Accepted Manuscript

Feeding of nano scale oats β-glucan enhances the host resistance against *Edwardsiella tarda* and protective immune modulation in zebrafish larvae

R.M.C. Udayangani, S.H.S. Dananjaya, Baldassare Fronte, Cheol-Hee Kim, Jehee Lee, Mahanama De Zoysa

PII: S1050-4648(16)30730-6

DOI: 10.1016/j.fsi.2016.11.035

Reference: YFSIM 4316

To appear in: *Fish and Shellfish Immunology*

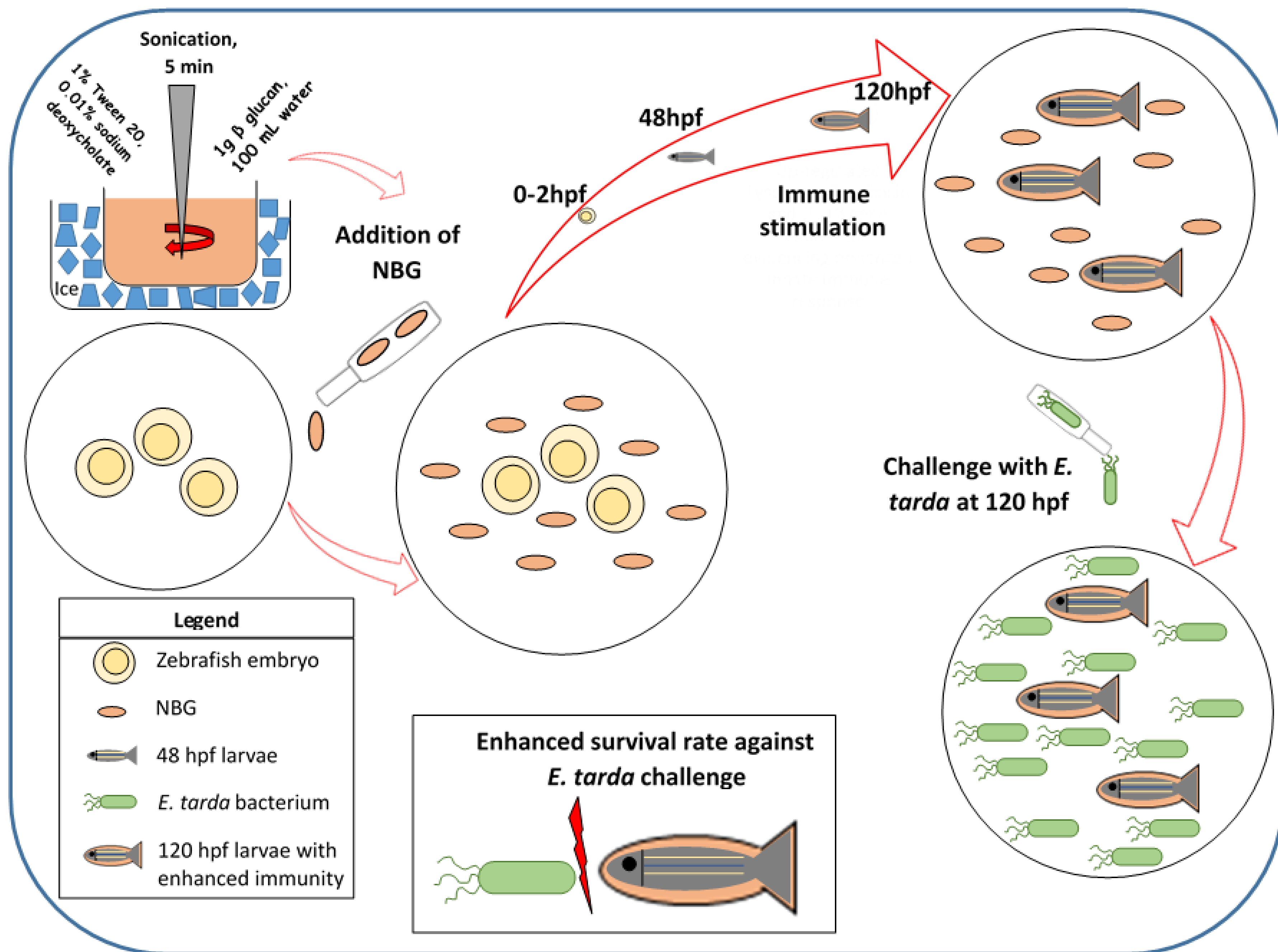
Received Date: 16 August 2016

Revised Date: 8 November 2016

Accepted Date: 12 November 2016

Please cite this article as: Udayangani RMC, Dananjaya SHS, Fronte B, Kim C-H, Lee J, De Zoysa M, Feeding of nano scale oats β-glucan enhances the host resistance against *Edwardsiella tarda* and protective immune modulation in zebrafish larvae, *Fish and Shellfish Immunology* (2016), doi: 10.1016/j.fsi.2016.11.035.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.


Sonication, 5 min
1% Tween 20, 0.01% sodium deoxycholate
1g β glucan, 100 mL water
Ice
Addition of NBG
0-2hpf
48hpf
120hpf
Immune stimulation
Challenge with E. tarda at 120 hpf
Legend
Zebrafish embryo
NBG
48 hpf larvae
E. tarda bacterium
120 hpf larvae with enhanced immunity
Enhanced survival rate against E. tarda challenge

ACCEPTED MANUSCRIPT

1 **Short Communication**

2 Feeding of nano scale oats β-glucan enhances the host resistance against
3 *Edwardsiella tarda* and protective immune modulation in zebrafish larvae

4

5 R.M.C. Udayangani[a], S.H.S. Dananjaya[a], Baldassare Fronte[b], Cheol-Hee Kim[c], Jehee Lee[d,e],
6 Mahanama De Zoysa[a,e*]

7
8 [a]College of Veterinary Medicine and Research Institute of Veterinary Medicine,
9 Chungnam National University, Yuseong-gu, Daejeon, 34134, Republic of Korea.
10
11 [b]Department of Veterinary Science, University of Pisa, viale delle Piagge 2, 56124 Pisa (I), Italy.
12
13 [c]Department of Biology, Chungnam National University, Yuseong-gu, Daejeon 34134,
14 Republic of Korea.
15
16 [d]Department of Marine Life Sciences, School of Marine Biomedical Sciences,
17 Jeju National University, Jeju Self-Governing Province 63243, Republic of Korea.
18
19 [e]Fish Vaccine Research Center, Jeju National University, Jeju Self-Governing Province
20 63243, Republic of Korea.
21
22
23

