Fish and Shellfish Immunology 84 (2019) 120–123

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

Adjuvant effects on protection and immune response of Japanese flounder immunized by the formalin-killed cells of *Edwardsiella tarda*

Hidehiro Kondo*[,1], Seangmin Chung[1], Eriko Hirosawa, Ikuo Hirono

Laboratory of Genome Science, Graduate School of Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku, Tokyo, 108-8477, Japan

ARTICLE INFO

Keywords:
Edwardsiellosis
Freund's adjutant
Paralichthys olivaceus
Vaccine

ABSTRACT

We evaluated the effects of Freund's adjuvants (FCA/FIA) on protection and immune response of Japanese flounder *Paralichthys olivaceus* immunized by the formalin-killed cell (FKC) of *Edwardsiella tarda*. Combination of FKC and FCA/FIA did not confer protection against the challenge, while they significantly induced higher antibody titers than that of FKC only. The suppression of FKC-dependent induction of interferon γ (IFNγ) mRNA levels by FCA/FIA was observed by gene expression profiling. Similarly, interleukin (IL)-12 p35 mRNA levels were not detected after FKC + FCA or + FIA. These results suggest that the mineral oil in Freund's adjuvants might suppress the signaling pathway(s) that induce IFNγ and IL-12 gene expression.

1. Introduction

Edwardsiellosis causes economic losses in many cultured fish species in Japan including Japanese eel *Aguilla japonica* [1], Japanese flounder *Paralichthys olivaceus* [2] and Red sea bream *Pagrus major* [3]. The causative agent, *Edwardsiella tarda*, is an intracellular pathogen. The bacteria can survive in phagocytes and resist against bactericidal activities of the host defense system [4–6].

The formalin-killed cells (FKC) vaccine does not protect Japanese flounder against edwardsiellosis. Mekuchi et al. [7] reported that the mortality of the fish immunized with the FKC, extracellular products or intracellular components of the bacteria was similar to the mortality of unimmunized fish. The relative percent survivals (RPSs) of Japanese flounder vaccinated with an attenuated strain or a natural avirulent isolate were significantly high [8,9]. On the other hand, vaccination with five avirulent strains provided only limited protection [10]. For example, the RPS obtained with one of these strains was only 45%. Therefore, the effectiveness of a vaccine can strongly depend on the strain. Moreover, some subunit- or DNA vaccines against bacteria provided protection against bacterial challenge [11].

In aquaculture, vaccine adjuvants can modify the immune responses of fish. They may enhance antibody responses and provide a long-lasting protection against diseases. Freund's complete/incomplete adjuvants (FCA/FIA) have been extensively used in the immunological studies, in which they have been shown to stimulate both humoral and cell-mediated adaptive immune responses [12]. FCA is a moisture of a mineral oil with heat-killed *Mycobacteria* cells, while FIA lacks the *Mycobacteria* cells. In *E. tarda* infection in Japanese flounder, a recombinant protein of major antigen Et49 combined with FIA showed an RPS of 81%, although the antigen by itself had an RPS of only 34% [13]. In contrast, FCA/FIA had only limited effects in other fish species [12].

In order to develop an effective vaccine by using adjuvants, it is necessary to understand how the adjuvants modulate fish immune responses. Here we elucidated the effects of the FCA/FIA on protection and immune response of Japanese flounder immunized by the *E. tarda* FKC. The adjuvants strongly enhanced antibody responses 2 weeks post infection (w.p.i.). A microarray analysis revealed that FCA/FIA suppressed the upregulation of interferon-γ mRNA levels.

2. Materials and methods

2.1. Fish sampling and challenge test

Fish, average total length of 12 cm, were intraperitoneally injected with phosphate buffered saline (PBS), 1×10^9 cells of FKC of *E. tarda* (strain NUS806), and 1×10^9 cells of FKC mixed with FCA (FKC + FCA) or that with FIA (FKC + FIA). The injected fish were reared at 18 °C, and then spleen was sampled at 6 h and on 3 d after immunization. The blood was taken from the fish at 2 w.p.i. to be assessed by ELISA. Ten of the fish were intraperitoneally challenged with 1.5×10^5 cells of *E. tarda* at 4 weeks after immunization and mortality was recorded.

