Fish and Shellfish Immunology 94 (2019) 558–565

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Novel pectin isolated from *Spirulina maxima* enhances the disease resistance and immune responses in zebrafish against *Edwardsiella piscicida* and *Aeromonas hydrophila*

S.L. Edirisinghe[a], S.H.S. Dananjaya[a], Chamilani Nikapitiya[a], T.D. Liyanage[a], Kyoung-Ah Lee[b,c], Chulhong Oh[b,c], Do-Hyung Kang[b,c,*], Mahanama De Zoysa[a,**]

[a] College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon, 34134, Republic of Korea
[b] Jeju Marine Research Institute, Korea Institute of Ocean Science and Technology (KIOST), Jeju Special Self-Governing Province, 63349, Republic of Korea
[c] Department of Ocean Science, University of Science and Technology (UST), Jeju Special Self-Governing Province, 63349, Republic of Korea

ARTICLE INFO

Keywords:
Aeromonas hydrophila
Mucin
Edwardsiella piscicida
Immunostimulant
Pectin
Spirulina maxima

ABSTRACT

In this study, we demonstrate the enhanced disease resistance and positive immunomodulation of novel pectin isolated from *Spirulina maxima* (SmP) in zebrafish model. Zebrafish larvae exposed to SmP had significantly ($p < 0.05$) higher cumulative percent survival (CPS) at 25 (44.0%) and 50 μg/mL (67.0%) against *Edwardsiella piscicida* compared to the control. However, upon *Aeromonas hydrophila* challenge, SmP exposed larvae at 50 μg/mL had slightly higher CPS (33.3%) compared to control group (26.7%). SmP supplemented zebrafish exhibited the higher CPS against *E. piscicida* (93.3%) and *A. hydrophila* (60.0%) during the early stage of post-infection (< 18 hpi). qRT-PCR results demonstrated that exposing (larvae) and feeding (adults) of SmP, drive the modulation of a wide array of immune response genes. In SmP exposed larvae, up-regulation of the antimicrobial enzyme (*lyz*: 3.5-fold), mucin (*muc5.1*: 2.84, *muc5.2*: 2.11 and *muc5.3*: 2.40-fold), pro-inflammatory cytokines (*il1β*: 1.79-fold) and anti-oxidants (*cat*: 2.87 and *sod1*: 1.82-fold) were identified. In SmP fed adult zebrafish (gut) showed > 2-fold induced pro-inflammatory cytokine (*il1β*) and chemokines (*cxcl18b*, *ccl34a.4* and *ccl34b.4*). Overall results confirmed the positive modulation of innate immune responses in larval stage and it could be the main reason for developing disease resistance against *E. piscicida* and *A. hydrophila*. Thus, non-toxic, natural and biodegradable SmP could be considered as the potential immunomodulatory agent for sustainable aquaculture.

1. Introduction

Edwardsiella piscicida [1] and *Aeromonas hydrophila* [2] are responsible for causing serious fish diseases "Edwardsiellosis" and "Motile Aeromonad Septicemia", respectively. The use of antibiotics and antiseptic agents are the major disease control strategies being applied in aquaculture. However, the use of antibiotics and antiseptic agents cause serious negative impacts such as residual effect, toxicity and development of multidrug resistant bacteria [3,4]. Moreover, higher mortality and slow growth rate during early larval stages are considered as a major challenge in aquaculture. This is mainly due to incomplete host defense system in early larval stages to compete with pathogens [5]. Therefore, the use of biodegradable and relatively non-toxic immunomodulatory agents is promising prophylactic approach to improve the host immune defense against pathogenic agents [6].

Pectin is one of the major components of the cell wall of terrestrial plants and it has a complex macromolecule structure [7]. Pectin isolated from different sources such as citrus, apple pomace has shown diverse biological functions including prebiotic effect [8], chemo preventive role in colon cancer [9], control of blood cholesterol [10], and immunomodulatory responses [11]. Structurally, pectin consists of various fragments of linear and ramified regions [11]. The linear region (backbone of pectin) consists of α-1, 4-D-galacturonan units and the ramified region is represented by different hetero-polysaccharides. The relationship between pectin and immunomodulatory activity has been described under different factors such as extraction technique,

* Corresponding author. Jeju Marine Research Institute, Korea Institute of Ocean Science and Technology (KIOST), Jeju Special Self-Governing Province, 63349, Republic of Korea.
** Corresponding author. College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon, 34134, Republic of Korea.
E-mail addresses: dohkang@kiost.ac.kr (D.-H. Kang), mahanama@cnu.ac.kr (M. De Zoysa).

https://doi.org/10.1016/j.fsi.2019.09.054
Received 10 May 2019; Received in revised form 18 September 2019; Accepted 20 September 2019
Available online 20 September 2019
1050-4648/

polysaccharide structure and degree of branching [11,12]. Therefore, identification of pectin from different sources would be able to introduce new pectin types with diversified biological functions.

Spirulina is a blue-green microalga (Cyanobacteria) belongs to family Oscillatoriaceae [13]. Gumbo and Nesamvuni [14] have described the nutritional composition of *Spirulina* with the presence of bioactive compounds such as essential amino acids, proteins, fatty acids, minerals and antioxidants. Also, therapeutic uses of *Spirulina* have been reported under antiviral [15], antibacterial, antifungal [16], antioxidant [17], anti-inflammatory and immune modulatory functions [13] *in vitro* and *in vivo*. The number of studies have been reported on food and medical benefits of *S. platensis* and *S. maxima* [18]. However, relatively few studies have been documented on the health benefits of *S. maxima* or its bioactive agents, which can be applied in the field of aquaculture. Thus, we isolated and characterized low methoxyl pectin from *S. maxima* and it was denoted as SmP. Based on our preliminary data and previous studies on pectin (extracted from citrus and apple), we hypothesized that SmP can activate immune responses and develop disease resistance in fish.

The aim of the present study was to determine the effect of the immunomodulatory role of pectin extracted from *S. maxima* against two fish pathogenic bacteria, *E. piscicida* and *A. hydrophila* in zebrafish (*Danio rerio*) model. Thus, pectin exposed (water immersion) larvae and pectin supplemented adult zebrafish (oral administration) were tested for immune protection with *E. piscicida* and *A. hydrophila.* Additionally, the antioxidant capacity of SmP was evaluated in larvae under *E. piscicida* challenge. Moreover, transcriptional analysis of immune response genes was conducted in SmP exposed larvae and SmP supplemented adult fish.

2. Materials and methods

2.1. Preparation and characterization of SmP

S. maxima based SmP was provided by Jeju Marine Research Institute, Korea Institute of Ocean Science and Technology (KIOST), Jeju Special Self-Governing Province 63349, Republic of Korea. Briefly, SmP (100 mg) was dissolved in deionized water (100 mL) and physicochemical characters such as particle size, zeta potential, morphology, and toxicity were analyzed. Particle size and zeta potential of SmP were measured by Zetasizer® Nano-ZS (Malvern instruments, Malvern, UK). The morphology of SmP was determined by field emission scanning electron microscopy (FE-SEM) (S-4800, Hitachi, Japan).