24 * Corresponding author:
25 Mahanama De Zoysa
26 College of Veterinary Medicine and Research Institute of Veterinary Medicine,
27 Chungnam National University, Yuseong-gu, Daejeon, 34134, Republic of Korea.
28
29 Tel:+82428216795; Fax:+82428218903
30 E-mail:mahanama@cnu.ac.kr (De Zoysa, M)

ACCEPTED MANUSCRIPT

31 **Abstract**

32 In this study, we prepared and characterized the oats origin of nano scale β-glucan (NBG) and
33 investigated the immunomodulatory properties in zebrafish larvae. Newly prepared NBG
34 (average particle size of 465 nm) was fully soluble in water. Zebrafish larvae survival rate was
35 increased against pathogenic bacteria *Edwardsiella tarda*, when NBG was added to the water
36 (500 μg/mL) compared to NBG non-exposed controls. Moreover, quantitative real time PCR
37 (qRT-PCR) results showed up-regulation of immune functional genes including TNF-α, IL-1β,
38 β-defensin, lysozyme, IL 10, IL 12 and C-Rel indicating higher survival rate could be due to
39 stronger immunomodulatory function of NBG (500 μg/mL). Thus, non-toxic, water soluble and
40 biodegradable NBG from oats could be considered as the potential immunostimulant for larval
41 aquaculture.

42

43 **Keywords:** Nano-scale beta glucan (NBG); immunostimulant, bio-degradable; *Edwardsiella*
44 *tarda*

45

46

47

48

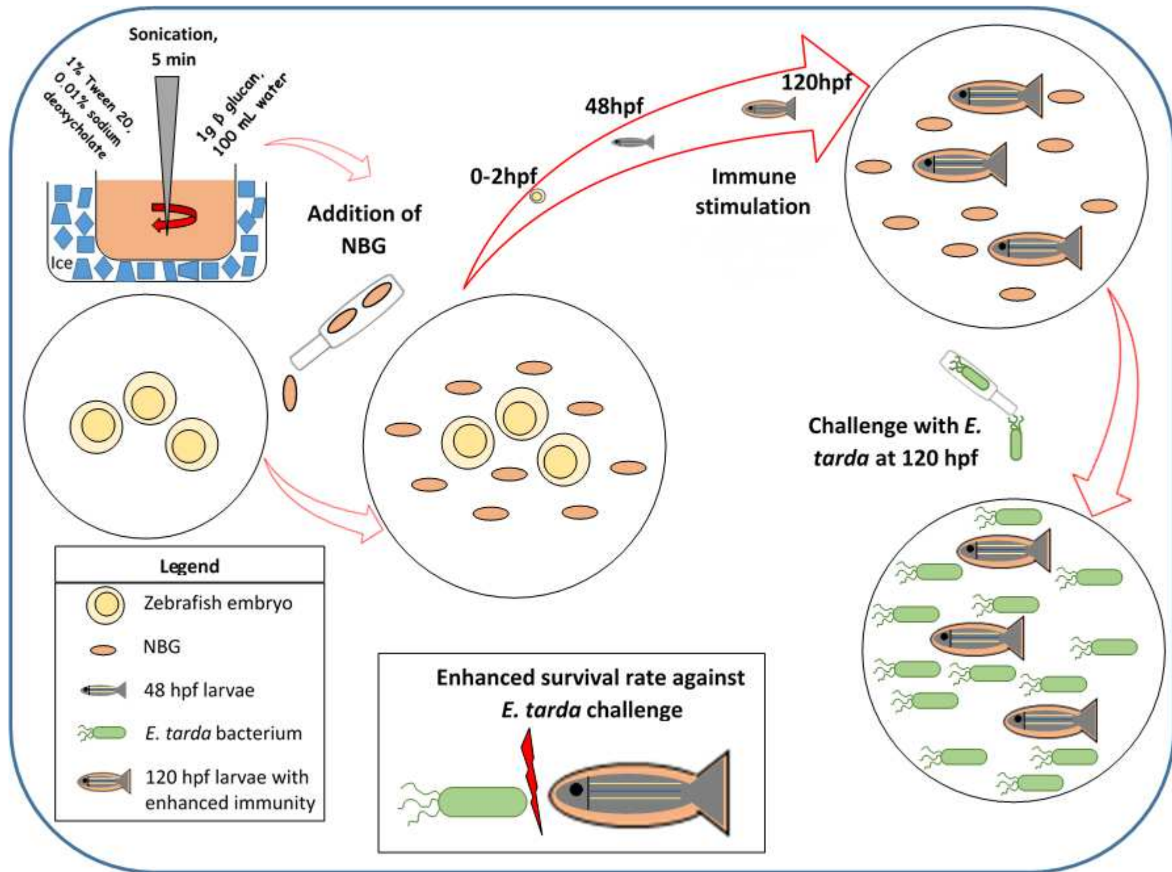
49

50

51

ACCEPTED MANUSCRIPT

52 **Graphical abstract**



53

54

55

56

57

58

59

60

ACCEPTED

61 **1. Introduction**

62 Immunomodulators or biological response modifiers (BRMs) are one of the most promising
63 prophylactic materials in aquaculture due to their ability of modulating the immune system.
64 Bricknell and Dalmo [1] strongly emphasized the importance of immuno-prophylactic measures
65 for the production of healthy fish larvae to improve the overall production of adult fish. Use of
66 immunomodulators has been increased in aquaculture due to several reasons such as continuous
67 rapid expansion of aquaculture sector and frequent disease outbreaks [2]. β-glucan is one of the
68 most applied natural imunostimulants in aquaculture. Different types of β-glucans are found in
69 bacteria, yeast, fungi (mushrooms), algae, and grains like oats, and barley [3,4], and their
70 molecular structures varied depending on the source. For instance, yeast (*Saccharomyces*
71 *cerevisiae*) β-glucan generally consists of β D (1→3) and or (1→4) linked anhydro D glucose
72 units as back bone and β D (1→6) linked branches, whereas oat β-glucan (OBG) consists of
73 unbranched (linear) polysaccharide with (1→3) and (1→4) β D glucan [5-7]. Thus, the immune
74 modulatory properties of β-glucan can be varied based on type and degree of branching,
75 solubility, molecular mass, tertiary structure, polymer charge and solution conformation [8].
76 Extensive information have been reported related to the immunomodulatory effects of β-glucan
77 derived from yeast (*S. cerevisiae*) in aquaculture species (Table 1). However, limited information
78 is available on application of oat β-glucan on different fish larval stages, where the disease
79 prevention by vaccination is restricted. Effectiveness of β-glucan immunomodulation depends
80 highly on its solubility levels, thus, oat β-glucan has considerable advantage compared to that of
81 yeast derived β-glucan, which is not fully soluble in water [15]. Moreover, direct exposure of β-
82 glucan in water would be one of the most cost-effective way of administration to the larval stages

83 of fish. Additionally, it was reported that globular and smaller size β-glucan particles have
84 stronger immune activation and thereby higher disease resistant capacity [16].

