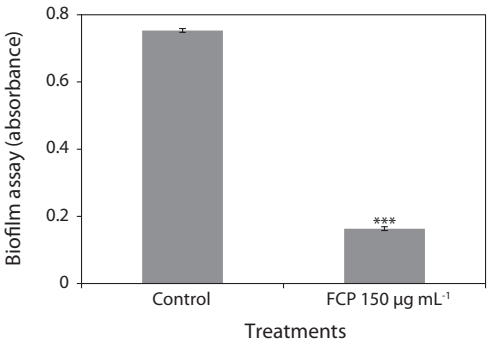

Fig. 1. Effect of fucose containing polymer (FCP)-treatment on biofilm formation in *Pseudomonas aeruginosa* PA14. Error bars indicate the standard error of the mean. Asterices indicate statistically significant differences between control and FCP-treatment, according to Student's t test (***p < 0.0001).

FCP inhibited *Pseudomonas aeruginosa* toxic metabolites

The highly diffusible, pigmented, phenazines, and two other (i.e., HCN and siderophores) toxic secondary metabolites, are known for their key roles in direct killing of infected organisms. The amount of pyocyanin present in the culture filtrate was much lower FCP-treated cultures (0.33 μg mL^{-1}), as compared to the control (0.45 μg mL^{-1}) (Fig. 2A). The HCN production of PA14 was reduced to 50% with FCP treatment (Fig. 2B). In addition, the FCP-treatment greatly reduced siderophore production ($p \leq 0.001$) (Fig. 2C).

FCP inhibited *Pseudomonas aeruginosa* secretary virulence factors

To further support the data, the effect of FCP on secretary virulence factors: i.e., protease, alkaline protease and elastase, of the pathogen PA14 were studied by supplementing bacterial growth medium with FCP. It was observed that proteolytic enzyme activity was reduced from 1.0 to 0.4 units (after 24 h) and 2.5 to 1.8 units (after 48 h) due to FCP-treatment ($p < 0.001$) (Fig. 3A). A similar trend in reduction was also noticed with alkaline protease activity ($p < 0.01$) (Fig. 3B). However, the elastase enzyme activity ($p < 0.01$) was found to be increased due to FCP treatment (Fig. 3C).

FCP suppressed the expression of *Pseudomonas aeruginosa* quorum sensing genes

Both the *las* and *rhl* quorum-sensing systems were

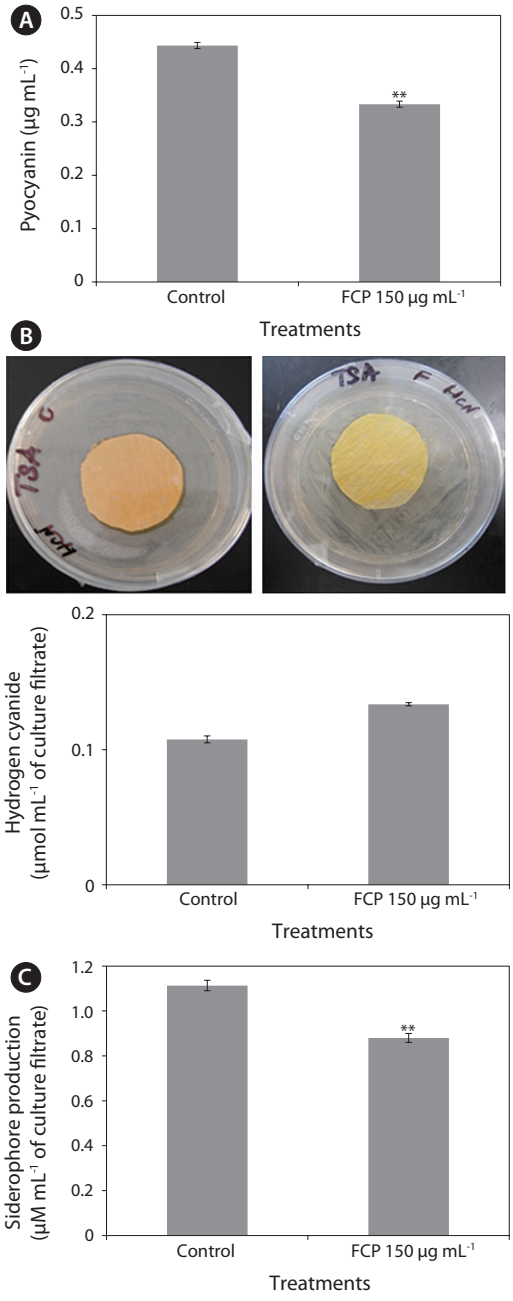

Fig. 2. Effect of fucose containing polymer (FCP)-treatment on the secreted virulence factors of *Pseudomonas aeruginosa* PA14: pyocyanin (A), hydrogen cyanide (B), and siderophore (C) production. Error bars indicate the standard error of the mean. Asterices indicate statistically significant differences between control and FCP-treated worms, according to Student's t test (**p < 0.001).