* Corresponding author. Laboratory of genome science, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku, Tokyo, 108-8477, Japan.
E-mail address: h-kondo@kaiyodai.ac.jp (H. Kondo).
[1] Equally contributed.

https://doi.org/10.1016/j.fsi.2018.09.071
Received 30 May 2018; Received in revised form 19 September 2018; Accepted 25 September 2018
Available online 27 September 2018

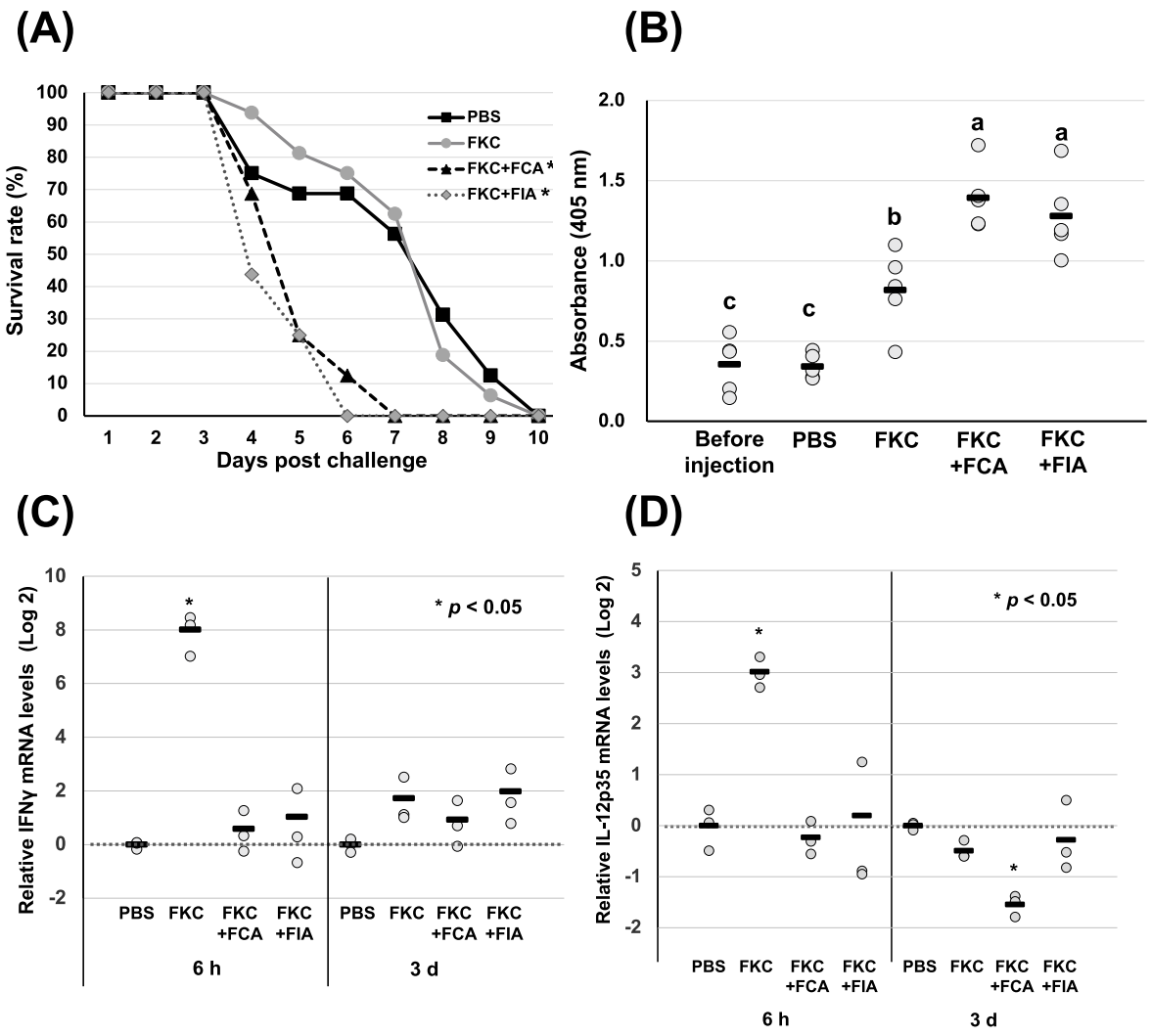

Fig. 1. (A) Cumulative survival of vaccinated fish after the challenge with *E. tarda*. (B) Antibody titers at 2 weeks after vaccination with FKC, FKC + FCA or FKC + FIA. Serum samples were collected from 5 fish/group, diluted 1:20, and then analyzed by ELISA. Each point represents an individual fish and black bar indicate the average of each group. Different letters represent significant differences as determined by Tukey's HSD ($P < 0.05$). (C, D) Expressions of IFNγ (C) and IL-12p35 (D) genes in the spleen from 3 fish/group at 6 h and on 3 d after PBS, FKC, FKC + FCA and FKC + FIA injection. Expression is expressed as Log2 values, relative to the average of the PBS group. Each point represents an individual fish and black bar indicate the average of each group. Asterisk indicates a significance difference by one-way ANOVA ($P < 0.05$).

2.2. Antibody titration using ELISA

The antibody activities were measured by the method of Taechavasonyoo et al. [14]. Briefly, the ELISA plate (Thermo) was prepared by placing 10^9 cells of the FKC in 100 µl per well and incubated at 4 °C overnight. The wells were washed three times with T-PBS (0.05% Tween 20 in PBS), covered with 100 µl of blocking solution (3% Skim milk) at room temperature for 2 h, washed three times with T-PBS, treated with 100 µl of primary fish antisera