85 Nano technology is one of the fastest developing fields in biomedical applications. However,
86 its potential to the aquaculture has not fully utilized yet. Nano based materials have been used in
87 aquaculture as antimicrobial agents [17], and drug delivery [18], for detecting pathogens in the
88 water and water purification systems [19]. Hence, biodegradable and nano size materials with
89 stronger immune stimulatory properties would be a novel addition to aquaculture for improving
90 the fish health by replacing the existing conventional products. The objective of this study was to
91 prepare nano scale, water soluble, biodegradable, and nontoxic β-glucan from oats with intention
92 to use as an effective immunomudulator in fish larval aquaculture. Prepared NBG was
93 characterized based on size, and tested for its immunomodulatory potential by investigating the
94 disease resistant capacity against *E. tarda* and immune functional gene responses in zebrafish
95 larvae.

96

97 **2. Materials and methods**

98 **2.1 Synthesis, characterization and fluorescent labeling of NBG**

99 Commercial oats β-glucan (Food Chem, China) was used to prepare the NBG. Briefly, oats β-
100 glucan (1 g) was dissolved in 100 mL of distilled water containing 1 % Tween 20, 0.01%
101 Sodium deoxycholate (Sigma, USA) and sonicated for 5 min using a probe sonicator (Sonic and
102 Material, USA). Sonicated β-glucan was tested for homogeneity, particle size and zeta potential
103 by Zetasizer S-90 Malvern instrument (Malvern, UK). Morphology of NBG was analyzed by
104 field emission scanning electron microscope (S-4800, Hitachi, Japan). DTAF, 5-(4,6-
105 dichlorotriazinyl aminofluorescein) is considered as a convenient and reliable agent for

106 fluorescent labeling of amino-enriched polysaccharides [20]. To track and visualize the NBG
107 intake by zebrafish larvae, NBG was labeled with DTAF according to method described by
108 Mccann et al., [21]. Briefly, 25 mg of NBG was suspended in 0.1M borate buffer and mixed with
109 10 mg of DTAF in 0.1M borate buffer (pH 10.8). The mixture was incubated at room
110 temperature for 16 h under gentle stirring. After incubation, unattached DTAF was removed by
111 washing with distilled water followed by ethanol precipitation. DTAF labeled NBG was
112 collected by centrifugation (4000 rpm, at 4 ^{o}C for 30 min). Final product of NBG was freeze
113 dried until further use.
114

115 **2.2 Zebrafish larvae culture and NBG exposure**

116 Wild type zebra fish were purchased from a commercial aquarium, Seoul, South Korea. Fish
117 were maintained in automated water circulated systems at 28 ±1 °C with 12/12 h light/dark cycle.
118 The water conductivity was maintained in 500±50 µS at all times. The fish were daily fed with
119 *artemia* (brine shrimp) 3 times per day at 4% body weight. For the *in vivo* trials, 2 NBG
120 concentrations (100 and 500 µg/mL) were prepared using embryo water containing 60 mg/L
121 aquarium salt. Embryo water was used as the control. Once the zebrafish embryos were received,
122 approximately 250 embryos at 2 h post fertilization (hpf) were transferred into separate beakers,
123 which contained pre prepared NBG concentrations. After confirming the active condition of
124 embryo using light microscope, 25 healthy embryos were selected and re-transferred in to 90×22
125 mm petri dishes containing 10 mL of each NBG solution. Embryos were maintained at 25 °C
126 with 12/12 light/dark cycle and media were renewed daily throughout the experiment. All the
127 treatments were conducted in triplicates. The hatched larvae were separated and maintained in
128 same NBG solutions for another 3 days. For the gene expression study, 75 larvae (n=75) of each

129 NBG exposed group for at least 100 h (continuously) were collected at the age of 120 hpf. Then
130 they were snap frozen in liquid nitrogen and stored at -80 °C until further used. Control larvae
131 (n=75) without NBG exposure were used as control group. To confirm the presence and
132 localization of NBG in zebrafish digestive tract, 5 larvae from each treatment groups were
133 randomly selected at 48 hpf and transferred into DTAF labeled NBG of similar concentrations
134 (100, and 500 µg/mL). At the age of 5 day post fertilization (dpf), DTAF labeled NBG treated
135 zebrafish larvae were observed, and images were taken under a camera attached fluorescence
136 microscope (OLYMPUS IX71 DP70, Japan).
137

138 **2.3 Immune challenge of NBG exposed zebrafish larvae against fish pathogenic *E. tarda***

139 Immune challenge experiment was conducted to investigate the immunomodulatory effect of
140 NBG on disease resistance capacity of zebrafish larvae. NBG exposed and control larvae at 120
141 hpf were randomly selected for the challenge experiment (n=30) and pathogenic *E. tarda* (KCTC
142 12267) was used to challenge fish. Briefly, a single colony of *E. tarda* was grown in brain heart
143 infusion broth at 25 ^{o}C with shaking at 160 rpm for 16 h. The overnight culture was then re-
144 inoculated into 20 mL of fresh brain heart infusion broth (1:100 dilutions) and allowed to grow
145 until ~ 0.6 OD_{600}. The, bacterial culture was pelleted by centrifugation (3500 rpm at 4 °C for 10
146 min) and re-suspended in ×1 phosphate buffered saline (PBS). NBG treated and untreated larvae
147 were immune challenged by exposing *E. tarda* at 5×10^{8} CFU/mL in larval culture plates. The
148 mortality was recorded at every 6 h intervals to determine the survival percentage. The
149 experiment was conducted in 3 replicates for each treatment.
150

151 **2.4 qRT-PCR analysis of immune genes of zebrafish larvae upon NBG exposure**