studied since the both play critical roles in *P. aeruginosa* pathogenicity, including synthesis and regulation of important virulence genes of the QS biosynthetic pathways, as well as production of secondary metabolites, believed to be toxic. It was observed that the growth and cell den-

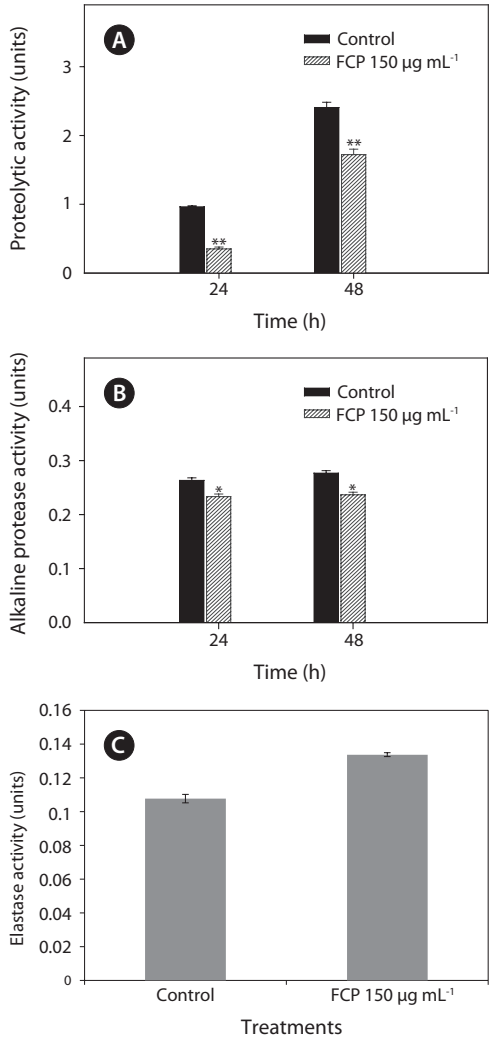

Fig. 3. Influence of fucose containing polymer (FCP) treated culture medium on the proteolytic enzyme activity (A), alkaline protease activity (B), and elastase activity (C) of *Pseudomonas aeruginosa* strain PA14. Error bars indicate the standard error of the mean. Asterices indicate statistically significant differences between control and FCP-treated worms, according to Student's t test (*p < 0.01, **p < 0.001).

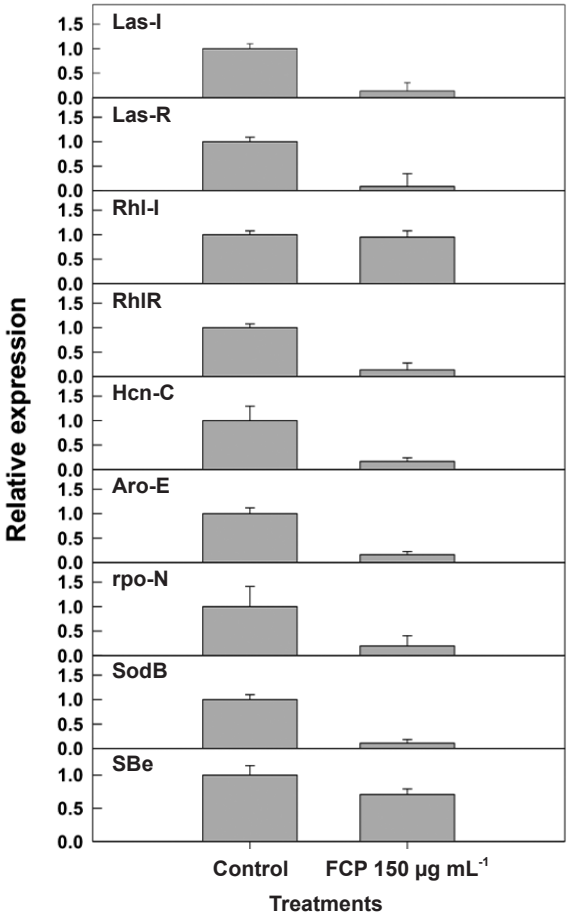

Fig. 4. Relative expression of quorum sensing and the virulence factor-related genes of *Pseudomonas aeruginosa* PA14 treated with fucose containing polymer (FCP) extract. Error bars indicate the standard error of the mean. Asterices indicate statistically significant differences between control and FCP-treated worms, according to Student's t test (**p < 0.001).

sity of *P. aeruginosa* were reduced considerably following FCP-treatment (data not shown).