diluted 20-times with PBS, incubated at room temperature for 1 h, washed three times with T-PBS, reacted with 1:3000 of anti-Japanese flounder IgM rabbit antiserum [14], washed three times with T-PBS, incubated with 1:1000 of anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) to detect the rabbit IgG, washed five times, incubated with phosphatase substrate at room temperature for 30 min, and mixed with 50 µl of 3M NaOH to stop the reaction. The absorbance at 405 nm was measured with Multiskan FC Microplate Photometer (Thermo Scientific, Japan).

2.3. Gene expression profiling by microarray

Gene expression was profiled by the method of Kaneshige et al. [15]. Total RNA was extracted from spleen samples (n = 3) using RNAiso Plus (Takara Bio, Japan) and purified with an RNeasy® Mini Kit (Qiagen, USA). RNA quality was evaluated using a 2200 TapeStation (Agilent, USA). The microarray was analyzed using three samples in each group, and each sample was selected based on RNA quality. cDNA was synthesized from 200 ng of RNA and labeled with cyanine 3-CTP using a one-color microarray-based gene expression analysis following instructions of the manufacturer (Agilent, USA). The labeled cDNA was subsequently hybridized at 65 °C for 17 h.

Microarrays were scanned with an Agilent G2565CA Microarray Scanner, and the images obtained from scanning were analyzed with Feature Extraction Software v9.5.3.1 (Agilent, USA). The data were normalized and analyzed using limma [16]. The fold-change differences were calculated against the average of those before treatments. Genes with a fold change of at least 8.0 and p value less than 1e-5 were considered to be differentially expressed.

Table 1
List of cytokines up-regulated after the treatments.