2.2. Zebrafish husbandry and exposure of SmP to embryo

Wild-type (AB) adult zebrafish were maintained in an automated water circulation system under 14 h light: 10 h dark photoperiod at 28 °C. After mating fish (~4 months old), embryos at 1 h post fertilization (1 hpf) stage were collected and rinsed with embryonic water. Viability of newly fertilized embryos was checked under the stereo microscope (Nikon, SMZ1000, Japan), prior to use for the experiments. First, embryos at 1–2 hpf stage were exposed to different concentrations of SmP (0–500 μg/mL) for 96 h to determine the toxicity level. After the determination of the non-toxic concentrations, embryos were exposed at 25 and 50 μg/mL of SmP to find out the effects of SmP on hatching process, disease resistance, detoxification of reactive oxygen species (ROS) and immunomodulation. Briefly, SmP exposure trial was consisted with three groups 1) control (0 SmP), 2) 25 and 3) 50 μg/mL of SmP exposed larvae. For testing the hatching effect of SmP, an experiment was conducted with 3 groups consisted of triplicates (10 embryos/replicate) in 6 well plates using 10 mL of embryo water. For the respective treatment groups, sterilized SmP (1 mg/mL stock solution) was added until final concentrations become 25 and 50 μg/mL. The control group was maintained in embryo water (10 mL). All the treatments and control embryos were incubated at 28 °C. The effects of SmP on embryos hatching were compared with the control group and the hatchability percentage (%) was determined at 48, 55 and 60 hpf. For analyzing the expression of immune response genes in larvae, SmP exposed (25 and 50 μg/mL) and control larvae were maintained in 3 groups as described above. To minimize the effect of early hatching on gene expression analysis, hatched larvae in control and SmP treated groups were selected separately at 55 to 60 hpf. At the 5 days (~120 hpf), total of 120 larvae from each group were collected in 3 replicates (40 larvae/replicate), snap frozen in liquid nitrogen and stored at −80 °C until RNA isolation. All experiments with zebrafish were conducted in accordance with the approved guidelines and regulations of the Animal Ethics Committee of Chungnam National University (CNU-00866).

2.3. Preparation of SmP supplemented diet, feeding and growth performance of zebrafish

To investigate the disease resistance and immunomodulatory effects of SmP in adult zebrafish, SmP supplemented diet (SmP 4%) was prepared using commercial feed. SmP supplemented diet was prepared by extruding after mixing of SmP (4%) with commercial feed (Dry basis). Control feed was prepared using commercial feed (100%) and both final products were oven dried (50 °C) for 12 h. The feeding trial was consisted with two groups, 1) control (commercial fish feed) and 2) SmP supplemented diet. Briefly, zebrafish (n = 140; mean weight 255 mg) were divided into four 20 L tanks to maintain two replicates per group. Prior to feeding trial, fish were acclimatized for one week. The prepared SmP supplemented and control diets were fed 3 times at 4% of body weight daily for 6 weeks. The daily feed intake was calculated based on the average body weight (4% of body weight) of each group. After 6 weeks of the feeding trial, the growth performance was calculated according to the following formulas: weight gain = W2 (g)-W1 (g); Specific growth rate (SGR) = 100 (lnW2-lnW1)/t, feed conversion ratio (FCR) = feed fed (g)/weight gain (g); where W1 is the initial weight, W2 is the final weight, and t is time duration of feeding trial. After feeding was terminated, 9 fish from each group (3 replicates/group) were randomly taken, and gut tissue was aseptically isolated. Collected gut tissues were snap frozen in liquid nitrogen, and then stored at −80 °C until RNA isolation. Remained fish (at 6 week) were used to challenge with *E. piscicida* and *A. hydrophila*.

2.4. Immune challenge of larvae and adult zebrafish against E. piscicida and A. hydrophila

2.4.1. Preparation of E. piscicida and A. hydrophila

Lab strains of *E. piscicida* and *A. hydrophila* were seeded onto Brain Heart Infusion (BHI) and Tryptic Soy Agar (TSA) plates, respectively, and plates were incubated overnight at 25 °C. A single colony from each plate was inoculated in to respective broth (4 mL) and incubated in shaker (180 rpm) at 25 °C for overnight. Then exponentially grown cultures were centrifuged at 3500 rpm at 4 °C for 10 min and bacterial pellets were harvested, washed twice with ice cold Phosphate Buffer Saline (PBS).

2.4.2. Exposure and intraperitonial (i.p.) injection of bacteria

To investigate the effect of SmP on disease resistance capacity, larvae were exposed to SmP for 5 days and adult zebrafish were fed with SmP supplemented diet for 6 weeks followed by bacteria challenge of *E. piscicida* and *A. hydrophila*. Briefly, the experiment was conducted using SmP exposed (25 and 50 μg/mL) and control larvae which maintained until 120 hpf (as described in 2.2). The control group was maintained under the same condition without SmP exposure. Prior to the bacteria challenge (120 hpf stage), all SmP exposed and control larvae were kept under heat stress (at 34 °C for 2 h) for facilitating the infection process. In larvae bacteria challenge, *E. piscicida* (5×10^7 CFU/mL) and *A. hydrophila* (3.3×10^7 CFU/mL) were

introduced into larvae culturing plates separately. The bacteria challenge of adult zebrafish was performed by intraperitoneal (i.p.) injection (20 μL) of *E. piscicida* (3.6×10^3 cells/fish) and of *A. hydrophila* (1.56×10^6 cells/fish). After the challenge, larvae and fish were maintained at 28 °C and mortality was recorded to determine the cumulative percentage survival (CPS).

2.4.3. Determination of ROS level in larvae with SmP exposure and bacteria challenge

To determine the effect of SmP on ROS production under bacteria challenge, larvae were stained using fluorescent dye, 2′7′dichloro-dihydro-fluorescein diacetate (H_2DCFDA) as described by Lackmann et al., [19]. Larvae were exposed to SmP as described previously (2.2) and then challenged with *E. piscicida* (2.4.2). Three groups of larvae were prepared namely, control (*E. piscicida* challenge without SmP exposure), SmP pre-exposed at 25 and 50 μg/mL with *E. piscicida* challenge. At 36 h post infection (hpi), nine larvae were selected from control and each treatment groups to quantify the ROS levels. H_2O_2 (5 mM) exposed larvae were used as a positive control. ROS detector, H_2DCFDA (Sigma-Aldrich) stock solution (1 mg/mL) was prepared using dimethyl sulfoxide (DMSO), and larvae were exposed to H_2DCFDA (5 μg/mL) for 30 min in dark at 28 °C. Excess dye was removed following the 3–4 times of washing steps. Images of the intracellular ROS levels were monitored by microscopy (Nikon SMZ1000, Japan) equipped with fluorescence filter (HIGHTSEA, USA). Quantitative ROS was analyzed by Image J software (Image J, version 1.6, USA).

2.5. RNA isolation, cDNA synthesis and qRT-PCR analysis

The total RNA was isolated from 40 larvae per replicate (3 replicates/group) and gut tissue (pool of 3 gut tissues/replicate) using TRIzol® (Invitrogen, CA) according to the manufacturer's instructions. The RNA concentration was quantified using NanoDrop One (Thermo Scientific, USA). The first strand cDNA was synthesized using 2.5 μg of total RNA by Prime Script™ first-strand cDNA synthesis kit (TaKaRa®, Japan) according to manufactures protocol. cDNA samples were diluted 40x, and stored at −20 °C. In order to analyze the mRNA expression, specific primers were designed based on the selected immune response genes as shown in Table 1. Briefly, it includes toll-like receptors (*tlr2*, *tlr4b* and *tlr5b*), transcriptional factor (*c-rel*), pro and anti-inflammatory (*il1β*, *tnfa*, *il6* and *il10*), chemokines (*cxcl8a*, *cxcl18b*, *ccl34a.4* and *ccl34b.4*), anti-microbial (*lyz, muc2.1*, *muc5.1*, *muc5.2* and *muc5.3*), heat shock protein (*hsp70*) and antioxidant enzymes (*cat* and *sod1*). The qRT-PCR was performed using Thermal Cycler Dice Real Time System (TaKaRa, Japan) and the reaction mixture was contained 3 μL cDNA template, 1 μL (10 μM) of each gene specific primer and 5 μL THUNDERBIRD® SYBR® qPCR mix (Toyobo, Japan). The relative mRNA expression level was determined by $2^{-\Delta\Delta Ct}$ method [20] and zebrafish β-actin was used as a house keeping gene. Fold change was calculated by dividing the average relative expression (fold) of SmP treatment by that of the respective control.