ACCEPTED MANUSCRIPT

152 Total RNA was isolated from 40 larvae of NBG treated and control samples using Trizol reagent
153 (Invitrogen) according to the manufacturer's protocol. Concentration and purity of RNA were
154 determined using a UV-spectrophotometer (BioRad, USA). Purified total RNA (2.5 μg) was
155 used to synthesize the first strand cDNA using PrimeScript™ first-strand cDNA synthesis kit
156 (TaKaRa, Japan) following the manufacturer's instructions. cDNA samples were diluted 40 ×
157 and they were stored at -20 °C for qRT-PCR analysis. To confirm the immunomodulatory effects
158 of NBG, transcriptional regulation of selected immune functional genes were analyzed by qRT-
159 PCR assay using a Thermal Cycler Dice Real Time System (TaKaRa, Japan). The selected genes
160 and specific primers used for this study is listed in the table 2, and these genes were selected
161 based on previous literature related to gene expression studies of zebrafish embryo or larvae. *β-*
162 *actin* gene was selected as an internal control. The 10 μL reaction was carried out in triplicates
163 consisting 3 μL of cDNA (1:40 dilution), 5 μL of 2 × SYBR premix (TaKaRa, Japan), 1 μL of
164 each primer (10 pmol/μL). The thermal reaction was included single cycle of 95 °C for 30 sec,
165 followed by 40 cycles of 95 °C for 5 sec, 58 °C for 20 sec, and 72 °C for 20 sec. To affirm the
166 melting curve in order to evaluate a specific PCR product was amplified, a cycle of 95 °C for 15
167 sec, 60 °C for 30 sec and 95 °C 15 sec was executed at end of the reaction. Finally, triplicate Ct
168 values for respective reactions were subjected to Livak method [22] to calculate the expression
169 fold. Data are expressed as the expression fold relative (normalized) to that of *β-actin*. Fold units
170 were calculated dividing the normalized expression values of the treatment by the normalized
171 expression values of the control.
172
173
174

175 **2.5 Statistical analysis**

176 All the statistical data analysis was performed using OriginPro (OriginLab Corporation 2015)
177 and one way analysis of variance (ANOVA) followed by Tukey's test was conducted for the
178 mean comparison. The differences were considered statistically significant at $p < 0.05$ and data
179 were presented as mean ± standard error of mean.

180

181 **3. Results and discussion**

182 Present study describes small size, non-toxic and bioactive NBG derived from oats that
183 enhances disease resistance capacity in zebrafish larvae against fish pathogenic *E. tarda*.
184 Activation of innate immune responses via up-regulation of immune genes could be one of the
185 main roles of NBG, for observed strong disease resistance in larvae.

186 Bricknell and Dalmo [1] emphasized that use of immunostimulants as dietary
187 supplements in fish larval aquaculture could enhance the protective innate immune defense in
188 developing fish until their adaptive immune system is fully functioned. Sasson et al., [23]
189 pointed out that reduction of particle size into nano-scale could improve the solubility, mobility
190 and efficacy of bioactive agents over larger particles. Therefore, we aimed to develop nano size
191 β-glucan from oats as an immunomodulatory and dietary supplement to be used in larval
192 aquaculture. To confirm the nano size of the newly synthesized NBG, physio-chemical
193 characteristics were studied. NBG solution showed a lower sedimentation rate with slow rate of
194 aggregation, and particles remain in suspension longer compared to the normal β-glucans of oats
195 (data not shown). Formation of β-glucan nano particles was detected by the peak at 260 nm using
196 UV-vis spectroscopy (Supplementary fig. 1). SEM analysis showed the morphology of NBG
197 particles, however, most of the particles had some levels of aggregation (Fig. 1A). Zetasizer

198 analysis result displayed the narrow particle size distribution of NBG with average size of
199 465 nm (Fig. 1 B), and Zeta potential of NBG was -23.6 mV. Formation of smaller size
200 β-glucan particles and lower sedimentation could be due to depolymerization via the
201 breakage of glyosidic bonds result from zonication as reported in previous studies [24].
202 Oyaebide et al., [25] have performed an experiment with zebrafish larvae to investigate
203 the immustimulatory efficacy of polysaccharide, and suggested that intake of β-glucan by
204 larvae was high between day 4 and 5 post exposure. Fluorescent DTAF labeled NBG
205 (Fig. 1C) was localized in the gut of zebrafish larvae at day 5 post exposure (120 hpf).
206 Moreover, higher fluorescence intensity was observed in intestinal tract at 500 µg/mL
207 (Fig.1D) than 100 µg/mL (Fig. 1E). Additionally, we observed that exposure of NBG up
208 to 5000 µg/mL to 120 hpf larvae for 24 h did not make any toxic effects to zebrafish
209 larvae (data not shown).
210 *E. tarda,* is a Gram negative bacterium belongs to the family *Enterobacteriaceae* and
211 highly pathogenic to wide range of culture fish such as carp, tilapia, eel, catfish, mullet, salmon,
212 trout and olive flounder as well as to the amphibians, reptiles and humans [26-28]. It is essential
213 to examine the efficacy of immunostimulants against *E. tarda* like serious fish pathogens to
214 enhance the disease resistance of fish larvae. Therefore, immunomodulatory function of newly
215 synthesized NBG was investigated based on the survival rate of immune challenged fish larvae
216 against *E. tarda*. NBG exposed zebrafish larvae had significantly higher survival rate compared
217 to control group against *E. tarda* challenge (Fig. 2). Observed immune stimulant properties of
218 synthesized NBG correlates with several previous studies of β-glucans of different origins. For
219 instance, zebrafish larvae mortality was decreased when β glucan exposed for 5 days before
220 *Vibrio anguillarum* challenge compared to un-exposed group [25].

221 When consider the early life stages of fish, the first 2-3days of larvae after hatching
222 basically depends on the innate immune system [29]. In general, the effectiveness of the
223 immunostimulants highly depends on the target cells recognizing the immunostimulants as
224 potential risk molecules followed by triggering the defense pathways [1]. For further
225 understanding of the effects of NBG on the innate immunity of fish larvae, we examined the
226 expression levels of 8 immune functional genes in zebrafish. Lysozyme [30] and β-defensin [31]
227 are the major antimicrobial components in first line of host defense, whereas TNF-α and IL-1β
228 are key pro-inflammatory cytokines [32], mainly produced by activated macropahges in different
229 immune activation pathways [33]. Oyarbide et al, showed induced mRNA transcriptional
230 responses of TNF-α, lysozyme, myeloperoxidase, and transferrin, indicating activated innate
231 immunity after 6 days exposure of β-glucan (50-150 µg/mL) in 144 hpf stage of zebrafish larvae,
232 by conducting similar experiment by exposing the zebrafish larvae to conventional β-glucan [25].
233 Although, β-glucan exposure levels and durations were not perfectly match with our
234 experimental design, qRT-PCR results of the present study clearly demonstrated the significant
235 ($P<0.05$) up-regulation of TNF-α, (42.2-fold), IL-1β (2.2-fold), IL 10 (72.1- fold), IL 12(193.9-
236 fold), β-defensin (4.9-fold), lysozyme (50.70-fold) and C-Rel (2.4-Fold) genes at the highest
237 exposure level of NBG (500 µg/mL), suggesting that NBG can activate the innate immune
238 responses in zebrafish larvae (Fig. 3). However, only 4 genes, namely TNF-α (2.9-fold), IL 10
239 (3.7- fold), IL 12 (5.3- fold) and lysozyme (3.1-fold) showed up-regulation at 100 µg/mL NBG
240 exposure and this notable differences in expression of immune genes could be the result of NBG
241 concentration that used and the particle amount that dispersed in the water. Availability of
242 dispersed NBG particles could be lower in 100 µg/mL than 500 µg/mL, thus the lower
243 concentration (100 µg/mL) could result in lower immune modulatory effects. This indicates that