			FKC 6h		FKC+FCA 6h		FKC 3d		FKC+FCA 3d	
Probe ID	Acc. No.	Product name	Log2FC	P_value	Log2FC	P value	Log2FC	P value	Log2FC	P value
CUST_12226_PI426483990[a]	AB427185.1	CC chemokine-like	4.8	6.1E-06	4.7	7.6E-06	1.4	9.4E-02	1.6	9.9E-03
CUST_12932_PI426483990[a]	AB427185.1	CC chemokine-like	6.4	3.3E-06	5.9	6.8E-06	2.4	1.8E-02	1.6	9.9E-03
CUST_351_PI426483993[a]	AB427185.1	CC chemokine-like	6.0	6.2E-05	5.5	1.5E-04	1.8	1.8E-01	2.1	9.2E-03
CUST_48_PI426483993[b]	BAG50577.1	Interferon gamma	6.4	3.9E-07	1.9	1.6E-02	2.1	8.8E-03	2.6	4.1E-02
CUST_49_PI426483993[b]	BAG50577.1	Interferon gamma	5.9	3.1E-06	1.3	1.7E-01	1.3	1.9E-01	4.2	1.3E-03
CUST_9251_PI426483990[b]	BAG50577.1	Interferon gamma	5.6	4.3E-06	1.2	2.0E-01	1.2	1.9E-01	3.2	7.2E-03
CUST_7075_PI426483990[c]	AB720983.1	Interleukin 1 beta	5.8	3.7E-05	5.6	5.5E-05	2.7	2.3E-02	2.6	4.1E-02
CUST_9569_PI426483990[c]	AB720983.1	Interleukin 1 beta	7.4	5.9E-06	7.3	6.8E-06	3.9	2.2E-03	0.9	1.8E-01
CUST_290_PI426483993	DQ267937.1	Interleukin 6	4.1	4.9E-06	2.6	4.5E-04	0.6	3.3E-01	3.1	3.3E-04
CUST_10972_PI426483990[d]	BAF91496.1	M17 homologue	4.4	1.7E-06	5.3	2.7E-07	0.5	3.5E-01	2.2	1.6E-02
CUST_310_PI426483993[d]	BAF91496.1	M17 homologue	4.6	7.4E-07	5.6	1.2E-07	0.9	1.8E-01	2.5	5.2E-03

Probes indicated by the same alphabet encode the same gene products. The values were compared with those before the treatments, and the Log2 transformed fold-change values (Log2FC) and P value were represented. both Log2FC $>$ 3.0 and P value $<$ 1.0E-05 were indicated by gray.

2.4. IFNγ and IL-12p35 genes expression profiling by qPCR

The primers for the IFNγ, IL-12p35 and elongation factor 1 alpha (EF-1α) genes are shown in Supplemental Table. Total RNA was extracted from the spleen of each sample (n = 3) using RNAiso (Takara, Japan) and 2 μg of total RNA was used for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The cDNA samples were diluted 5-times with distilled water. qRT-PCR was performed using 5 μl cDNA in a THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) using an ABI7300 Real-time PCR system (Applied Biosystems, USA). The expression levels of the target genes were normalized to the expression level of an internal control gene (EF-1α) and the relative mRNA levels were calculated by the 2-ΔΔCt method [17], and standardized to the average value of the PBS-injected group on the corresponding sampling points. The significance of differences between values on the standard and the others were evaluated by ANOVA followed by Dunnett's post hoc test.

3. Results and discussion

3.1. Effects of Freund's adjuvants on FKC vaccine

Fish mortality was observed on day 4 after the challenge (Fig. 1A). All of the fish immunized with FKC + FCA died by 7 days after the challenge and all of the fish immunized with + FIA died by 6 days after the challenge. Unimmunized fish and fish immunized with FKC survived longer than those immunized with FKC + FCA and + FIA. Jiao et al. [13] reported that the relative percent survival (RPS) of fish immunized with an antigen with FIA was 81%, and that the RPS of fish immunized with only the antigen was 34%. Tang et al. [18] also claimed that the antigens mixed with FIA showed higher RPS than the controls. In this study, we used FKC but not certain antigens, and thus the immune-response after the vaccination might be different from the response of those immunized with antigens mixed with FIA.

3.2. Effects of Freund's adjuvants on the antibody titer

Antibody levels in fish immunized with FKC, FKC + FCA or FKC + FIA were significantly higher than those in the PBS group (Fig. 1B). Moreover, the FKC + FCA and FKC + FIA groups showed higher antibody levels than the FKC group. Although the antibody levels in the fish immunized with FKC + FCA and + FIA were higher than those in the other groups, these fish were susceptible to the bacterial challenge (see Fig. 1A). The specific antibody, which opsonize the bacteria, enhance adhesion of *E. tarda* to phagocytes, where the bacteria is able to survive and replicate [6]. Therefore, the specific antibodies elicited by the FKC with the adjuvants did not confer to the protection against the bacteria.