2.6. Statistical analysis

All the statistical data analysis was performed using GraphPad Prism software version 5 (GraphPad Software Inc. USA) and one-way analysis of variance (ANOVA). Tukey's test was applied for the mean comparison. The differences were considered as significant at $p < 0.05$ and data were presented as mean ± SD for triplicate.

3. Results

3.1. Physicochemical properties and toxicity of SmP

Physicochemical characterization and toxicity of SmP were evaluated to ensure the properties of the tested sample. SmP was moderately soluble in water (1 mg/mL) and showed a slightly basic pH (8.2). Average particle size and zeta potential were determined as 202 nm and −29.2 mV, respectively (data not shown). SEM analysis results confirmed that SmP particles were aggregated with irregular shapes (Fig. 1A). Toxicity of SmP was examined only at higher exposure concentrations (≥400 μg/mL) in larvae and its LD_{50} was determined as 330 μg/mL (Fig. 1B).

3.2. SmP exposure induced the hatching of embryos

We examined the effect of SmP exposure on the hatching process of zebrafish embryos at lower concentrations (25 and 50 μg/mL) at different time intervals (48, 55 and 60 hpf). SmP exposed embryos hatched early compared to control embryos (Fig. 2). Hatching % was significantly higher ($p < 0.05$) in 25 (20%) and 50 μg/mL (36%) of SmP exposed larvae at 48 hpf than control group (7%). With the time hatching % was increased significantly ($p < 0.05$) in both SmP exposed 25 (62%) and 50 μg/mL (76%) compared to control (28%) at 55 hpf. The hatchability % was its maximum (~100%) at 60 hpf for all the groups and hatched larvae had no visual toxicity or deformities.

3.3. SmP exposed larvae displayed greater disease resistance and low ROS levels under bacteria challenge

Upon *E. piscicida* challenge, at 96 hpi, SmP exposed larvae had significantly ($p < 0.05$) higher CPS of 44 (25 μg/mL) and 67% (50 μg/mL) compared to control (0%) group (Fig. 3A). However, after *A. hydrophila* challenge, CPS was 26.67, 26.67 and 33.33% in control, SmP at 25 μg/mL and 50 μg/mL, respectively. The CPS values were slightly higher in SmP exposed larvae, but not significantly ($p > 0.05$) different among groups (Fig. 3B). High levels of ROS can increase the cellular redox state towards the oxidative stress followed by oxidation of molecules such as lipids, DNA, proteins, and as a result, oxidative stress generates cell death, pathological and disease conditions. To investigate whether SmP could reduce the oxidative stress under bacteria (*E. piscicida*) exposure, the level of ROS was measured in larvae. As expected, the highest ROS level (215%) was observed in H_2O_2 treated larvae (positive control) compared to the control group (100%) with no SmP exposure. Interestingly, ROS level was gradually decreased in concentration dependent manner in SmP exposed larvae with 79 (25 μg/mL) and 67% (50 μg/mL) (Fig. 4). Overall results confirmed that SmP can reduce the oxidative stress caused by *E. piscicida,* and thereby it increases CPS in SmP exposed larvae.

3.4. Growth performances and disease resistance of SmP supplemented zebrafish

Growth performances of adult zebrafish after six weeks of SmP supplementation were summarized (S. Table 1). SmP (4%) supplemented adult zebrafish exhibited slightly reduced body weight than the control group, however it was not statistically significant ($p < 0.05$). Additionally, FCR was increased in SmP fed adults due to lower weight gain compared to control fish. Moreover, SGR (Sup. Table 1) was not changed considerably. Adult zebrafish were challenged with *E. piscicida* and *A. hydrophila* after oral administration of SmP for six weeks to find out the effects of SmP on disease resistance as an immunomodulatory agent. Under *E. piscicida* challenge, a slightly high CPS survival rate was observed during 0–24 hpi in the SmP fed group than the control group (Fig. 5A). On the other hand, *A. hydrophila* challenged zebrafish showed slightly high CPS throughout the examined period (Fig. 5B). Moreover, there was no significant difference ($p > 0.05$) of CPS between the *E. piscicida* and *A. hydrophila* challenge.

3.5. Immune responses of larvae and adults upon SmP administration

To understand the immune activation of larvae and adults by exposure and feeding of SmP, twenty genes were selected under different

Table 1
Description of primers used in this study.

Gene	Accession number	Primer name	Primer sequence (5′-3′)
Toll like receptor 2 (*tlr2*)	NM_212812.1	tlr-2-F	TCTCCGTCTTGGTTTCAC
		tlr-2-R	GGTCCCACAGTTGAGTATG
Toll like receptor 4 (*tlr4b*)	AY388400.1	tlr-4-F	GGAATAATGGGCAGCCGTAAG
		tlr-4-R	AGCGACACCAACTATCAATG
Toll like receptor 5 (*tlr5b*)	BC163185.1	tlr-5-F	GAAACATTCACCCTGGCACA
		tlr-5-R	CTACAACCAGCACCACCAGAATG
c-rel	AY163837	c-rel-F	ACTACAGCTCCCAACAGCCTCAAA
		c-rel-R	AAACTGGTAGCCCGTTGCTAGTGA
Interleukin-1β (*il1β*)	AY340959.1	il-1β-F	TCAAACCCCAATCCACAGAG
		il-1β-R	TCACTTCACGCTCTTGGATG
Tumor necrosis factor- α (*tnfα*)	AY427649	tnf- α-F	AGAAGGAGAGTTGCCTTTACCGCT
		tnf- α-R	AACACCCTCCATACACCCGACTTT
Interleukin-6 (*il6*)	JN698962.1	il-6-F	TCAACTTCTCCAGCGTGATG
		il-6-R	TCTTTCCCTCTTTTCCTCCTG
Interleukin-10 (*il10*)	AY887900.1	il-10-F	CCCTATGGATGTCACGTCATG
		il-10-R	CATATCCCGCTTGAGTTCCTG
Chemokine ligand 8a (*cxcl8a*)	XM_009306855.2	cxcl-8a-F	CTTCCCTCCAAGCCCACAC
		cxcl-8a-R	GATCCGGGCATTCATGG
Chemokine ligand 18b (*cxcl18b*)	NM_001115060	cxcl-18b-F	CTGCTGCTGCCGGTAGTTTA
		cxcl-18b-R	TCAACTTTGTCGCAGTTTGG
CC-chemokine (*ccl34a.4*)	BC162421.1	ccl-34 a.4-F	TGCAGCTCAACCAGAAGATG
		ccl-34 a.4-R	CTTTGACGCATGGAGGATTT
Chemokines CCL-C24 (*ccl34b.4*)	NP_001108521	ccl-34b.4-F	TCGAGTTGGAGTAACATGTG
		ccl-34b.4-R	GTACCTTTCCTTCTCTGCGTAG
Lysozyme-C (*lyz*)	AF402599	lyz c-F	AAGCAGGTTTAAGACCCACCGAGT
		lyz c-R	AAGTCTGAACAGGCCACTTTGCAC
Mucin 2.1 (*muc2.1*)	NC_007136.7	muc 2.1-F	AATATGCCTTGCGGAACAAC
		muc 2.1-R	GTGCTGAGGTTGCAGAATGA
Mucin 5.1 (*muc5.1*)	XM_009297795.1	muc 5.1-F	TGGCAACTTGGCTGATGATA
		muc 5.1-R	TCGTCACACGGACCAGTAGA
Mucin 5.2 (*muc5.2*)	XM_009297793.1	muc 5.2-F	GGTGTCTGTTCCGATCAATC
		muc 5.2-R	TCATCCTTGTCGCCATTGTA
Mucin 5.3 (*muc5.3*)		muc 5.3-F	GGGGAAAACTACACCAGCAA
		muc 5.3-R	TGTGAATTCTGTGCCAGAGC
Heat shock protein (*hsp70.1*)	AB062116.1	hsp70-F	CATGGTCCTGGTGAAGATGAA
		hsp70-R	GTCTGTGGGACTCGTTGAAATA
Catalase (*cat*)	NM_130912.2	cat-F	CCAAGGTCTGGTCCCATAAAG
		cat-R	GCTCAACCTCCGCGAAATA
Super oxide dismutase (*sod1*)	NM_131294.1	sod-F	AGGTGACTGGTGAAATTACTGG
		sod-R	GTCTCACACTATCGGTTGGC
β actin	AF025305	β actin- F	AATCTTGCGGTATCCACGAGACCA
		β actin- R	TCTCCTTCTGCATCCTGTCAGCAA