To validate the results from the biochemical analysis of secondary metabolite production, following FCP-treatment, gene expression studies were conducted using quantitative RT-PCR. The relative expression of major QS (i.e., *lasI*, *lasR*, *rhlI*, and *rhlR*) and virulence factors (i.e., *hcnC*, *aroE*, *rpoN*, *sbe*, *sodB*, *phz*, and *pyoS3a*) was considerably reduced due to FCP treatment. The observed reduction in the gene expression was about 5- to 7-fold ($p < 0.001$), except *rhlI* (where there was no significant difference between control and treatment) ($p > 0.05$) and *sbe* (where there was only a 2-fold reduction relative to the control ($p = 0.028$) (Fig. 4).

FCP-treatment affected *Pseudomonas aeruginosa* motility

Since adherence, motility, biofilm formation, virulence and pathogenesis are associated and are involved in pathogenesis, we studied the effect of FCP-treatment on PA14 motility. FCP-treatments significantly affected swimming, swarming and the "twitching" motility of PA14 (Fig. 5). The direct effect of FCP-treatments on PA14 motility might, at least in part, contribute to reduced virulence on *C. elegans*.

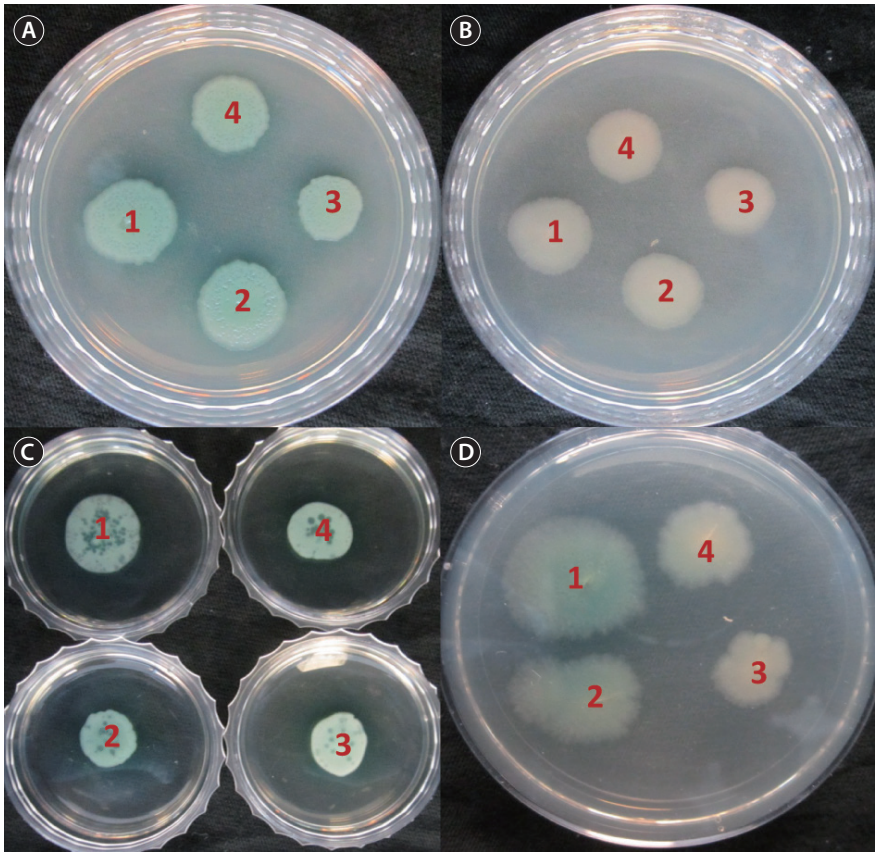

Fig. 5. Effect of fucose containing polymer (FCP)-treatment on the motility of *Pseudomonas aeruginosa* PA14: swarming (1) on Luria-Bertani (LB) agar (A), slow killing agar (B), swimming (C), and twitching (D) motility.

