Feeding with poly(I:C) induced long-term immune responses against bacterial infection in turbot (Scophthalmus maximus)

Jing He a, Zhuang Wang a, Yanbo Zhao a, Jin Yang a, Yuanxing Zhang b,c, Qin Liu a,c, Dahai Yang b,c n

a State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China
b Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Zhuhai 519000, China
c Shanghai Engineering Research Center of Maricultured Animal Vaccines, Shanghai 200237, China

ARTICLE INFO

Keywords:
Poly(I:C)
Anti-oxidant stress
Intestinal immune responses
Bacterial-infection turbot

ABSTRACT

Poly(I:C) is a kind of chemosynthetic double-stranded RNA (dsRNA) analogue which could act as TLR3 agonist and induce IFN production. It is widely applied in anti-virus treatment and immunoregulation, as well as vaccine adjuvant in farmed animals. However, whether poly(I:C) could activate innate immune response to defense against bacterial infection remains unclear. In this study, we established a feeding trial model with different dose of poly(I:C) in turbot larvae, then challenged with Edwardsiella piscicida after 3–7 weeks resting period. The results show that feeding turbot with poly(I:C) exhibited a stronger inflammatory response and antioxidant stress ability, and significantly elevated the survival rate within the decreased bacterial loads. Importantly, the bacterial infection-induced white feces in hindgut of turbot were significantly alleviated after poly(I:C) feeding, and this administration induced protection could last for about 7 weeks. Taken together, these findings indicate that feeding turbots with poly(I:C) could enhance a long-term intestinal mucosal immunity in response to bacterial infection, suggesting that poly(I:C) might be a promising immunostimulant in aquaculture.

1. Introduction

Polyriboinosinic-polyribocytidilic acid (poly(I:C)) is a kind of chemosynthetic double-stranded RNA (dsRNA) analogue [1], which could mimic the structure of virus RNA and as a potent ligand of TLR3 to induce IFN production [2]. It has been widely applied as an effective immunostimulant for human diseases, as well as the adjuvant utilized in vaccines and cancer treatment [3, 4]. The role of poly(I:C) in activating antiviral immune response has been revealed in teleost fish. For example, poly(I:C) vaccination could effectively protect zebrafish liver cells [5], as well as promote the anti-viral response in zebrafish liver cells [6]. Moreover, it was suggested that poly(I:C) could induce the expression of IFNa3 through the TLR/TRIF and RLRs/MAVS pathways in the macrophages of large yellow croaker [7]. However, as a potent immunostimulant, whether poly(I:C) could protect host from bacterial infection remains largely unknown.

In recent years, the farmed turbots (Scophthalmus maximus) is mainly threatened by the bacterial pathogens (e.g., the Edwardsiella piscicida (previously known as Edwardsiella tarda) [8] and Aeromonas salmonicida [9]), causing an enormous economic loss in this industry. To avoid the antibiotics resistant, and develop an effective immuno-therapy to prevent diseases is urgent. To this end, the effective vaccine design and the immunostimulants administration are the promising strategies. For example, we have successfully developed the live vaccine against Edwardsiella tarda, and the genetically engineered live vaccine against Vibrio anguillarum in turbot [10, 11]. However, these vaccines’ inoculation should be utilized as intraperitoneal injection, which is limited by the weight of the fish. Thus, the immunostimulants development should be a promising supplement of vaccination.

There are several kinds of immunostimulants, which have been applied as the diet additive to improve the innate immune responses of fish. For example, dietary feeding with β-glucan could elevate the innate immune responses and improve the disease resistance effects in Nile tilapia [12]. Administrated with probiotics could modulate the immune response against tilapia lake virus infection [13]. Moreover, dietary feeding with Spirulina platensis could activate the mucosal immune responses in rainbow trout [14]. In this study, we proposed that poly(I:C) could be utilized as dietary additive for turbot, and developed a
short-term, low-dose feeding of poly(I:C) strategy, and revealed its anti-bacterial infection effects, through provoking the non-specific and long-lasting protection, as well as maintaining the intestinal health of turbot. Taken together, these results suggest that feeding with poly(I:C) might be a promising immunopotentiator in aquaculture.