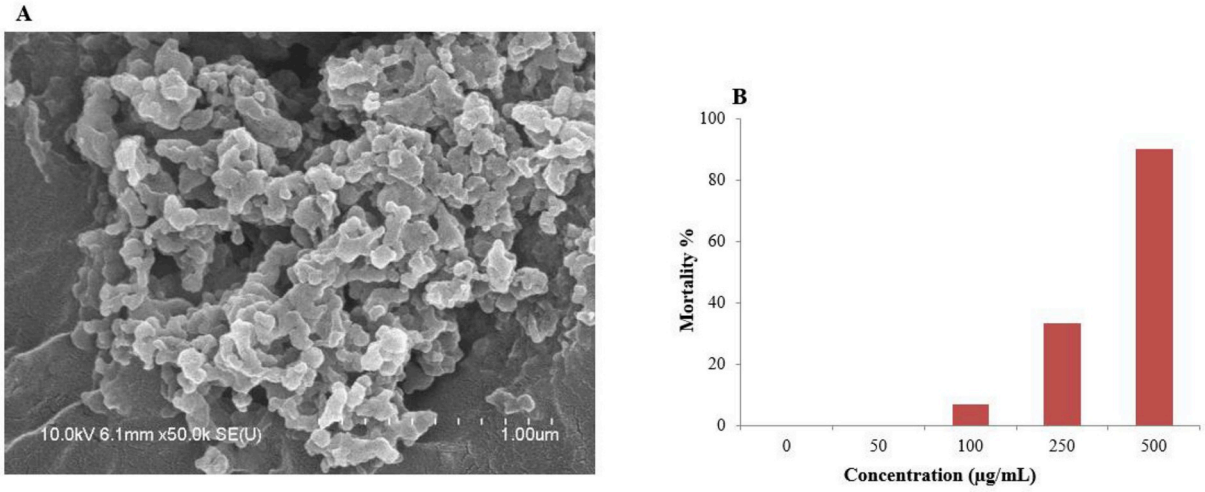

Fig. 1. Morphology of SmP particles and its toxicity on zebrafish larvae. (A) FE-SEM image showing the nano scale SmP particles. (B) Mortality of zebrafish larvae exposed to SmP (0–500 µg/mL).

immune functional categories namely, toll like receptor, transcription factor, pro and anti-inflammatory, chemokine, anti-microbial, heat shock protein, and antioxidants/enzyme. qRT-PCR analysis was conducted in SmP exposed larvae for 5 days and in the gut of SmP supplemented adult zebrafish (Fig. 6). In SmP exposed larvae five genes were induced (> 1.5-fold) at lower concentration (25 µg/mL), and they were *tlr5b* (2.72-fold), *lyz* (2.69-fold), *muc5.2* (1.64-fold), *cat* (1.73-fold) and *sod1* (1.78-fold). When larvae exposed at higher SmP

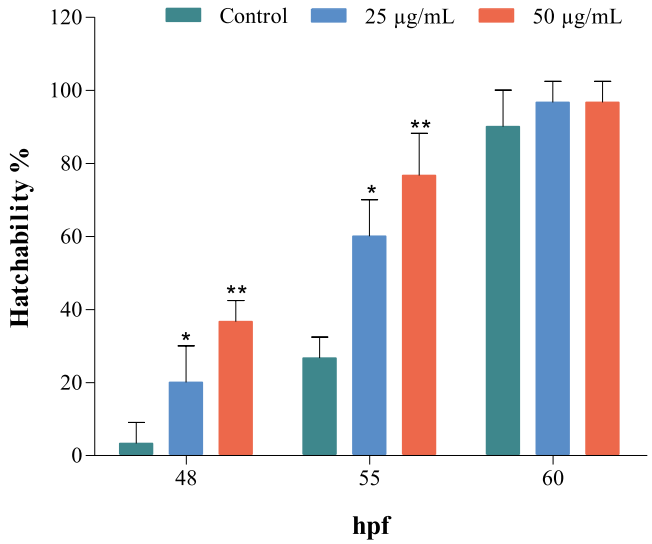

Fig. 2. Effect of SmP exposure on hatching of zebrafish embryos. Graph indicates the hatching percentage at 25 and 50 µg/mL of SmP exposure with respective control. Values are mean ± SD of triplicates and representative of two independent experiments; *$p < 0.05$, **$p < 0.01$.

concentration (50 µg/mL), eight genes were induced (> 1.5-fold), which were namely *tlr5b* (3.57-fold), *il1β* (1.79-fold), *lyz* (3.50-fold), *muc5.1* (2.84-fold), *muc5.2* (2.11-fold), *muc5.3* (2.40-fold), *cat* (2.87-fold) and *sod1* (1.82-fold). Interestingly, all five genes which induced at 25 µg/mL were further induced at 50 µg/mL. In contrast, none of the selected pro and anti-inflammatory (*tnfα*, *il6* and *il10*) and chemokine genes (*cxcl8a*, *cxcl18b*, *ccl34a.4,* and *ccl34b.4*) were induced in SmP exposed larvae. In the gut of SmP fed zebrafish, a total of twelve genes showed over 1.5-fold up regulation. Those induced genes were *c-rel* (2.52-fold), *il1β* (2.07-fold), *tnfα* (1.89-fold), *lyz* (3.12-fold), *cxcl8a* (1.99-fold), *cxcl18b* (2.62-fold), *ccl34a.4* (3.0-fold), *ccl34b.4* (3.34-fold), *muc5.1* (3.26-fold), *muc5.3* (7.48-fold), *cat* (4.61-fold) and *sod1* (4.40-fold). Among all the tested genes, *muc5.3* showed the highest level of induction (7.48-fold) in the gut. Interestingly, total six genes (*il1β*, *lyz*, *muc5.1*, *muc5.3*, *cat* and *sod1*) were induced (> 1.5-fold) in both SmP treated larvae (25 µg/mL) and gut of the adult zebrafish.