244 observed immune enhancing properties could be a direct result of NBG, which exhibits ability to
245 trigger the innate immune system. However, apart from our observations, previous reports show
246 inconsistent effects of β-glucans on immune modulatory genes especially on the cytokines (TNF-
247 α, IL-1β, IL10, and IL12) suggesting the complexity and the shallowness of the current
248 knowledge related to the immune modulation properties of β-glucans [30]. In conclusion our
249 synthesized NBG shows strong disease resistance capacity against pathogenic *E. tarda* and able
250 to increase the transcript levels of key genes involved in immune responses in zebrafish larvae.
251 Altogether, these findings demonstrate that NBG could be a promising immunostimulant for the
252 larval stages of fish, which has potential to use in aquaculture industry.
253

254 **Acknowledgments**
255 This work was supported by a National Research Foundation of Korea (NRF) grant funded by
256 the Korea government (MSIP) (2014R1A2A1A11054585), and Nano Material Technology
257 Development Program through the National Research Foundation of Korea (NRF) funded by the
258 Ministry of Science, ICT and Future Planning (2009-0082580).
259

260 **Conflict of interest**
261 No conflict of interest
262

263 **Submission declaration**
264 The authors of this manuscript certify and declare that this work has not been previously published,
265 that is not under consideration for publication elsewhere and that it has been approved for publication
266 by all of the authors and our affiliated organizations.

ACCEPTED MANUSCRIPT

267 **References**

268 [1] I. Bricknell, R.A. Dalmo, The use of immunostimulants in fish larval aquaculture, Fish &
269 Shellfish Immunol. 19(5) (2005) 457–472.

270 [2] D.K. Meena, P. Das, S. Kumar, S.C. Mandal, A.K. Prusty, S.K. Singh, S.C. Mukherjee, Beta-
271 glucan: An ideal immunostimulant in aquaculture (a review), Fish Phy. & Biochem. 39(3)
272 (2013) 431–457.

273 [3] R.A. Dalmo, J. Bogwald, Beta-glucans as conductors of immune symphonies, Fish &
274 Shellfish Immunol. 25(4) (2008) 384–396.

275 [4] A. Estrada, C.H. Yun, A. Van Kessel, B. Li, S. Hauta, B. Laarveld. Immunomodulatory
276 activities of oat β-glucan *in vitro* and *in vivo*, Microbiol. Immunol. 41(12) (1997) 991–998.

277 [5] D. El Khoury, C. Cuda, B.L. Luhovyy, G.H. Anderson. Beta glucan: health benefits in
278 obesity and metabolic syndrome, J. Nut. Metabol. 2012 (2012) ID 851362 28pages.

279 [6] M. McIntosh, B.A. Stone, V.A. Stanisich. Curdlan and other bacterial (1→3)-β-D-glucans,
280 Appl. Microbiol Biotechnol. 68(2) (2005) 163–173.

281 [7] O. Rop, J. Mlcek, T. Jurikova. Beta-glucans in higher fungi and their health effects, Nutr.
282 Rev. 67(11) (2009) 624–631.

283 [8] J.J. Volman, J.D. Ramakers, J. Plat. Dietary modulation of immune function by beta-glucans,
284 Physiol. Behav. 94(2) (2008) 276–284.

285 [9] J.J. Miest, C. Arndt, M. Adamek, D. Steinhagen, T.B.H. Reusch, Dietary β-glucan
286 (MacroGard®) enhances survival of first feeding turbot (*Scophthalmus maximus*) larvae by
287 altering immunity, metabolism and microbiota, Fish & Shellfish Immunol. 48 (2016) 94–104.

288 [10] J. Skjermo, T.R. Størseth, K. Hansen, A. Handå, G. Øie, Evaluation of β-(1 → 3, 1 → 6)-
289 glucans and high-M alginate used as immunostimulatory dietary supplement during first
290 feeding and weaning of Atlantic cod (*Gadus morhua* L.), Aquaculture 261(3) (2006) 1088–
291 1101.

292 [11] H. Al-Gharabally, A. Al-Marzouk, I.S. Azad, Role of β-glucans and levamisole on the
293 enhancement of the survival rate, immune response and disease resistance of blue-fin porgy
294 *Sparidentax hasta* (Sobaity) larvae, Res. J. Biotechnol. 8(7) (2013) 18-23.

295 [12] B.K. Das, C. Debnath, P. Patnaik, D.K. Swain, K. Kumar, B.K. Misrhra, Effect of β-glucan
296 on immunity and survival of early stage of *Anabas testudineus* (Bloch), Fish & Shellfish
297 Immunol. 27(6) (2003) 678–683.

298 [13] S. Ramzani, M. Soltani, H. Gholipourkanani, Influence of dietary dinamune® on growth
299 performance and lysozyme activity in rainbow trout (*Oncorhynchus mykiss*) fry, Agric. Tech.
300 Biol. Sci. 11(10) (2013) 11:865–869.

301 [14] S. Efthimiou, Dietary intake of beta-1, 3/1, 6 glucans in juvenile dentex (*Dentex dentex*),
302 Sparidae: effects on growth performance, mortalities and non-specific defense mechanisms, J.
303 Appl. Ichol. 12(1) (1996) 1-7.

304 [15] L. Du, X. Zhang, C. Wang, D. Xiao, Preparation of water soluble yeast glucan by four kinds
305 of solubilizing processes, Engineering 4(10B) (2012) 184–188.

306 [16] Y. Tabata, Y. Ikada, Effect of the size and surface charge of polymer microspheres on their
307 phagocytosis by macrophage, Biomaterials 9(4) (1988) 356-362.