2. Materials and methods

2.1. Ethical statement

All animal experiments in this study were conformed to the guidelines for the care and use of laboratory animals and ethically approved by the Laboratory Animal Ethical Committee of East China University of Science and Technology (Protocol number 2,006,272). Turbot larvae were anesthetized using tricaine (200 μg/ml, Aladdin, China) before dissection, and all efforts were made to minimize suffering.

2.2. Turbot feeding trial and maintaining condition

Turbot larvae (3–5 g) were used for feeding trial. Poly(I:C) (Niujia-Bio, China) was emulsified with 10% fish oil and 3% phosphatidylcholine in water to generate emulsion, then mixed into the basic pellet diet to the final dose of 0.005% (FD-H) and 0.00125% (FD-L), the control group fed the emulsion mixed pellet diet without poly(I:C) (FD-N). After 4-weeks daily feeding, the turbot larvae were maintained as the control groups. For the maintenance of turbot larvae, the temperature was kept at 18 °C, total ammonia-nitrogen was <0.5 mg/L and dissolved oxygen was >5.0 mg/L.

2.3. Bacterial challenge

At 3-, 6- and 7-weeks post feeding (wpf), turbot larvae were challenged with Edwardsiella piscicida. The bacteria were cultured overnight in trypticase yeast broth (TYB) medium with 50 μg/ml Colistin (Col) at 30 °C, 200 rpm shaker. Then the bacteria were inoculated into fresh medium as the ratio of 1:100 and cultured overnight. The concentration of the bacteria was calculated by NanoDrop (Thermo, UAS). Thirty fish

| Table 1 | q-PCR primers used in this study. |
|---------|----------------------------------|
| Primers | Forward sequence (5′→3′) | Reverse sequence (5′→3′) |
| ii-1β  | CTCCCAACCGCTGCTACTCACG       | GGCAGACTGCATCTCATGAGC |
| ii-6   | CTCCTAACGGTGCTCCTACA          | GAAAGCAGTCACACGACTCC |
| mfsA   | CCGCTACCGGTCCTGACGCA          | GAGTACAGGATATCTATGTT |
| hk1    | AGATATGACCGTGCTGCTG           | CATGCCGCTACACATTTCCT |
| pfk    | GCACCTTTCCTCCTTGTAGAT         | ATGGTATCTGTGAGGTTGG |
| ldh    | ATGCTCTTTCCTGCTTTG           | TCATCCTCAGGGGATAGG |
| gapdh  | AAGGCCATTCTGCGATAC             | CATGACACACGCCTGACAAAG |

Fig. 1. Poly(I:C) feeding elevates the gene expression of innate immune responses during bacterial infection. (A) Schematic of the poly(I:C) feeding trial and experimental design. (B) q-PCR analysis of cytokine genes (including ii-1β, ii-6 and mfsA) and metabolic enzymes related genes (including hk1, pfk and ldh) transcripts in different tissues (including gill, intestine, liver, spleen and kidney) of infected turbot larvae (including FD-H, FD-L and FD-N group) at 3dpi. Data are presented as mean ± SD of three independent experiments; ns, no significance, * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.
from each group were randomly picked and immersed with $2 \times 10^8$ CFU/ml bacteria for 2 h in 6-L tank. The survival rate of turbot larvae was observed every day after bacterial challenge.

2.4. Bacteria enumeration

After 3 wpf, nine larvae from corresponding groups were randomly picked at 7 dpi of bacterial challenge and divided triplet, the tissues were collected and sufficiently grinded in 200 $\mu$l PBS. Then pipetting 20 $\mu$l grinding fluid to 180 $\mu$l PBS for a 10-fold dilution and series dilution were prepared in the same way. For bacterial enumeration, 10 $\mu$l of serial diluents were plated on the deoxycholate hydrogen sulfide lactose (DHL) agar plate (Col$^+$) and enumerated the black colonies after culturing in 37 $^\circ$C incubator for 24 h.