4. Discussion

Among the natural polymers, pectin is widely considered as biocompatible, biodegradable and non-toxic polysaccharide [21]. Biological activity of pectic polysaccharides is mainly related to its physicochemical properties (particle size, net charge, solubility, etc.), structure and additional modifications as well as applied dose. Therefore, we first determined the particle properties and toxicity of SmP using zebrafish embryos. Isolated SmP had nano size particle size (202 nm), negative charge (−29.2 mV) and irregular shape. Jiao et al. [22], described nanoparticles have unique physical and chemical properties which enable them to transport and deliver to the target tissue. Furthermore, nanoparticles are able to interact with the immune system for modulating its function. Therefore, we suggest SmP isolated from *S. maxima* may have specific biological functions including immunomodulation due to its nano size and negative charge. Before the immunomodulation of larvae, we determined the toxicity of SmP by exposing zebrafish eggs. LD_{50} of SmP was determined as 330 µg/mL for zebrafish embryos after 96 h post exposure and then non-toxic doses (25 and 50 µg/mL) of SmP were selected based on zero mortality and absent of visual morphological defects in either embryo or larvae. Toxicity of pectin has not been investigated with fish larvae to compare our results, however, *in vitro* cytotoxicity results revealed that high methoxylated pectin nanoparticles prepared from citrus have IC_{50} up to 18.8 mg/mL against HepG2 cells [23].

Yamagami [24] described that hatching of fish embryos is a sequential process which breakdown the egg envelope (chorion) and allows embryos to leave from it. The hatching process is directly regulated by hatching gland cells, which produce the hatching enzyme. Furthermore, it was described that there are some other processors such as formation and hardening of the fish envelope which can indirectly related to hatching. Several studies discussed about the hatching delay of fish embryos with the exposure to metal nanoparticles and agrochemicals [25,26]. Moreover, temperature is an external factor for regulating hatching process. Okunsebor et al. [27], reported significantly higher hatching % in *Heterobranchus bidorsalis* embryos at 28 °C and 30 °C compared to 26 °C. In this study, we noticed early hatching of SmP exposed embryos in a concentration dependent manner. Furthermore, mRNA expression of hatching enzyme also induced in SmP exposed embryos. Hence, it can be suggested that SmP may enhance the production of hatching enzyme in the hatching gland cells. Practically, if all the embryos are hatched at specific time point without any growth deformities, it could be much advantageous and

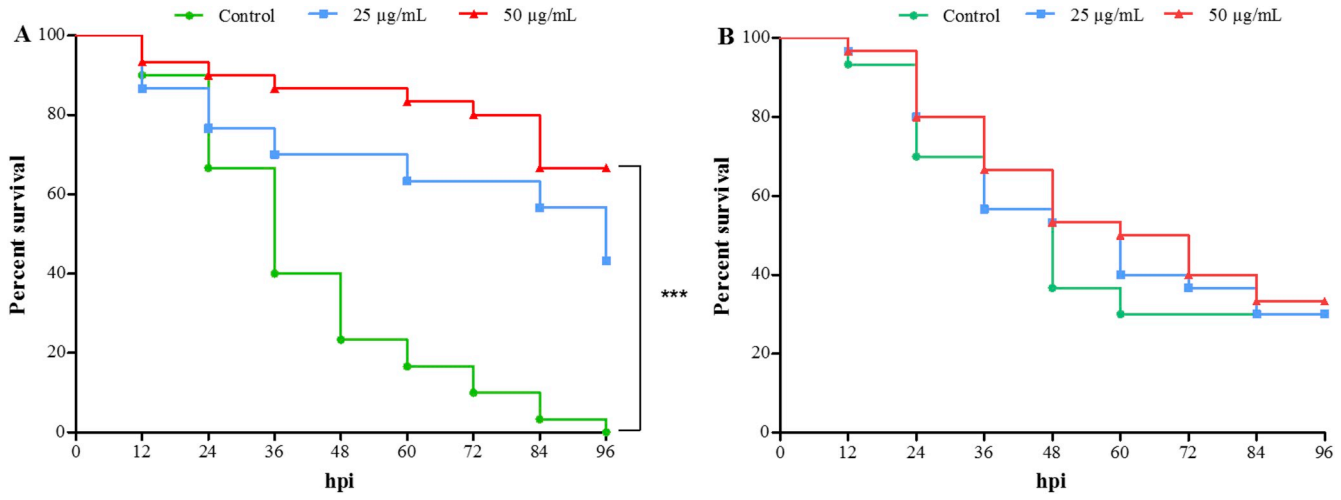

Fig. 3. Disease resistance of SmP exposed zebrafish larvae upon *E. piscicida* and *A. hydrophila* challenge. CSP of (A) *E. piscicida* and (B) *A. hydrophila* until 96 hpi. Values are mean ± SD of the triplicates. Significance difference (Kaplan–Meier, Wilcoxon $p \leq 0.001$) between SmP exposed versus control were marked with *** in graph A.