308 [17] S.H.S. Dananjaya, G.I. Godahewa, R.G.P.T. Jayasooriya, J. Lee, M. De Zoysa,
309 Antimicrobial effects of chitosan silver nano composites (CAgNCs) on fish pathogenic
310 *Aliivibrio* (Vibrio) *salmonicida*, Aquaculture 450 (2016) 422–430.

311 [18] L, Li, S.L. Lin, L. Deng, Z.G. Liu, Potential use of chitosan nanoparticles for oral delivery
312 of DNA vaccine in black seabream Acanthopagrus schlegelii Bleeker to protect from Vibrio
313 parahaemolyticus. J. Fish Dis. 36 (2013) 987–995.

314 [19] R.D. Handy, FSBI Briefing Paper : Nanotechnology in fisheries and aquaculture. Fish Soc.
315 British Isles 2012 01–29.

316 [20] S. Prigent-Richard, M. Cansell, J. Vassy, A. Viron, E. Puvion, J. Jozefonvicz, D. Letourneur,
317 Fluorescent and radiolabeling of polysaccharides: binding and internalization experiments on
318 vascular cells. J. Biomed. Mat. Res. 40(2) (1998) 275–281.

319 [21] F. McCann, E. Carmona, V. Puri, R.E. Pagano, A.H. Limper, Macrophage internalization of
320 fungal beta-glucans is not necessary for initiation of related inflammatory responses. Infec.
321 Immun. 73(10) (2005) 6340–6349.

322 [22] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time
323 quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25(4) (2001) 402-408.

324 [23] Y. Sasson, G. Levy-Ruso, O. Toledano, I. Ishaaya, Nanosuspensions: emerging novel
325 agrochemical formulations, in: I. Ishaaya, R. Nauen, A.R. Horowitz (Eds.), Inseticides
326 Ddesign Using Advanced Technologies, Springer publications, Berlin, 2007, PP. 1-39.

327 [24] K.W. Hunter, R.A. Gault, M.D. Berner, Preparation of microparticulate beta-glucan from
328 *Saccharomyces cerevisiae* for use in immune potentiation, L. Appl. Microbiol. 35(4) (2002)
329 267–271.

330 [25] U. Oyarbide, S. Rainieri, M. Pardo, Zebrafish (*Danio rerio*) larvae as a system to test the
331 efficacy of polysaccharides as immunostimulants, Zebrafish 9(2) (2012) 74–84.

ACCEPTED MANUSCRIPT

332 [26] J. Zheng, K.Y. Leung, Dissection of a type VI secretion system in *Edwardsiella tarda*, Mol.
333 Microbiol. 66(5) (2007) 1192–1206.

334 [27] S. Park, T. Aoki, T. Jung, Pathogenesis of and strategies for preventing *Edwardsiella tarda*
335 infection in fish, Vet. Res. 43(67) (2012) 1–11.

336 [28] B.R. Mohanty, P.K. Sahoo, Edwardsiellosis in fish: a brief review, J. Biosci. 32(7) (2007)
337 1–14.

338 [29] B. Magnadottir, Innate immunity of fish (overview), Fish & Shellfish Immunol. 20(2) (2006)
339 137–151.

340 [30] C. Hall, M.V. Flores, T. Storm, K. Crosier, P. Crosier, The zebrafish lysozyme C promoter
341 drives myeloid-specific expression in transgenic fish, BMC Dev. Biol. 7(42) (2007) 7-42.

342 [31] J. Zou, C. Mercier, A. Koussounadis, C. Secombes, Discovery of multiple beta-defensin like
343 homologues in teleost fish, Mol. Immunol. 44(4) (2007) 638–647.

344 [32] J. Watzke, K. Schirmer, S. Scholz, Bacterial lipopolysaccharides induce genes involved in
345 the innate immune response in embryos of the zebrafish (*Danio rerio*), Fish & Shellfish
346 Immunol. 23(4) (2007) 901–905.

347 [33] N.L. Vujanovic, Role of TNF superfamily ligands in innate immunity, Immunol Res. 50(2-3)
348 (2011) 159-174.

349

350

351

352

353

354

355

356

357

ACCEPTED MANUSCRIPT

Table 1: Selected studies of yeast and oats β-glucans as immunomodulators for early stages of fish.

Type of β glucan	Fish species	Administration mode	Immunomodulatory effects	Reference
Yeast (*S. cerevisiae*) β glucan β -1,3/1,6-glucans	Turbot (*Scophthalmus maximus*)	Oral/feed	Enhancement of survival rate	[9]
Yeast (*S. cerevisiae*) β glucan β -1,3/1,6-glucans	Atlantic cod (*Gadus morhua*)	Oral/feed	No significant enhancement in survival or growth rate	[10]
β-(1→3, 1→6)-glucan of marine origin (*Chaetoceros mülleri*)	Atlantic cod (*Gadus morhua*)	Oral/feed	Enhancement of survival rate Increased growth rate	
Yeast (*S. cerevisiae*) β glucan β -1,3/1,6-glucans	Blue-fin porgy (*Sparidentex hasta*)	Oral/feed	Improvement in the growth rate Enhanced lysozyme activity, bacterial agglutining and haemagglutining	[11]
β- glucan of barley origin	Climbing perch (*Anabas testudineus*)	Immersion	Enhanced innate immune response and disease resistance against *Aeromonas hydrophila*	[12]
Yeast (*S. cerevisiae*) β glucan β -1,3/1,6-glucans	Rainbow trout (*Oncorhynchus mykiss*)	Oral/feed	Enhanced survival rate Reduced feed conversion rate Enhanced specific growth rate	[13]
Yeast (*S. cerevisiae*) β glucan β -1,3/1,6-glucans	Common dentex (*Dentex dentex*)	Oral/feed	Enhanced survival rate	[14]

Table 2. Description of the selected immune functional genes of zebrafish and specific primers used in this study.