3.3. Gene expression profiles after the vaccination

The gene expression profiles in the spleen were compared between fish immunized with FKC + FCA and fish immunized with only FKC. The microarray data were deposited into the gene expression omnibus database under accession number GSE111115. In the samples at 6 h after FKC immunization, the intensities of 222 spots were at least 8- fold higher ($p < 10^5$) than they were before immunization, while in the samples at 6 h after FKC + FCA immunization, the intensities of 213 spots were at least 8- fold higher. Similarly, the intensities of 214 spots were higher at 3 d after immunization. The mRNA levels of some cytokines (CC chemokine, interleukin (IL)-1β and IL-6) increased at 6 h in the FKC immunized group (Table 1). Almost all of the spots showed similar patterns between the FKC and FKC + FCA immunization (data not shown). In Japanese flounder, the genes induced by the injection of certain bacterial FKC were not significantly different, while the magnitude of up- or down regulation was distinct between the bacterial species [19]. Although mycobacteria cell components in FCA have been thought to influence gene expression in mammals [20,21], they may have limited effects in fish.

Only the intensities of the spots corresponding to interferon γ (IFNγ) were significantly higher in the FKC-immunized fish, but they were not significantly different in the FKC + FCA group (Table 1). It should be noted that only the IFNγ gene was differently regulated between FKC and FKC + FCA groups. The IFNγ mRNA level at 6 h after FKC immunization was significantly higher than it was in the other groups, while the levels at on 3d were not significantly changed (Fig. 1C). The IFNγ mRNA levels at 6 h also did not increase in FKC + FIA group. Therefore, regulation of the IFNγ gene expression might be caused by mineral oil forming an emulsion but not by the cellular components of mycobacteria.

In mammals, interleukin (IL)-12 upregulates IFNγ transcription. IL-

12 is an inflammatory cytokine, composed of p35 and p40 subunits [22,23]. Because the microarray did not have spots for the IL-12 subunits, we further investigated changes IL-12p35 subunit mRNA levels by qPCR. As in the case of IFNγ, the IL-12p35 mRNA level at 6 h after FKC immunization was significantly higher than the other groups (Fig. 1D). Interestingly, at 3 d, the IL-12p35 mRNA level was significantly lower in the FKC + FCA-immunized fish than in the control. In the amberjack, FKC vaccine with recombinant IL-12 showed effective against the intracellular bacterium *Nocardia seriolae* [24]. IL-12 and IFNγ regulate the balance between the Th1 and Th2 responses, which act against intracellular bacteria and regulate antibody production, respectively [22,23], and induce cell mediated immunity [24]. Therefore, the fish immunized with FKC + FCA/FIA, which showing lower IL-12 and IFNγ mRNA levels might show lower cell mediated immune responce.

In conclusion, adding Freund's adjuvants to the vaccines resulted in higher antibody titers and suppress the upregulation of IL-12 and IFNγ mRNA levels. Our data raise the possibility that the signaling pathways involved in IL-12 and IFNγ induction might be distinct from the other inflammatory pathways. Because IL-12 and IFNγ are important in the Th1 response, further studies are needed to characterize the signaling pathways regulating IL-12 and IFNγ expression to develop effective vaccines against intracellular bacteria.

Acknowledgements

This research was supported in part by grants from JSPS KAKENHI Grant Number 25292125.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.09.071.