Table 1. Effect of dietary treatment of FCP on the survival of *Caenorhabditis elegans* (wild type N2) challenged with *Pseudomonas aeruginosa* PA14

Sample No.	Treatments	Total No. of worms	Censored	Mean ± SE survival (h)
Experiment No. 1[a] (anti-infective assay)				
1	Control	113	8	83.1 ± 1.12
2	FCP 50 μg mL^{-1}	98	4	87.3 ± 0.60
3	FCP 150 μg mL^{-1}	122	7	98.0 ± 1.39
4	FCP 250 μg mL^{-1}	89	9	95.2 ± 1.48
Experiment No. 2[b] (anti-bacterial assay)				
1	Control	113	6	83.1 ± 1.12
2	FCP 50 μg mL^{-1}	105	7	89.0 ± 2.51
3	FCP 150 μg mL^{-1}	94	0	100.4 ± 1.40
4	FCP 250 μg mL^{-1}	99	11	97.5 ± 2.54
Experiment No. 3[c] (combination of both anti-infective and bacterial assay)				
1	Control	113	6	83.1 ± 1.12
2	FCP 50 μg mL^{-1}	108	8	102.4 ± 2.18
3	FCP 150 μg mL^{-1}	117	2	111.3 ± 1.56
4	FCP 250 μg mL^{-1}	130	4	105.0 ± 1.43

Fucose containing polymer (FCP) at concentrations of 50, 150, and 250 μg mL^{-1} was used in each of the three experiments. SE indicates the standard error mean.

[a]The dietary treated worms were exposed to a bacterial lawn cultured without FCP treatment.

[b]The worms cultured on modified nematode growth agar without dietary treatment were exposed to a bacterial lawn cultured with different concentrations of FCP.

[c]The worms with FCP-treated diet were exposed to a bacterial lawn cultured with different concentrations of FCP.

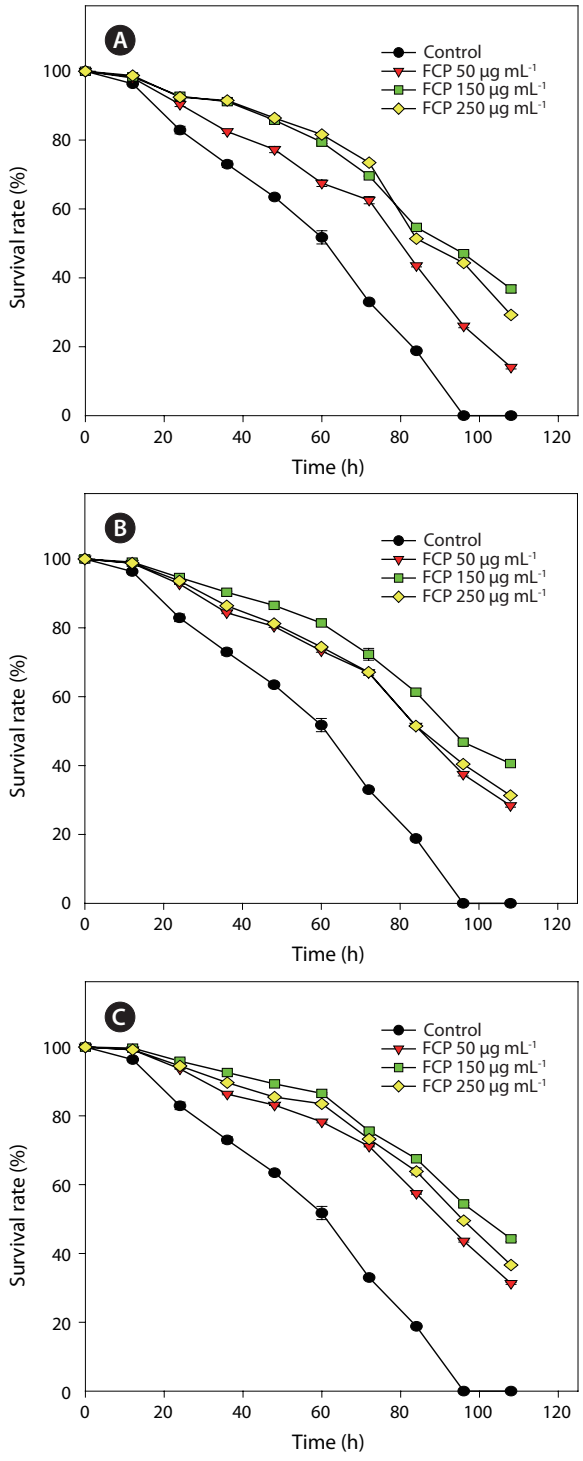

Fig. 6. (A) Effect of fucose containing polymer (FCP)-treatment on the survival of *Caenorhabditis elegans* (wild type N2), challenged with *Pseudomonas aeruginosa* PA14. (B) The worms cultured on modified nematode growth agar, without dietary treatment, were exposed to a bacterial lawn, cultured with different concentrations of FCP. (C) The worms with a FCP-treated diet, were exposed to a bacterial lawn cultured with different concentrations of FCP. Error bar indicates the standard error of the mean among the individual worms scored. The p-value was <0.01 at each time point of observation according to Student's t test.