ACCEPTED MANUSCRIPT

Gene and accession number	Primer name	Primer sequence (5'-3')
TNF-α (AF025305)	TNF- α-F	AGAAGGAGAGTTGCCTTTACCGCT
	TNF- α-R	AACACCCTCCATACACCCGACTTT
IL-1 β (AY427649)	IL-1 β-F	ACGTCATCATCGCCCTGAACAGAA
	IL-1 β-R	TGTAAGACGGCACTGAATCCACCA
Interleukin-10 (AY887900.1)	IL- 10-F	CCCTATGGATGTCACGTCATG
	IL- 10-R	CATATCCCGCTTGAGTTCCTG
Interleukin-12 p40 subunit (AB183002.1)	IL- 12-F	CTCAGGGAAACAGGATTACGG
	IL- 12-R	GATCTTCCTAAAGCTCCACTGG
β Defensin like 1 (NM_001081553)	DEFB1- F	TGTGCAAGTCTCAGTGGTGTTTGC
	DEFB1- R	TTTGCCACAGCCTAATGGTCCGAA
Lysosyme (AF402599)	Lysosyme C-F	AAGCAGGTTTAAGACCCACCGAGT
	Lysosyme C-R	AAGTCTGAACAGGCCACTTTGCAC
C-Rel (AY163837)	C-Rel-F	ACTACAGCTCCCAACAGCCTCAAA
	C-Rel-R	AAACTGGTAGCCCGTTGCTAGTGA
β actin (AF025305)	β actin-F	AATCTTGCGGTATCCACGAGACCA
	β actin-R	TCTCCTTCTGCATCCTGTCAGCAA

376
377
378
379
380
381 **Figure legends**

ACCEPTED MANUSCRIPT

382 **Figure 1.** Characterization and labeling of NBG (A) FE-SEM image showing the NBG particles
383 (B) Size distribution by intensity of synthesized NBG showing the average particle size 465 nm
384 (C) DTAF labeled NBG under fluorescence microscope (D) Merged image of fluorescence
385 microscopic and light microscopic images of DTAF labeled NBG (100 µg/mL) ingested larvae
386 (E) Merged image of fluorescence microscopic and light microscopic images of DTAF labeled
387 NBG (500 µg/mL) ingested larvae.

388

389 **Figure 2.** Average survival percentage (%) of zebrafish larvae exposed to NBG. Zebrafish
390 embryos are exposed to different NBG concentrations (0, 100 and 500 µg/mL) and challenged
391 with *E. tarda* (CFU $5x10^8$) at 120 hpf. The symbols represent the mean survival rate of the 3
392 replicates and the error bars indicate the standard error of the means. The data points bearing
393 different letters were statistically different for given time points ($p < 0.05$).

394

395 **Figure 3.** Transcriptional analysis of selected immune functional genes upon continuous NBG
396 treatment (100 and 500 µg/mL) relative to the control group (untreated) at 120 hpf. The asterisk
397 mark was used to indicate statistical significance compared to non-treated control ($p < 0.05$).