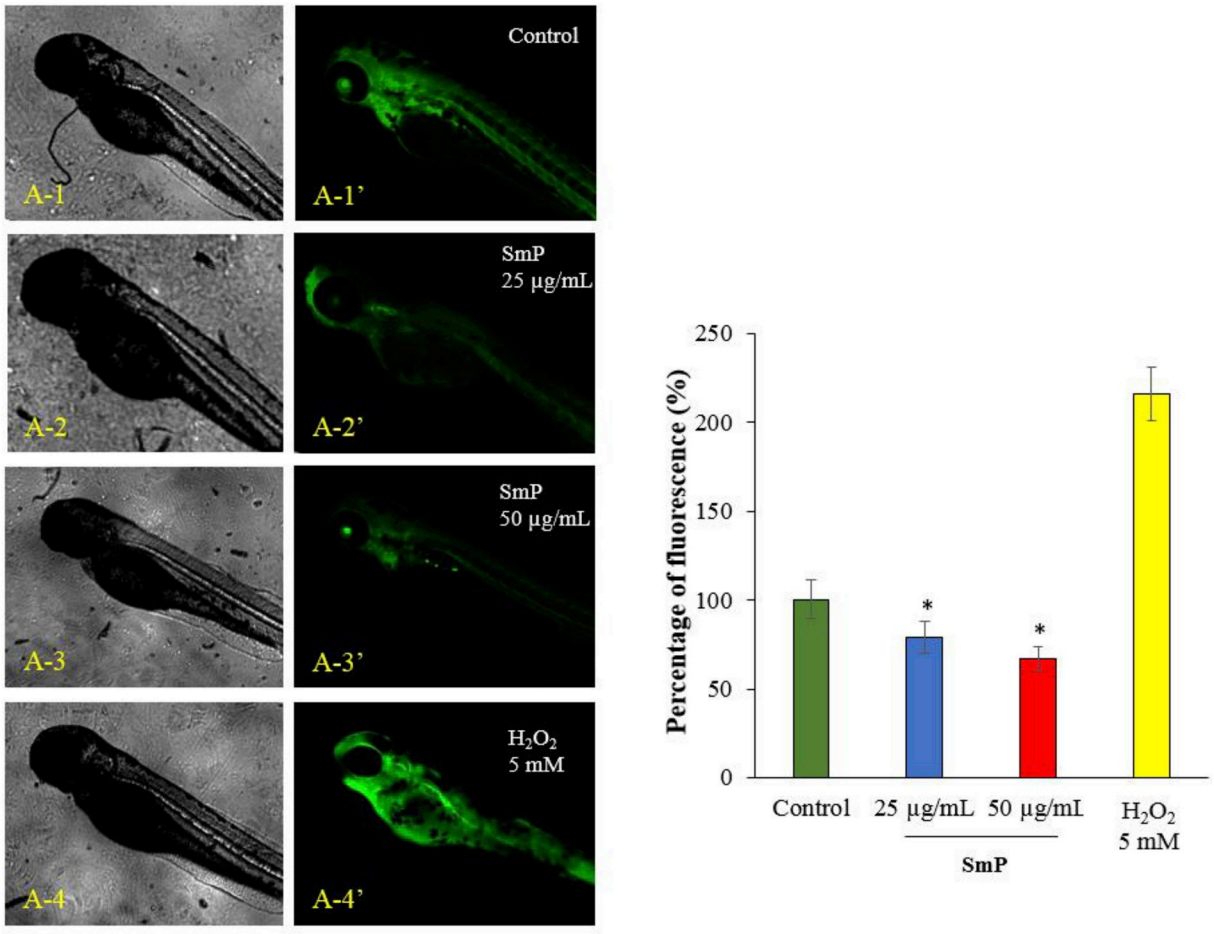

Fig. 4. ROS production after SmP exposure to zebrafish larvae. The data represent relative fluorescence intensity of *E. piscicida* induced ROS generation. Quantified ROS were normalized to control (as 100%). Respective bright field and fluorescence images were control: A-1, A-1′; SmP (25 µg/mL): A-2, A-2′; SmP (50 µg/mL): A-3, A-3′ and H_2O_2 (5 mM): A-4, A-4′. The bar chart shows (mean ± SD) the quantified fluorescence (%) from three independent experiments (n = 9, *$p < 0.05$).

easier for culturing all the larvae at uniform growth stage for breeding purpose and/or for using them, for experimental purposes. Other than that early hatching is also important for rapid transition from innate to adaptive immunity by activating the complement genes along the development stages [28]. According to our main objective, we examined the effect of SmP on immune gene activation and disease resistant capacity of zebrafish. However, effect of early hatching on maturation of adaptive immune components was not analyzed since it may not the direct effect of SmP. Moreover, SmP exposed and control larvae were separated during a specific period 55–60 hpf and maintained till

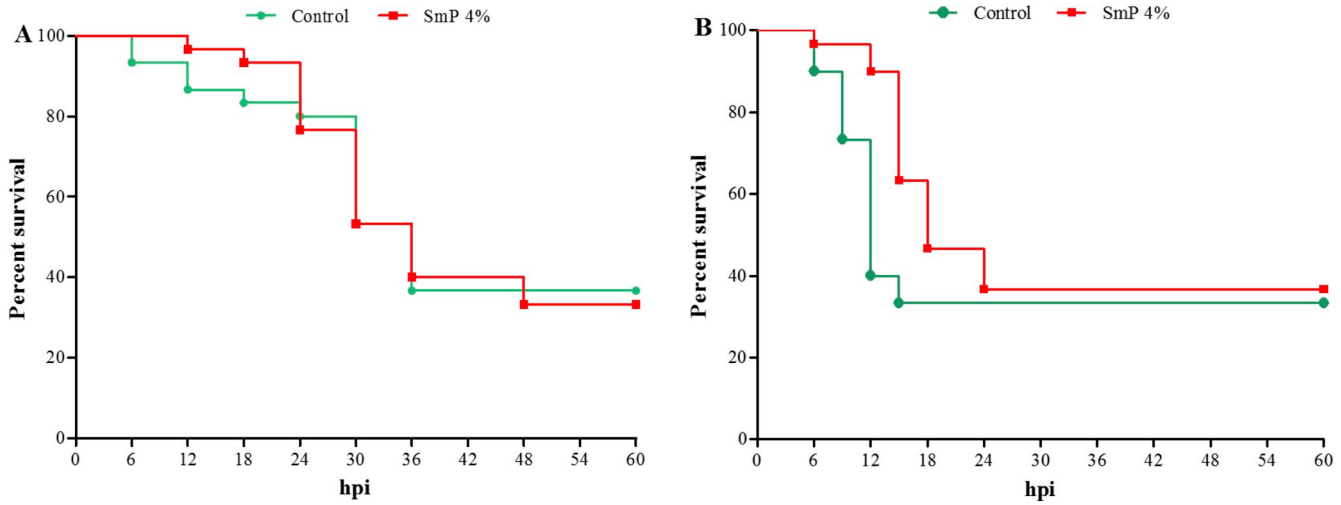

Fig. 5. Disease resistance of SmP supplemented zebrafish upon *E. piscicida* and *A. hydrophila* challenge. Fish were injected (i.p.) with 20 µL of (A) *E. piscicida* (3.6×10^3 cells/fish) and (B) *A. hydrophila* (1.56×10^6 cells/fish). Values are not significantly different ($p > 0.05$) in both challenge experiments compared to respective control group.

Gene category	Gene name	Larvae SmP exposure 25 μg/mL	Larvae SmP exposure 50 μg/mL	Adult SmP oral administration (4%)
Toll like receptors	*tlr2*	1.05±0.15	1.11±0.03	0.48±0.09
	tlr4b	0.98±0.20	1.03±0.02	0.08±0.31
	tlr5b	2.72±0.14	3.57±0.17*	1.24±0.17
Transcription factors	*c-rel*	0.82±0.03	1.32±0.14*	2.52±0.07*
	il1β	1.35±0.35	1.79±0.22	2.07±0.25
Pro and anti-inflammatory	*tnfα*	0.68±0.17	1.16±0.08	1.89±0.37
	il6	0.62±0.08	0.72±0.24	0.51±0.36
	il10	0.88±0.27	1.16±0.06	0.07±0.01
Chemokines	*cxcl8a*	0.89±0.00	0.61±0.02	1.99±0.16
	cxcl18b	1.19±0.16	0.94±0.13	2.62±0.41*
	ccl34a.4	0.97±0.07	0.98±0.10	3.00±0.17*
	ccl34b.4	1.06±0.06	0.84±0.06	3.34±0.22**
Anti-microbial genes	*lyz*	2.69±0.05*	3.50±0.11*	3.12±0.27
	muc2.1	1.07±0.16	0.65±0.17	1.18±0.47
	muc5.1	1.26±0.24	2.84±0.03*	3.26±0.15*
	muc5.2	1.64±0.08*	2.11±0.09	0.46±0.21
	muc5.3	1.39±0.40*	2.40±0.42	7.48±0.05**
Heat shock protein	*hsp70*	0.67±0.03	1.26±0.27	0.42±0.62
Antioxidant enzymes	*cat*	1.73±0.01	2.87±0.37*	4.61±0.13*
	sod1	1.78±0.01	1.82±0.20	4.40±0.21*

<0.5
0.5-0.79
0.8-0.99
1
1.01-1.2
1.21-2
>2

Fig. 6. Transcriptional response of immune genes in zebrafish larvae exposed to SmP (25 and 50 μg/mL) for 5 days and SmP supplemented (4%) adults for 6 weeks. Values are mean ± SD of the triplicates. Asterisk marks are used to indicate statistical significance compared to SmP treated vs non-treated control (one way ANOVA, $*p < 0.05$, $**p < 0.01$). Basal level, upregulated and down regulated expressions are considered as 1, > 1.21 and < 0.79-folds, respectively.

sampling at 120 hpf to minimize any effect related to early hatching as secondary impact on immune responses.