FCP protects *Caenorhabditis elegans* from pathogen infection

Three concentrations of FCP were tested to identify the role of the sulphated fucans in the *C. elegans* immune responses and direct anti-bacterial potential against PA14. The addition of FCP, in all three experiments significantly ($p < 0.01$) reduced PA14-inflicted killing, as compared to the control (Table 1, Fig. 6). A significant number ($p < 0.01$) of surviving worms were recorded in FCP treatments, beyond the point (96 h) where control worms were completely dead. A much reduced killing rate was recorded with pre-treatment of the worms, as well as the pathogen, with added FCP (Table 1, Fig. 6C). FCP 150 µg mL^{-1} found more effective among all three concentrations tested. Neither developmental abnormalities, nor death of *C. elegans* were observed when the diet of the worm was treated with FCP (data not shown).

FCP alters *Caenorhabditis elegans* immune gene expression against pathogen infection

The expression of genes present in immune response pathways varies widely, according to host innate immunity. We chose to test the modulation in the level of expression of seven key genes, i.e., lipases, lysozyme, saponin-like protein, thaumatin-like protein, matridin SK domain protein, anti-bacterial protein, and lectin family protein from different immune response pathways (Appendix 3) based on FCP dietary treatment. FCP treatment caused a substantial up-regulation of all the genes tested. Expression of *tlp* was increased14-fold ($p < 0.0001$), while *lectin* increased 5-fold ($p < 0.01$), *abf*-1 increased 4-fold ($p < 0.001$). Also, the expression of *msk-1*, *ssp*-1, *lys*-1, and *lipase* were increased by at least 3-fold ($p < 0.01$) (Fig. 7).

FCP protects worms against pathogen infection by altering functional pathways

From the previous experiment, it was inferred that FCP, at 150 µg mL^{-1}, was the most effective in protecting to *C. elegans* worms against pathogen stress. Under such stress FCP-treated worms survived longer, as compared to control. To test if FCP-induced protection is mediated by conserved stress pathways; *skn-1*, *daf-2*, and *pmk-1*, we used *C. elegans skn-1*, *daf-2*, and *pmk-1*, deletion mutants. FCP-treatment did not rescue *skn-1*, *daf-2*, and *pmk-1* mutants. There was no clear difference between FCP-treated and control on the duration of survival of

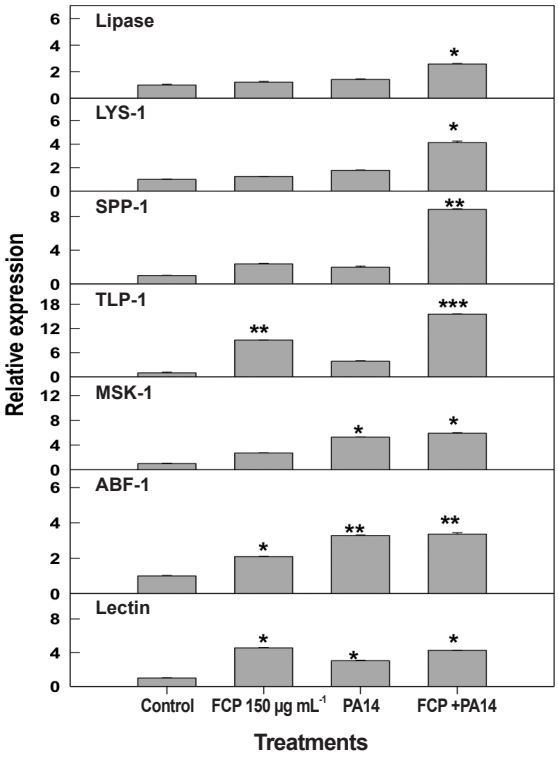

Fig. 7. Relative expression of immune response genes in fucose containing polymer (FCP)-treated *Pseudomonas aeruginosa* PA14, as compared to the control. Error bars indicate the standard error of the mean. Asterices indicate statistically significant differences between control and FCP-treated worms, according to Student's t test (*p < 0.01, **p < 0.001, ***p < 0.0001).

stressed worms. Although, the treatment offered initial protection against the pathogen infection, the effect dissipated rapidly (Fig. 8, Appendix 2), and the survival rates were similar to that of the untreated worms.