398

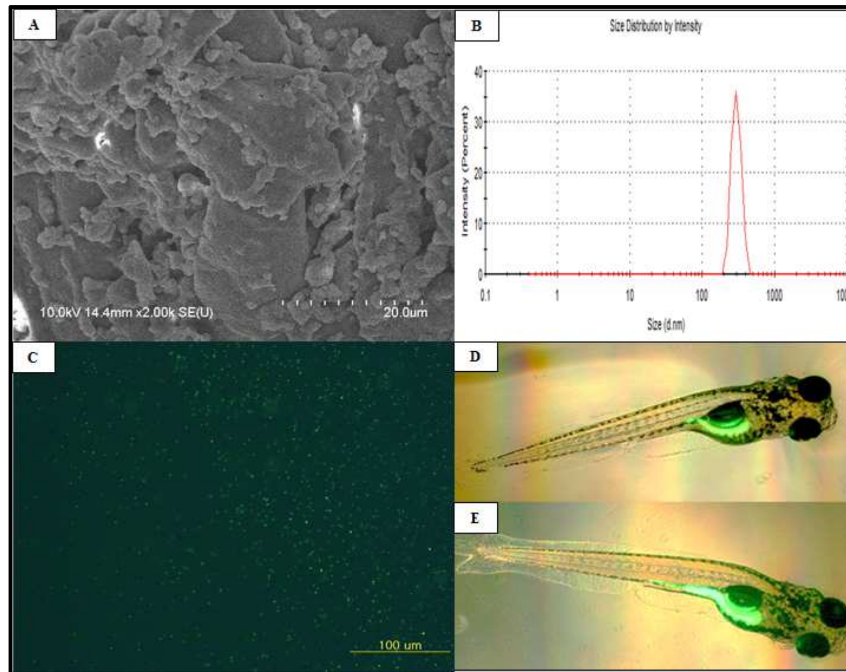
399

400

401

ACCEPTED MANUSCRIPT

402



403 **Fig. 1.**

404

405

406

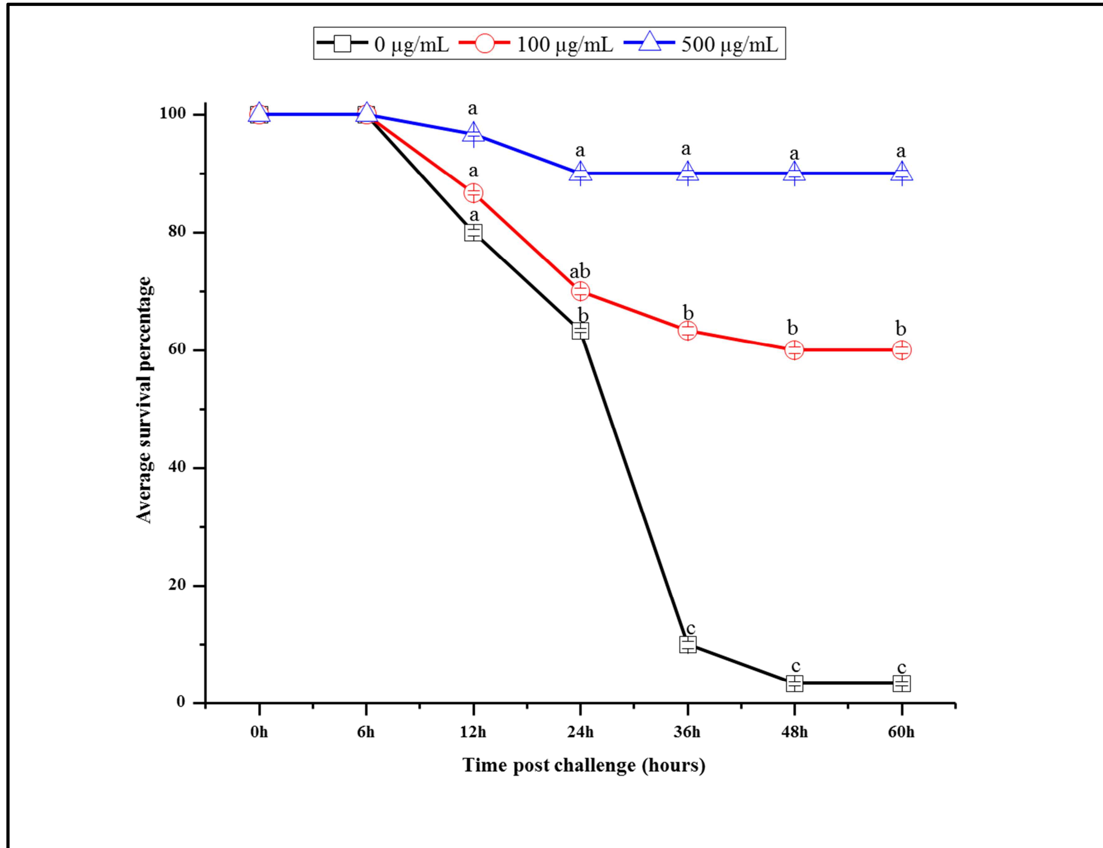
407

408

409

410

ACCEPTED MANUSCRIPT



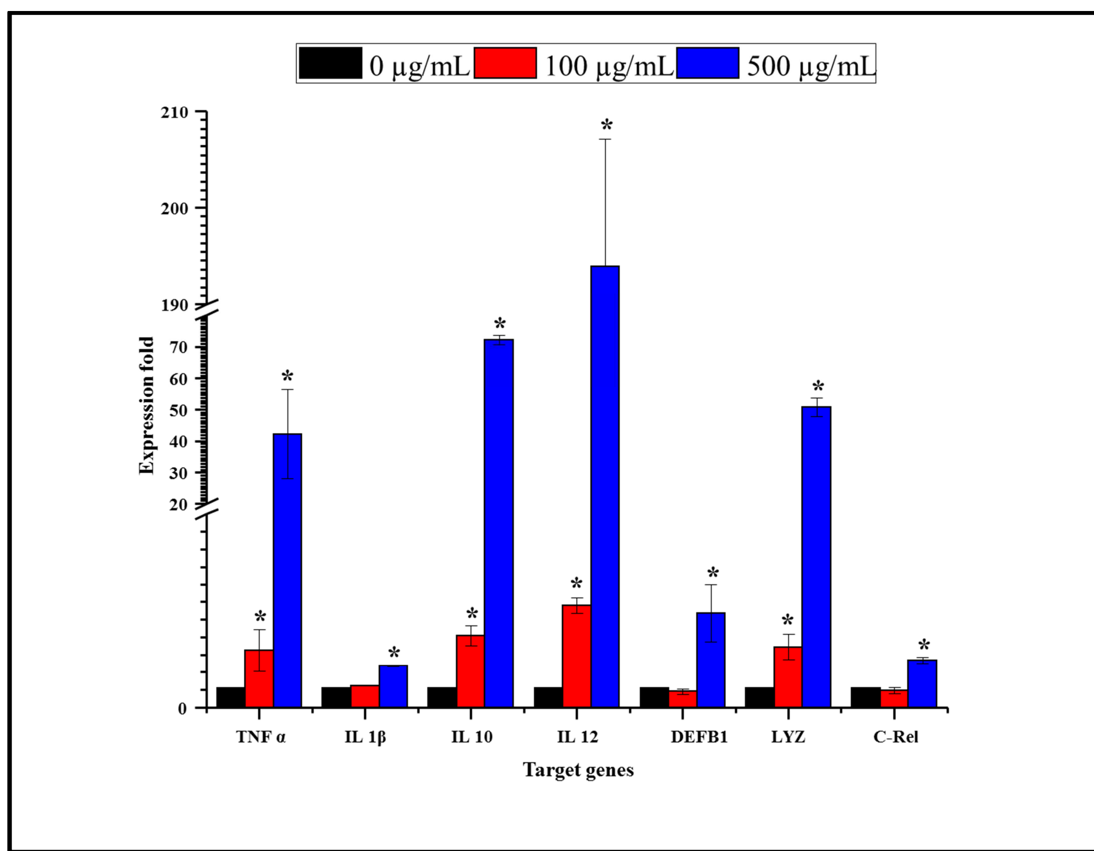
411

412 **Fig. 2.**

413

414

415



416

417 **Fig. 3.**

418

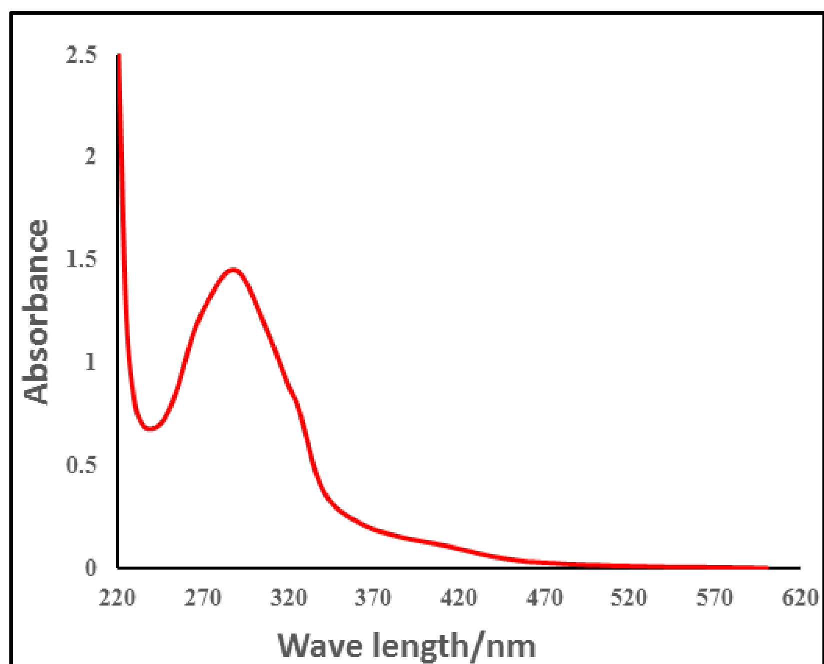
419

420

421

422

ACCEPTED MANUSCRIPT



423

424 **Supplementary Fig 1.**

425 UV spectrum of NBG. The absorption band of the NBG solution (in 0.5 NaOH) was observed at
426 280 nm, which was ascribed to carbonyl groups.

427

428

429

ACCEPTED MANUSCRIPT

Highlights

> Nano size beta glucan of oats origin can enhance the diseases resistance in zebrafish larvae.

>Nano size beta glucan up-regulates the immune functional genes in zebrafish larvae.

> Non-toxic and biodegradable nano size beta glucan from oats would be a better immunostimulant for larval aquaculture.

ACCEPTED MANUSCRIPT