References

[1] S. Egusa, Some bacterial diseases of freshwater fishes in Japan, Fish Pathol. 10 (1976) 103–114.
[2] T. Nakatsugawa, *Edwardsiella tarda* isolated from cultured young flounders, Fish Pathol. 18 (1983) 99–101.
[3] N. Yasunaga, S. Ogawa, K. Hatai, Characteristics of the fish pathogen *Edwardsiella* isolated from several species of cultured marine fishes, Bull Nagasaki Pref Inst Fish 8 (1982) 57–65.
[4] T. Iida, H. Wakabayashi, Resistance of *Edwardsiella tarda* to opsonophagocytosis of eel neutrophils, Fish Pathol. 28 (1993) 191–192.
[5] T. Miyazaki, N. Kaige, Comparative histopathology of Edwardsiellosis in fishes, Fish Pathol. 20 (1985) 219e27.
[6] P.S. Srinivasa Rao, T.M. Lim, K.Y. Leung, Opsonized virulent *Edwardsiella tarda* strains are able to adhere to and survive and replicate within fish phagocytes but fail to stimulate reactive oxygen intermediates, Infect. Immun. 69 (2001) 5689–5697.
[7] T. Mekuchi, T. Kiyokawa, K. Honda, T. Nakai, K. Muroga, Vaccination trails in the Japanese flounder against Edwardsiellosis, Fish Pathol. 30 (1995) 251–256.
[8] S. Cheng, Y.H. Hu, M. Zhang, L. Sun, Analysis of the vaccine potential of a natural avirulent *Edwardsiella tarda* isolate, Vaccine 28 (2010) 2716–2721.
[9] Y. Sun, C.S. Liu, L. Sun, Isolation and analysis of the vaccine potential of an attenuated *Edwardsiella tarda* strain, Vaccine 28 (2010) 6344–6350.
[10] T. Takano, T. Matsuyama, N. Oseko, T. Sakai, T. Kamaishi, C. Nakayasu, M. Sano, T. Iida, The efficacy of five avirulent *Edwardsiella tarda* strains in a live vaccine against Edwardsiellosis in Japanese flounder, *Paralichthys olivaceus*, Fish Shellfish Immunol. 29 (2010) 687–693.
[11] S.B. Park, T. Aoki, T.S. Jung, Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish, Vet. Res. 43 (2012) 67.
[12] C. Tafalla, J. Bøgwald, R.A. Dalmo, Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives, Fish Shellfish Immunol. 35 (2013) 1740–1750.
[13] X.D. Jiao, S. Cheng, Y.H. Hu, L. Sun, Comparative study of the effects of aluminum adjuvants and Freund's incomplete adjuvant on the immune response to an *Edwardsiella tarda* major antigen, Vaccine 28 (2010) 1832–1837.
[14] A. Taechavasonyoo, I. Hirono, H. Kondo, The immune-adjuvant effect of Japanese flounder *Paralichthys olivaceus* IL-1β, Dev. Comp. Immunol. 41 (2013) 564–568.
[15] N. Kaneshige, W. Jirapongpairoj, I. Hirono, H. Kondo, Temperature-dependent regulation of gene expression in Japanese flounder *Paralichthys olivaceus* kidney after *Edwardsiella tarda* formalin-killed cells, Fish Shellfish Immunol. 59 (2016) 298–304.
[16] M.E. Ritchie, B. Phipson, D. Wu, Y. Hu, C.W. Law, W. Shi, G.K. Smyth, Limma powers differential expression analyses for RNA-sequencing and microarray studies, Nucleic Acids Res. 43 (2015) e47.
[17] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[18] X. Tang, W. Zhan, X. Sheng, H. Chi, Immune response of Japanese flounder *Paralichthys olivaceus* to outer membrane protein of *Edwardsiella tarda*, Fish Shellfish Immunol. 28 (2010) 333–343.
[19] H. Kondo, Y. Kawana, Y. Suzuki, I. Hirono, Comprehensive gene expression profiling in Japanese flounder kidney after injection with two different formalin-killed pathogenic bacteria, Fish Shellfish Immunol. 41 (2014) 437–440.
[20] M.A. Behr, M. Divangahi, Freund's adjuvant, NOD2 and mycobacteria, Curr. Opin. Microbiol. 23 (2015) 126–132.
[21] E. Ishikawa, D. Mori, S. Yamasaki, Recognition of mycobacterial lipids by immune receptors, Trends Immunol. 38 (2017) 66–76.
[22] W.T. Watford, M. Moriguchi, A. Morinobu, J.J. O'Shea, The biology of IL-12: coordinating innate and adaptive immune responses, Cytokine Growth Factor Rev. 14 (2003) 361–368.
[23] S. Zundler, M.F. Neurath, Interleukin-12: functional activities and implications for disease, Cytokine Growth Factor Rev. 26 (2015) 559–568.
[24] M. Matsumoto, K. Araki, K. Hayashi, Y. Takeuchi, K. Shiozaki, H. Suetake, A. Yamamoto, Adjuvant effect of recombinant interleukin-12 in the Nocardiosis formalin-killed vaccine of the amberjack *Seriola dumerili*, Fish Shellfish Immunol. 67 (2017) 263–269.