DISCUSSION

In recent years the pharmaceutical industry have shown considerable interest in the biological activity of seaweed-derived sulphated polysaccharides (SPs) (Craigie 2011, Fitton 2011). Large proportions of SPs are L-fucose; sulphates along with small amount of other sugars such as xylose, galactose, mannose, etc. (Bilan et al. 2006). SPs have shown to possess anti-angiogenic, anti-tumor (Koyanagi et al. 2003), cell-mediated immune cell modulation (Cumashi et al. 2007), anti-metastatic

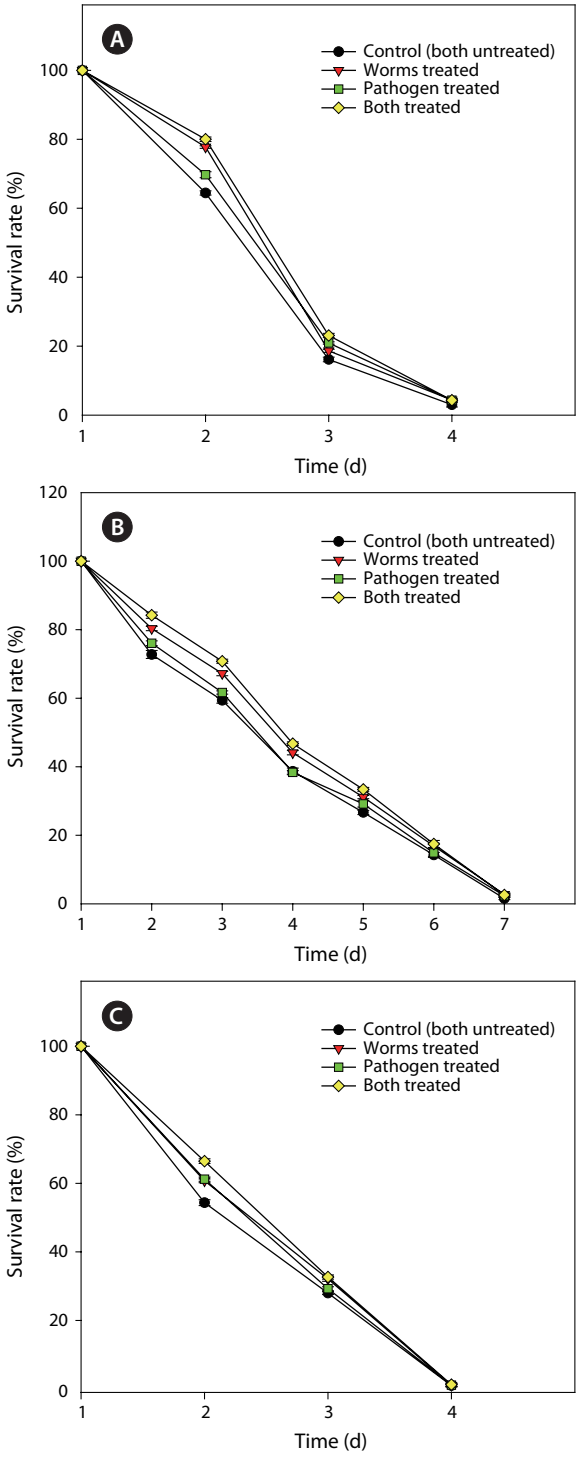

Fig. 8. Effect of fucose containing polymer (FCP)-treatment on the survival of *Caenorhabditis elegans* mutants challenged with *Pseudomonas aeruginosa* PA14; *skn-1* (A), *daf-2* (B), and *pmk-1* (C) mutant. Error bars indicate the standard error of the mean amongst the individual worms scored. The p-value was ≥0.05 at each time point of observation according to Student's t test.

fibrino-lytic (Alekseyenko et al. 2007) and anti-thrombotic properties (Cumashi et al. 2007). Anti-viral activities of fucoidan(s) from selected brown algae were well demonstrated by Hayashi et al. (2008) and Trinchero et al. (2009). Reilly et al. (2008) reported the the benefical effects of SPs in weaning pigs. SPs are effective free-radical scavengers that prevent oxidative damage and a wide range of age-related and degenerative conditions (Fujiki et al. 1992). It was recently reported that the FCP (Kandasamy et al. 2014) and water extract of Tasco® (a commercial product from *A. nodosum*) contributed to thermal stress tolerance of *C. elegans* (Kandasamy et al. 2011) and improved the immunity of the worms against *P. aeruginosa* infections (Kandasamy et al. 2012). Treatment with a hot-water extract from the brown alga *Sargassum duplicatum* increased the immunity and resistance of cultured shrimps against *Vibrio alginolyticus* infection (Ghaednia et al. 2011). The extract of *A. nodosum* was active against some pathogenic and non-pathogenic organisms of both Gram-positive and Gram-negative types (Vacca and Walsh 1954).