Immunomodulation can secure a high survival rate in the larval stage of fish until they develop the adaptive immune system completely. One of the main constraints of aquaculture is high mortality during the larval stage [29]. Ringo et al. [30] described that fish larvae primarily rely on non-specific immune responses. Immunostimulants develop high disease resistance by enhancing the non-specific immune responses, and thereby more stable growth could be achieved especially in larvae [6]. In the present study, disease resistance of larvae was assessed by bacteria challenge after immunomodulation with SmP. Results demonstrated that CPS was increased in larvae upon 25 and 50 μg/mL SmP exposure against *E. piscicida* infection at 96 hpi. Additionally, *A. hydrophila* challenged larvae also showed increased CPS except for 48 and 96 hpi than the control. Collectively, our data suggest that both concentrations (25 and 50 μg/mL) were effective in developing diseases resistance against *E. piscicida* and *A. hydrophila*, and difference CPS pattern could be due to exposure doses and variation of virulence factors of bacteria species.

Moreover, we can confirm that the efficacy of SmP as an immunomodulatory agent in larvae culture is dose dependent. Furthermore, pectin acts as an antioxidant [31], and it involves to detoxify the ROS for protecting animals from oxidative stresses. In the present study, ROS level was suppressed in SmP exposed larvae in a dose dependent manner after *E. piscicida* challenge. Therefore, it could be suggested that the antioxidant activity of SmP promotes the reduction of bacteria induced oxidative stress, which may lead to have high disease resistance. However, the immunomodulatory effect of SmP was not high in adults as shown in larvae, which may be due to low feeding dose in the diet (SmP 4%) or short administration period (six weeks). Vidal et al. [32] have summarized the responses of fish to various diets supplemented with immunostimulants from plants, herbs, algae and pathogen associated molecular patterns (PAMPs). Moreover, they suggested an understanding of possible pathways related to immune functions in fish is difficult without having gene expression profiles under the standard administration of immunostimulants with optimized doses. Well-known immunostimulants such as β-glucan, lipopolysaccharide (LPS) and peptidoglycans have been widely tested in fish [33–35]. Popov and Ovodov [11] have stated the immune reactivity of pectin determines differences in the structure of the branched region. As an example, pectin pocess immunostimulatory effects, when it has less than 75% galacturonic acid residues, ramified region, rhamnogalacturonan (RG-I), and apiogalacturonan regions. Very recently, Doan et al. [36] have shown the enhanced serum immunological responses (lysozyme and serum peroxidase) and disease resistance (against *Streptococcus agalactiae*) of orange peels derived pectin in Nile tilapia. In this study, immune responses were analyzed at different growth stages (larvae and adult) and two modes of administrations (exposure and oral). Based on upregulated pattern recognition receptor (*tlr5b)*, and antibacterial genes (*lyz*, *muc5.1*, *muc5.2,* and *muc5.3)*, we confirm the activation of innate immune responses in larvae by SmP. Moreover, transcription factor (*c-rel*), interleukin (*il1β*), chemokines (*cxcl18b*, *ccl34a.4,* and *ccl34b.4*), anti-microbial genes (*lyz*, *muc5.1* and *muc5.3*), and antioxidant enzymes (*cat* and *sod1*) were upregulated in adult zebrafish, representing that innate immune responses could be modulated by SmP. Considering the above facts, we further confirm that disease resistance might have attributed to immune modulatory properties of SmP. Collectively, it could be suggested that SmP has an immunomodulatory function, which could activate gut immune responses directly or indirectly.

As a summary, SmP enhances the early hatching of zebrafish embryos, suggesting that *S. maxima* pectin could be developed as a hatching enhancer to obtain larvae at a specific stage. We demonstrated SmP exposure to larvae can have a beneficial immune stimulatory effect than the adults with high disease resistance when fish were challenged with *E. piscicida* than *A, hydrophila*. Therefore, SmP has the potential to be used in aquaculture as an immunostimulant or as non-toxic dietary supplements at any life stages of the fish, especially during the larval stage.

Acknowledgements

This work was supported by a research funds (PE99621 and PE99722) of Jeju Marine Research Institute, Korea Institute of Ocean Science and Technology (KIOST), Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.054.

References

[1] N. Buján, A.E. Toranzo, B. Magariños, *Edwardsiella piscicida*: a significant bacterial pathogen of cultured fish, Dis. Aquat. Org. 131 (2018) 59–71 https://doi.org/10.3354/dao03281.

[2] H. Xia, T. Ying, L. Fenghui, L. Yushuang, Y. Pinhong, W. Wenbin, et al., The effect of *Aeromonas hydrophila* infection on the non-specific immunity of blunt snout bream (*Megalobrama amblycephala*), Cent. Eur. J. Immunol. 42 (2017) 239–243 https://doi.org/10.5114/ceji.2017.70965.

[3] N.D. Friedman, E. Temkin, Y. Carmeli, The negative impact of antibiotic resistance, Clin. Microbiol. Infect. 22 (2016) 416–422 https://doi.org/10.1016/j.cmi.2015.12.002.

[4] M.G. Rasul, B.C. Majumdar, Abuse of antibiotics in aquaculture and it's effects on human, aquatic animal and environment, Saudi J. Life Sci. 2 (2017) 81–88 http://scholarsmepub.com/wp-content/uploads/2017/07/SJLS-2381-88.

[5] H.R.C. Angel, I.C. Leonardo, M.M.B. Juan, Immunostimulation and trained immunity in marine fish larvae, Fish Shellfish Immunol. 80 (2018) 15–21 https://doi.org/10.1016/j.fsi.2018.05.044.

[6] I. Bricknell, R.A. Dalmo, The use of immunostimulants in fish larval aquaculture, Fish Shellfish Immunol. 19 (2005) 457–472 https://doi.org/10.1016/j.fsi.2005.03.008.

[7] P. Srivastava, R. Malviya, Source of pectin, extraction and its applications in pharmaceutical industry, Indian J. Nat. Prod. Resour. 2 (2011) 10–18 http://hdl.handle.net/123456789/11534.

[8] W.S.F. Chung, M. Meijerink, B. Zeuner, J. Holck, P. Louis, A.S. Meyer, et al., Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon, FEMS Microbiol. Ecol. 93 (2017) 43–57 https://doi.org/10.1093/femsec/fix127.

[9] S. Wang, P. Li, S.M. Lu, Z.Q. Ling, Chemoprevention of low-molecular-weight citrus pectin (LCP) in gastrointestinal cancer cells, Int. J. Biol. Sci. 12 (2016) 746–756 https://www.ncbi.nlm.nih.gov/pubmed/27194951.

[10] F. Brouns, E. Theuwissen, A. Adam, M. Bell, A. Berger, R.P. Mensink, Cholesterol-lowering properties of different pectin types in mildly hyper-cholesterolemic men and women, Eur. J. Clin. Nutr. 66 (2012) 591–599 https://www.ncbi.nlm.nih.gov/pubmed/22190137.