2.5. RNA isolation and q-PCR

After 3 wpf, nine turbot larvae were randomly picked from corresponding groups after bacterial challenge and divided triplet, the tissues, including gill, intestine, liver, spleen and kidney, were collected from the larvae and placed into 200 $\mu$l RNAstore Reagent (Tiangen, China) and stored at $-80^\circ$C. RNA of individual sample was isolated by Trizol (Invitrogen, USA) and chloroform. The cDNA was synthesized by FastQuant RT Kit (Tiangen), then analyzed the gene expression with QuantStudio 3 (Thermofisher, USA) by using SuperReal PreMix Plus (SYBR Green) (Monard, China) according to procedures. The results were analyzed by $2^{-\Delta\Delta C_{t}}$ method. Primers used in this study were listed in Table 1. The reference gene was gapdh.

2.6. MDA, SOD and CAT activity assay

After 3 wpf, nine turbot larvae were randomly picked at 7 dpi of bacterial challenge and divided triplet, the tissues including intestine, liver and spleen were collected and added 200 $\mu$l RIPA lysis buffer (Beyotime, China) to lyse the tissues by grinding. Total protein concentration in different tissues were measured by Enhanced BCA Protein Assay Kit (Beyotime). The contents of superoxide dismutase (SOD), catalase (CAT) and Malondialdehyde (MDA) were measured through commercial assay kits (Beyotime) according to manufacturer’s recommendations. The absorbance of the reaction mixture was measured by Spectra Max M5 micro plate reader (Molecular Devices, USA).

2.7. LZM activity assay

Lysozyme (LZM) activity was measured by Micrococcus Lysodeikticus Cells (Sangon Biotech, China). The Micrococcus Lysodeikticus Cells suspension was prepared by PBS and the final concentration was 0.2 g/L. 200 $\mu$l suspension and 10 $\mu$l cell lysis solution or 10 $\mu$l RIPA lysis buffer was added to 96-well plate and immediately mixed by pipetting. Lysozyme activity was calculated through the decrease of absorbance in A$_{450}$ nm within 3 min, using the formula applied in previous study [15]: $U = \left(\frac{\Delta A_{450 \text{nm}}}{3/0.001}\right) \times \text{dilution ratio}$.

2.8. Histopathological analysis

After 3 wpf and 7 wpf, the tissues including liver, spleen and intestine from 3 fish at 7 dpi in different groups were randomly collected and fixed with 4% paraformaldehyde (PFA), then dehydrated using ethanol and vitrificated by dimethylbenzene. The organs were embedded in paraffine blocks and sliced with paraffine slicing hematoxylin and eosin (H&E) staining and stereomicroscopy were used for histopathological observation.

2.9. Statistical analysis

The results were performed by Prism 7.0 (Graphpad, USA). The data were presented as mean $\pm$ SD with three independent experiments. The statistical significance was analyzed by two-way ANOVA or Students’ t-
3. Results

3.1. Poly(I:C) feeding elevates the gene expression of innate immune responses during bacterial infection

To evaluate the protective effects of poly(I:C) as dietary additive for turbot, we performed a 4-weeks feeding trial with different dose of poly(I:C) (FD-L and FD-H) in turbot larvae, then the bacterial challenge experiments were performed after 3 weeks of resting period (Fig. 1A). We analyzed the expression of several inflammatory cytokines (including il-1β, il-6 and tnf-α) in different tissues at 3 dpi, as well as the enzymes involved in aerobic glycolysis pathways (including hk1, pfk and ldh) which has been reported to mediate immune response through enhancing the transcription of cytokines [16]. We found that the transcriptional levels of metabolic genes (hk1, pfk and ldh) in FD-H and FD-L larvae were remarkably up-regulated in all immuno-organs, comparing with that in FD-N larvae (Fig. 1B). Meanwhile, the expression of inflammatory cytokines (il-1β, il-6 and tnf-α) in FD-H and FD-L larvae were also dynamically up-regulated in most of the immuno-organs, but not in intestine (Fig. 1B). Taken together, these results suggest that poly(I:C) feeding could enhance the innate immune response during bacterial infection in turbot.