C. elegans is a well-defined laboratory model to study the origin, function and evolution of innate immunity in higher animals. In present study, 150 μg mL^{-1} of FCP showed the best protection against worm killing by PA14, while higher or lower concentrations were less efficient. However, the higher concentration (250 μg mL^{-1}) also showed protection in all the treatments and was significantly better than control. This indicated that the higher concentration (250 μg mL^{-1}) did not negatively affect the growth and development and was not toxic to the worms. Moreover, 150 μg mL^{-1} was identified as the optimum FCP concentration for protecting the worms against PA14 infection showed a sigmoidal dose-dependent response of FCP protecting in *C. elegans* against the lethal pathogen PA14. Previously, brown and red seaweed extracts have shown similar dose-response in immune related studies in *C. elegans* (Liu et al. 2013). *A. nodosum* extracts have been demonstrated to significantly reduce coliform bacteria in the ileum and caecum (Vacca and Walsh 1954). Here, we observed a considerable reduction in the killing rate of wild type worms following FCP-treatment. Braden et al. (2007) also reported that *A. nodosum* reduced the toxicity and enhanced the immunity of endophyte-infected tall fescue forage. Results of the present studies clearly demonstrated the effect of FCP-treatment on the suppression of PA14 virulence factors.

Conserved signal transduction in *C. elegans*, in response to pathogen stress, depends mainly on four important regulatory pathways: 1) the p38 MAP-kinase pathway, 2) the programmed cell-death pathway, 3) transforming growth factor β pathway, and 4) DAF-2 insulin / IGF-I like signaling pathway. The current study analyzed the effect of FCP on the immunity of *C. elegans* against pathogen stress by observing the survival of worms during pathogen stress in the presence of FCP and also by measuring the expression of selected innate immune response genes involved in various signaling pathways. To better explain the mechanism, the same killing experiment was conducted with mutant *C. elegans*. FCP-treatments protected the wild type worms, but not to *skn-1*, *daf-2*, or *pmk-1* mutants. These results indicated that, *P. aeruginosa* infection used host-effector molecules, along with pathogen virulence factors, to suppress the immune function in *C. elegans*. Possibly, it activated the DAF-2 insulin signaling pathway, which translocates DAF-16 proteins from the nuclei, leading to down-regulation of immune responsive transcriptional targets (*lys-1*, *spp-1*, *tlp-1*, *abf-1*, etc.) (Braden et al. 2007). The results suggest FCP-treatments upregulated the expression of immune response genes (i.e., *lectin*, *abf-1*, *msk-1*, *ssp-1*, *lys-1*, and *lypases*) indicating that FCP suppressed pathogen-mediated activation of the DAF-2 and other signaling pathways (Evans et al. 2008). Besides these, there are other mechanisms, which mainly rely on the mitogen-activated protein kinase pathway. *C. elegans* needs both innate immunity (through the activation of *pmk-1*, *skn-1*, related genes and pathways, etc.), as well as acquired immunity system to protect against pathogen infection.

We can conclude that treatment with FCP, even at a low concentration, induced the innate immune system of *C. elegans* and suppressed the worm's susceptibility to infection. Further, FCP suppressed PA14 secreted virulence factors, toxic metabolites and biofilm forming ability, through the suppression of bacterial quorum sensing. Thus, FCP offers both direct and indirect protection against pathogen infection. This study not only explained the molecular mechanisms of the present hypothesis, but also elucidated a better understanding of the brown seaweed and its extract-mediated protection, as observed in other host-pathogen interaction systems, studied earlier (Allen et al. 2001, Braden et al. 2004, Cheng et al. 2005, Ghaednia et al. 2011, Immanuel et al. 2012). In general, the extracts derived from marine algae, particularly *A. nodosum*, have tremendous potential to provide significant impacts in the nutraceutical, pharmaceutical and cosmeceutical industries.