[11] S.V. Popov, Y.S. Ovodov, Polypotency of the immunomodulatory effect of pectins, Biochemistry (Mosc.) 78 (2013) 823–835 https://www.ncbi.nlm.nih.gov/pubmed/24010844.

[12] P.A. Markov, S.V. Popov, I.R. Nikitina, R.G. Ovodova, Y.S. Ovodov, Anti-inflammatory activity of pectins and their galacturonan backbone, Russ. J. Bioorganic Chem. 37 (2011) 817–821 https://link.springer.com/article/10.1134/S1068162011070132.

[13] Q. Wu, L. Liu, A. Miron, B. Klímová, D. Wan, K. Kuca, The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: an overview, Arch. Toxicol. 90 (2016) 1817–1840 https://www.ncbi.nlm.nih.gov/pubmed/27259333.

[14] J.R. Gumbo, C.N. Nesamvuni, A Review: Spirulina a source of bioactive compounds and nutrition, J. Chem. Pharm. Sci. 10 (2017) 1317–1325 https://www.jchps.com/issues/Volume%2010.../20171025_074344_0090417.

[15] A.H. Corona, I. Nieves, M. Meckes, G. Chamorro, B.L. Barron, Antiviral activity of Spirulina maxima against herpes simplex virus type 2, Antivir. Res. 56 (2002) 279–285 https://www.ncbi.nlm.nih.gov/pubmed/12406511.

[16] G. Usharani, G. Srinivasan, S. Sivasakthi, P. Saranraj, Antimicrobial activity of *Spirulina platensis* solvent extracts against pathogenic bacteria and fungi, Adv. Biol. Res. 9 (2015) 292–298 https://pdfs.semanticscholar.org/1642/5fbdd0c682a681c6883b39ec2523adbe6a35.

[17] M.M.S. Ismaiela, Y.M. El-Ayoutyb, M. Piercey-Normorea, Role of pH on antioxidants production by *Spirulina* (Arthrospira) *platensis*, Braz. J. Microbiol. 47 (2016) 298–304 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4874622/.

[18] M. Ghaeni, L. Roomiani, Review for application and medicine effects of Spirulina, *Spirulina platensis* microalgae, JOAAT 3 (2016) 114–117 http://www.joaat.com/uploadfile/2016/0628/20160628033646784.

[19] C. Lackmann, M.M. Santos, S. Rainieri, A. Barranco, H. Hollert, P. Spirhanzlova, et al., Novel procedures for whole organism detection and quantification of fluorescence as a measurement for oxidative stress in zebrafish (*Danio rerio*) larvae, Chemosphere 197 (2018) 200–209 https://doi.org/10.1016/j.chemosphere.2018.01.045.

[20] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-DDCT} method, Methods 25 (2001) 402–408 https://doi.org/10.1006/meth.2001.1262.

[21] S.T. Minzanova, V.F. Mironov, D.M. Arkhipova, A.V. Khabibullina, et al., Biological activity and pharmacological application of pectic Polysaccharides: a Review, Polymers 10 (2018) 1–10 https://doi.org/10.3390/polym10121407.

[22] Q. Jiao, L. Li, Q. Mu, Q. Zhang, Immunomodulation of nanoparticles in nanomedicine applications, BioMed Res. Int. 2014 (2014) 1–19 https://doi.org/10.1155/2014/426028.

[23] C. Chittasupho, M. Jaturanpinyo, S. Mangmool, Pectin nanoparticle enhances cytotoxicity of methotrexate against HepG2 cells, Drug Deliv. 20 (2013) 1–9, https://doi.org/10.3109/10717544.2012.739214.

[24] K. Yamagami, Studies on the hatching enzyme (choriolysin) and its substrate, egg envelope, constructed of the precursors (choriogenins) in *Oryzias latipes*: a sequel to the information in 1991/1992, Zool. Sci. 3 (1996) 331–340 https://doi.org/10.2108/zsj.13.331.

[25] X. Zhu, L. Zhu, Z. Duan, Q. Ruiqi, Y. Li, Y. Lang, Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage, J. Environ. Sci. Heal. A. 43 (2008) 278–284 https://doi.org/10.1080/10934520701792779.

[26] J.F. De la Paz, N. Beiza, S. Paredes-Zúñiga, M.S. Hoare, Triazole fungicides inhibit zebrafish hatching by blocking the secretory function of hatching gland cells, Int. J. Mol. Sci. 18 (2017) 1–16 https://doi.org/10.3390/ijms18040710.

[27] S.A. Okunsebor, P.C. Ofojekwu, D.G. Kakwi, B.S. Audu, Effect of temperature on fertilization, hatching and survival rates of *Heterobranchus bidorsalis* eggs and hatchlings, Br. J. Appl. Sci. Technol. 7 (2015) 372–376, https://doi.org/10.9734/BJAST/2015/9505.

[28] R. Castro, L. Jouneau, L. Tacchi, D.J. Macqueen, A. Alzaid, et al., Disparate developmental patterns of immune responses to bacterial and viral infections in fish, Sci. Rep. 5 (2015) 1–12 http://doi.org/10.1038/srep15458.

[29] E.D. Houde, Patterns and trends in larval-stage growth and mortality of teleost fish, J. Fish Biol. 51 (1997) 52–83 https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1095-8649.1997.tb06093.x.

[30] E. Ringo, T.H. Birkbeck, P.O. Munro, O. Vadstein, K. Hjelmeland, The effect of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L.) larvae, J. Appl. Microbiol. 81 (1996) 207–211 https://doi.org/10.1111/j.13652672.1996.tb04502.x.

[31] V.V. Smirnov, V.V. Golovchenko, F.V. Vityazev, et al., The Antioxidant properties of pectin fractions isolated from vegetables using a simulated gastric fluid, J. Chem. 2017 (2017) 1–10 https://doi.org/10.1155/2017/5898594.

[32] E.V. Vidal, F.R. López, M. Teles, S. MacKenzie, The response of fish to immunostimulant diets, Fish Shellfish Immunol. 56 (2016) 34–69 https://doi.org/10.1016/j.fsi.2016.06.028.

[33] C.K. Misra, B.K. Das, S.C. Mukherjee, P. Pattnaik, Effect of long term administration of dietary β-glucan on immunity, growth and survival of *Labeo rohita* fingerlings, Aquaculture 255 (2006) 82–94 https://doi:10.1016/j.aquaculture.2005.12.009.

[34] S.M. Paulsen, H. Lunde, R.E. Engstad, B. Robertsen, In vivo effects of beta-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar* L.), Fish Shellfish Immunol. 14 (2003) 39–54 https://doi:10.1006/fsim.2002.0416.

[35] L. Sahoo, J. Parhi, C. Debnath, K.P. Prasad, Effect of feeding Lipopolysaccharide as an immunostimulant on immune response and immune gene expression of *Labeo bata*, Vet. Immunol. Immunopathol. 188 (2017) 48–58 https://doi.org/10.1016/j.vetimm.2017.04.012.

[36] H.V. Doan, S.H. Hoseinifar, P. Elumalai, S. Tongsiri, et al., Effects of orange peels derived pectin on innate immune response, disease resistance and growth performance of Nile tilapia (*Oreochromis niloticus*) cultured under indoor biofloc system, Fish Shellfish Immunol. 80 (2018) 56–62 https://doi.org/10.1016/j.fsi.2018.05.049.