3.2. Poly(I:C) feeding elevates the anti-oxidative and bactericidal enzymatic activity

Since the cytotoxic malondialdehyde (MDA) could be accumulated during oxidative stress, while the superoxide dismutase (SOD) and catalase (CAT) could inhibit the overwhelming of oxidative stress [17], we further analyzed the anti-oxidative stress and bactericidal enzymatic activity during bacterial challenge with/without poly(I:C) feeding in turbot. We found that the content of MDA in the liver of FD-H and FD-L larvae was significantly less than that in FD-N larvae (Fig. 2A). Moreover, the enzyme activity of SOD and CAT in the spleen of FD-H and FD-L larvae were apparently higher than that in FD-N larvae, but not in tested liver and intestine (Fig. 2B and 2C). In addition, the LZM activity in different immuno-organs of FD-H and FD-L larvae are all comparatively higher than that in FD-N larvae, especially in intestine (Fig. 2D). These results suggest that poly(I:C) feeding could improve the anti-oxidative stress activity in liver and spleen, and elevate the bactericidal activity in intestine.

3.3. Poly(I:C) feeding alleviates the immuno-organ injury and strengthen the anti-infection ability

Furthermore, we analyzed the histopathological changes in different immuno-organs, and found that the liver vacuolization caused by oxidative stress after bacterial infection was obviously alleviated in FD-
H and FD-L larvae, which is consistent with the result in Fig. 2A. Besides, the inflammatory infiltration in spleen and intestine was also relieved in FD-H and FD-L larvae compared to FD-N larvae, and intestinal microvilli in FD-H larvae were more intact and thicker than that in FD-N larvae (Fig. 3A). Moreover, to verify whether poly(I:C) feeding could improve the anti-bacterial effects in turbot, we analyzed the bacterial infection-induced survival rate and bacterial loads, and found that the survival rate in FD-H and FD-L larvae were higher than that in FD-N larvae (Fig. 3B), and the bacterial colonization in intestine and gills were significantly decreased in FD-H and FD-L larvae (Fig. 3C). Taken together, these results suggest that poly(I:C) feeding could play an important role in maintaining intestinal integrity, which is essential for anti-bacterial infection in turbot.

3.4. Poly(I:C) feeding induces long-term effects in protecting turbot from bacterial infection-induced white feces

Based on the results above, we further analyzed the long-lasting protective effects of poly(I:C) feeding in turbot, through extending the resting period of poly(I:C) feeding to 6- and 7-weeks post administration (Fig. 4A). Firstly, The intestinal white feces [18], a common symptom of enteritis caused by bacterial infection, was detected at 14 dpi to evaluate the long-term effects of poly(I:C) feeding. We found that bacterial infection caused white feces in hindgut was significantly decreased in FD-H and FD-L larvae, until 6-weeks post poly(I:C) feeding (Fig. 4B and 4C). However, at 7-wpf, although the bacterial infection-induced white feces in FD-H and FD-L larvae exhibited no difference with FD-N larvae, the intestinal histopathological changes in FD-H larvae remains more intact, and exhibits less inflammatory infiltration, comparing with that in FD-N larvae (Fig. 4D). Furthermore, the bacterial loads in FD-H and FD-L larvae remain less than that in FD-N larvae during E. piscicida infection at 7-wpf (Fig. 4E). Taken together, these results suggest that poly(I:C) feeding as dietary additive is an effective way to keep turbot larvae from bacterial infection with a long-term protection.

4. Discussion

Mucosal immunity plays critical roles in immune defense of fish. Mucosal tissues including gills, skin and intestine, are the important immune barriers against microbial infection [19]. Among these organs, the intestinal health of fish is a major concern in aquaculture. Intestinal oxidation stress and inflammatory injury would cause high mortality in farmed fish [20], thus maintaining the homeostasis of intestine is crucial for healthy farming. Intact intestinal barrier could prevent the bacteria invade into the circulation and cause systemic infection [21], and intestinal epithelium could release antimicrobial peptides and alarmins.
onto the mucosal surface to kill the microbial directly or recruit the immune cells [22]. Moreover, the intestinal microbiota might also contribute to antibacterial infection process [23]. In our study, we found that feeding with poly(I:C) could not only elevate the immune response in peripheral immune organs, including liver and spleen, but also alleviate the intestinal oxidation stress and inflammatory symptoms caused by bacterial infection in mucosal organs. These results suggest that poly(I:C) feeding could effectively activate the mucosal immunity in turbot, which provide a feasible strategy to maintain the intestinal health of aquaculture animals. However, the molecular mechanisms of poly(I:C) feeding induced mucosal immune responses remains to be elucidated.