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Appendix 1. The PCR primers designed for the quorum sensing and virulence gene expression

Sequence list	Primer list	Sequence of oligonucleotides (5′ → 3′)
1	*lasI-F*	GCTCCTTGAACACTTGAGCA
	lasI-R	GCGCGAAGAGTTCGATAAAA
2	*lasR-F*	CCGCCGAATATTTCCCATA
	lasR-R	GATATCGGTTATCTGCAACTGCT
3	*rhlI-F*	GGAGCGCTATTTCGTTCG
	rhlI-R	GTCTCGCCCTTGACCTTCT
4	*rhlR-F*	TGCGTTGCATGATCGAGT
	rhlR-R	CGGGTTGGACATCAGCAT
5	*hcnC-F*	GCCTGGACAGTTGGTAGGC
	hcnC-R	GAACAGAACCTATGACATCGTGA
6	*aroE-F*	TTCTTCGAGCAGGGCAAG
	aroE-R	CAATTCGTCCACCAGACGAT
7	*rpoN-F*	ATACCTTCATGCGCAACCA
	rpoN-R	GGCTCTGCAGGCTCTTGAT
8	*sbe-F*	CTCGTTGGTCTCCTCGAGTT
	sbe-R	CCATCTACCAGCGTGAAGG
9	*sodB-F*	GTTCAAGGAAGAGTTCACCAAGA
	sodB-R	GTCGGCCTTCTTCACCAG
Endogenous control	*16S rRNA-F*	GATTAACGCTTGCACCCTTC
	16S rRNA-R	TAAGCACCGGCTAACTTCGT

PCR, polymerase chain reaction.

Appendix 2. The immune response primers used in this study

Sequence list	Primer list	Sequence of oligonucleotides (5′ → 3′)
1	*ZK6.7-F*	CGAATTCCTCCCAAACAACT
	ZK6.7-R	GAATAGGACGTTGTCGCAGA
2	*lys-1-F*	TTCGGATCTTTCAAGAAGGC
	lys-1-R	TGGGATTCCAACAACGTAAA
3	*spp-1-F*	TGAACATCGGAACTCTTTGC
	spp-1-R	TCAGCTCTTCCTCACACTCG
4	*F28D1.3-F*	AATCTGGATGCCTCGGATAC
	F28D1.3-R	CATCTGAGCAGTTGCAGAGC
5	*T20G5.7-F*	ATGTTCTCCCTCAAGACCGT
	T20G5.7-R	CGGAAGTGTAAACGACGAAG
6	*abf-1-F*	TGCCTTCTCCTTGTTCTCCT
	abf-1-R	ATCCTCTGCATTACCGGAAC
7	*F38A1.5-F*	CTGGGCCGGTATTAATTTGT
	F38A1.5-R	GTCTTCTTCGTCACGCACAT
Endogenous control	*ama-1-F*	CTGACCCAAAGAACACGGTGA
	ama-1-R	TCCAATTCGATCCGAAGAAGC

Appendix 3. Effect of FCP on the survival of different functional mutant, of *Caenorhabditis elegans* challenged with *Pseudomonas aeruginosa* PA14

Sample No.	Treatments (FCP 150 μg mL^{-1})	Total No. of worms	Censored	Mean ± SE survival (d)
skn-1				
1	Control	110	3	1.23 ± 0.057
2	Anti-infective effect[a]	112	4	2.06 ± 0.076
3	Anti-bacterial effect[b]	101	5	2.00 ± 0.043
4	Anti-bacterial / infective combined effect[c]	107	6	2.03 ± 0.012
daf-2				
1	Control	96	6	5.12 ± 0.048
2	Anti-infective effect[a]	95	5	5.10 ± 0.069
3	Anti-bacterial effect[b]	99	3	5.18 ± 0.095
4	Anti-bacterial / infective combined effect[c]	110	7	5.17 ± 0.089
pmk-1				
1	Control	112	5	2.18 ± 0.063
2	Anti-infective effect[a]	98	1	2.16 ± 0.059
3	Anti-bacterial effect[b]	104	4	2.20 ± 0.097
4	Anti-bacterial / infective combined effect[c]	109	8	2.24 ± 0.073

Fucose containing polymer (FCP) at concentrations of 150 μg mL^{-1} was used in each of the three experiments. SE indicates the standard error mean.

[a]The dietary supplemented worms were exposed to a bacterial lawn cultured without FCP treatment.

[b]The worms cultured on modified nematode growth agar without dietary supplementation were exposed to a bacterial lawn cultured with different concentrations of FCP.

[c]The worms with FCP-supplemented diet were exposed to a bacterial lawn cultured with different concentrations of FCP.