Trained immunity (TI), a kind of innate immune memory has been increasingly recognized in recent years [24]. Specifically, the stimuli, including β-glucan and bacille Calmette-Guerin (BCG), could be recognized by the cell membrane receptors (e.g., dectin-1 and NOD2) to activate the host innate immunity through epigenetic and metabolic reprogramming, exhibiting a long-term, non-specific immune responses and disease resistance of Nile tilapia (Oreochromis niloticus L.), J. Fish Dis. 41 (2018) 1579–1588.

Acknowledgements

This work was supported by the Natural Science Foundation of Shanghai (20ZR1415500), the Innovation Group Project of Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai) (311020005), and the Fundamental Research Funds for the Central Universities (222020112729).

References

[1] D.R. Davies, Polyinosinic plus polycytidylic acid: a crystalline polynucleotide complex, Nature 186 (1960) 1030–1031.
[2] M. Matsumoto, T. Seya, TLR3: interferon induction by double-stranded RNA including poly(I:C), Adv. Drug. Deliv. Rev. 60 (2008) 805–812.
[3] R. Ammi, J. De Waede, V. Willemsen, I. Van Brussel, D.M. Schrijvers, E. Lion, E. L. Smits, Poly(I:C) as cancer vaccine adjuvant: knocking on the door of medical breakthroughs, Pharmacol. Ther. 146 (2015) 120–131.
[4] A.M. Hafner, B. Correthny, H.P. Merkle, Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant, Adv. Drug. Delivar. Rev. 65 (2013) 1386–1399.
[5] M.J. Oh, I. Takami, T. Nishihara, W.S. Kim, C.S. Kim, S.R. Kim, M.A. Peak, Field tests of Poly(I:C) immunization with nervous necrosis virus (NNV) in sevenbend grouper, Epinephelus septemfasciatus (Thunberg), J. Fish Dis. 35 (2012) 187–191.
[6] A. Rayra, D. Torralba, D. Morera, L. Tort, S. Mackenzie, N. Roher, Zebrafish liver (ZFL) cells are able to mount an anti-viral response after stimulation with Poly (I:C), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 182 (2015) 55–63.
[7] Q. Li, M. Wu, K. Cui, S. Zhu, K. Mai, Q. Ai, Characterization of antiviral immune response induced by poly(I:C) in macrophages of farmed large yellow croaker, Fish Shellfish Immunol. 104 (2020) 663–672.
[8] X. Wang, Q. Wang, J. Xiao, Q. Liu, H. Wu, L. Xu, Y. Zhang, Edwardsiella tarda TSS component epp1 is regulated by ear1 and iron, and plays essential roles in the invasion of fish, Fish Shellfish Immunol. 27 (2009) 469–477.
[9] P. Wang, J. Li, T.T. He, N. Li, Z.L. Mo, P. Nie, H.L. Xie, Pathogenic characterization of Aeromonas salmonicida subsp. marcula turbot isolate from China, J. Fish Dis. 43 (2020) 1145–1154.
[10] Y. Gao, H. Wu, Q. Wang, J. Qu, Q. Liu, J. Xiao, Y. Zhang, A live attenuated combination vaccine evokes effective immune-mediated protection against Edwardsiella tarda and Vibrio anguillarum, Vaccine 32 (2014) 5937–5944.
[11] Y. Wang, Q. Wang, W. Yang, Y. Liu, B. Zhang, Functional characterization of Edwardsiella tarda twin-arginine translocation system and its potential use as biological containment in live attenuated vaccine of marine fish, Appl. Microbiol. Biotechnol. 97 (2013) 3554–3557.
[12] F.Y. Yamamoto, F.J. Satulii, M. Hume, D.M. Gatlin 3rd, The effect of beta-1,3-glucan derived from Dugene gralicis (AlgamuneTM) on the innate immune responses of Nile tilapia (Oreochromis niloticus L.), J. Fish Dis. 41 (2018) 1579–1588.
[13] P. Walsamitra, M.A. Zoral, A. Saengtienchai, A. Luengnaruemitchai, O. Decamp, B. Gorgoglione, W. Surachetpong, Probiotics modulate tilapia resistance and immune response against tilapia lake virus infection, Pathogens 9 (2020) 919.
[14] N. Sheikhzadeh, S. Mousavi, G. Hamidian, M. Firouzamandi, A.K. Osshani, K. Mardani, Role of dietary Spirulina platensis in improving mucosal immune responses and disease resistance of rainbow trout (Oncorhynchus mykiss), Aquaculture 510 (2019) 1–8.
[15] J.F.A. Koch, C.A.F. de Oliveira, F.S. Zanuzu, Dietary beta-glucan (MacroGard®) improves innate immune responses and disease resistance in Nile tilapia regardless of the administration period, Fish Shellfish Immunol. 112 (2021) 56–63.
[16] S.C. Cheng, J. Quintin, R.A. Cramer, K.M. Sheppardson, S. Sared, V. Kumar, E. J. Giamarellos-Bourboulis, J.H. Martens, N.A. Rao, A. Aghajani, G.R. Manjeri, Y. Li, D.C. Iirim, R.J. Arts, B.M. van de Veer, P.M. Deen, C. Logie, E. O'Neill, P. Willems, F.L. van de Veerdonk, J.W. van der Meer, A. Lopez Nadal, W. Ikeda-Ohtsubo, D. Sipkema, D. Peggs, C. McGurk, M. Forlenza, C. G.F. Wiegertjes, S. Brugman, Feed, microbiota, and gut immunity: using the zebrafish model to understand fish health, Front. Immunol. 11 (2020) 114.
[17] H. Schug, Y. Yue, R. Krese, S. Fischer, T.M. Kortner, K. Schirmer, Time- and concentration-dependent expression of immune and barrier genes in the RTgutGC zebrafish model to understand fish health, Front. Immunol. 11 (2020) 114.
[18] J.M. Allaire, S.M. Crowley, H.J. Ko, B.A. Vallance, The intestinal epithelium: central coordinator of mucosal immunity, Trends Immunol. 39 (2018) 677–696.
[19] Y.A. Yap, E. Marino, An insight into the intestinal web of mucosal immunity, microbiota, and diet in inflammation, Front. Immunol. 9 (2018) 2617.
[20] M.G. Netea, J. Quintin, J.W. van der Meer, Trained immunity: a memory for innate infection affords protection against reinfection via functional reprogramming of T cells, Cell Host Microbe 23 (2018) 89–98.
[21] J. Wiegertjes, S. Brugman, Feed, microbiota, and gut immunity: using the zebrafish model to understand fish health, Front. Immunol. 11 (2020) 114.
[22] X. Luo, Y. Wang, X. Sun, Correlation between bacteria in feed pellets and diseases of cultured turbot Scophthalmus maximus, South China Fish. Sci. 5 (2009) 13–21.
[23] T. Somamoto, T. Nakanishi, Mucosal delivery of fish vaccines: local and systemic immune following mucosal immunisations, Fish Shellfish Immunol. 99 (2020) 199–207.
[24] A. Lopez Nadal, W. Ikeda-Ohtsubo, D. Sipkema, D. Peggs, C. McGurk, M. Forlenza, G.F. Wiegertjes, S. Brugman, Feed, microbiota, and gut immunity: using the zebrafish model to understand fish health, Front. Immunol. 11 (2020) 114.
[25] H. Schug, Y. Yue, R. Krese, S. Fischer, T.M. Kortner, K. Schirmer, Time- and concentration-dependent expression of immune and barrier genes in the RTgutGC fish intestinal model following immune stimulation, Fish Shellfish Immunol. 88 (2019) 308–317.
[26] J.M. Allaire, S.M. Crowley, H.T. Law, S.Y. Wang, H.J. Ko, B.A. Vallance, The intestinal epithelium: central coordinator of mucosal immunity, Trends Immunol. 39 (2018) 677–696.
[27] A. Lopez Nadal, W. Ikeda-Ohtsubo, D. Sipkema, D. Peggs, C. McGurk, M. Forlenza, G.F. Wiegertjes, S. Brugman, Feed, microbiota, and gut immunity: using the zebrafish model to understand fish health, Front. Immunol. 11 (2020) 114.
[28] H. Schug, Y. Yue, R. Krese, S. Fischer, T.M. Kortner, K. Schirmer, Time- and concentration-dependent expression of immune and barrier genes in the RTgutGC fish intestinal model following immune stimulation, Fish Shellfish Immunol. 88 (2019) 308–317.