Fish and Shellfish Immunology 90 (2019) 431–439

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Valuable method for production of oral vaccine by using alginate and chitosan against *Lactococcus garvieae/Streptococcus iniae* in rainbow trout (*Oncorhynchus mykiss*)

Mostafa Halimi[a,*], Mojtaba Alishahi[a], Mohammad Reza Abbaspour[b], Masoud Ghorbanpoor[c], Mohammad Reza Tabandeh[d]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
[b] Targeted Drug Delivery Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Department of Microbiology and Immunology, Faculty of Veterinary Medicine,Shahid Chamran University of Ahvaz, Ahvaz, Iran
[d] Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

ARTICLE INFO

Keywords:
Oral vaccination
Chitosan-alginate
Ionotropic gelation
Lactococcus garvieae
Streptococcus iniae
Rainbow trout

ABSTRACT

The effectiveness of ionotropic gelation method (by combining alginate and chitosan) vaccine against *Lactococcus garvieae* and *Streptococcus iniae* was examined in rainbow trout. Fish were separated into four groups and fed the distinctive examined feeds. Our groups were included: A) fish immunized by chitosan-alginate coated vaccine, B) fish immunized by non-coated vaccine, C) fish feed by chitosan-alginate coated pellets without vaccine and D) fish feed by basic diet (non-coated and without vaccine). In groups A and B, the vaccination was carried out for 14 days. Fish of group C, like groups A and B were fed 14 days with pellets covered with chitosan-alginate without vaccine and a short time later they were fed with control diet. On day 0, 20, 40 and 60 of the trial, serum samples were extracted. Fish were challenged with *L. garvieae* and *S. iniae* after 60 days of research. Innate immunity components containing complement activity, total protein and IgM appeared no significant changes nearly in all groups during the 60 days that the examination finished. Although, bactericidal activity and lysozyme activity demonstrated a significant increase on days 20, 40 and 60 in group A compared to control groups (C and D) ($P < 0.05$) and similar results about the blood respiratory burst activity just on days 20 and 40 were obtained. Also, the relative expression of IL-6 of group A, was significantly higher compared to all of other groups (B, C and D) on days 20 and 60 of experiment ($P < 0.05$). The same results were obtained about the relative expression of IgM. The serum ELISA antibody titer against *L. garvieae,* increased significantly on days 20 and 40 of experiment in fish immunized by chitosan-alginate coated vaccine (Group A) compared to control groups (C and D)($P < 0.05$) while the result of ELISA test against *S. iniae* was significantly higher on days 40 and 60 of experiment in group A compared to groups B, C and D ($P < 0.05$). After challenge with these two live bacteria (*S. iniae* and *L. garvieae*), a survival rates of 76.67 ± 5.77% (challenged with *S. iniae*) and 66.67 ± 5.77% (challenged with *L. garvieae*) were seen in group immunized with chitosan-alginate coated vaccine (Group A), which were higher than survival rates gotten in other trial groups ($P < 0.05$). The consequences of the present experiment show that the oral vaccination of rainbow trout with improved chitosan-alginate (via ionotropic procedure) (group A) properly secures this important fish against *Lactococcus garvieae* and *Streptococcus iniae.*

1. Introduction

Vast variety of diseases are of the fundamental constraining issues identified with serious culture of sea life forms. Amid the most recent decade, plague and sporadic of Gram-positive cocci have been accounted for from various parts of the world [1]. *Streptococcus iniae* and *Lactococcus garvieae* are known as two noteworthy types of streptococcal diseases in many fish species especially salmonid fishes. Streptococcosis and Lactococcosis illnesses are brought about by *Streptococcus iniae* and *Lactococcus garvieae* separately which as a rule rise when the water temperature is over 15 °C [1,2].

Lactococcosis is a sort of streptococcosis with a wide scope of

* Corresponding author.
E-mail addresses: m_halimi82@yahoo.com, m-halimi@phdstu.scu.ac.ir (M. Halimi).

https://doi.org/10.1016/j.fsi.2019.05.020
Received 16 March 2019; Received in revised form 8 May 2019; Accepted 9 May 2019
Available online 11 May 2019

clinical signs including anorexia, melanosis, dormancy, sporadic swimming, exophthalmia, injuries in the vascular endothelium, aggregation of ascitic liquid in peritoneal depression, surface (in the base of fins, opercula-bucal locale) and interior hemorrhages (in the swim bladder, intestine, liver, spleen and kidney) and furthermore rot in the liver and spleen [1,3–5]. It must be indicated that this pathogen causes genuine monetary misfortunes because of raised rates of mortality (up to half) and diminishing fish development rate. Like *Lactococcus garvieae*, there are numerous reports with respect to the flare-ups of *S. iniae* contamination with high death rates in aquaculture [6]. The clinical indications of *S. iniae* are most like *L. garvieae*, for example, anorexia, laziness, hemorrhage and exophthalmia [7].

It is very much perceived that vaccination could be a compelling methodology to immunize fish against pathogens [8–10]. Fish vaccines can be utilized by injection, immersion and oral administration. Typically, the injection and immersion techniques are favored for vaccination because of their high viability [11–14]. However, these techniques are not appropriate for broad aquaculture on account of taking care of pressure and high work costs. Conversely, the oral immunization is by all accounts a perfect option, since it is less tedious, more practical and permits mass vaccination of fish [11,15–17]. Notwithstanding, till now, oral vaccination has not been as viable as injection or immersion immunization.

Today, numerous investigations have concentrated on covering of oral vaccines to upgrade the efficacy of vaccine delivery [18–21]. In such manner, polymers have been effectively used to keep vaccines against the acidic situation of stomach [22]. Chitosan-alginate containers are of productive materials for covering of oral immunizations in aquaculture.

Chitosan is a biopolymer of glucosamine and N-acetyl glucosamine deposits, acquired from the losses of seafoods, for example, shrimp, crab shell and furthermore cell wall of fungi [23,24]. As regular polymer, nanoparticles of chitosan have indicated suitable adequacy in covering of oral fish vaccines [25–27]. Notwithstanding chitosan, alginate microparticles were likewise utilized for covering of an oral vaccine. Alginates are polysaccharides removed from brown seaweeds and have comprised of chains of ß-D-mannuronic (M) and ar-L-guluronic (G) acids [20,21,23].

In the present study, we have investigated for the first time a chitosan-alginate-coated oral vaccine against *Streptococcus iniae* and *Lactococcus garvieae* in rainbow trout. Our trial was trailed by estimating immunological components (bactericidal activity, lysozyme activity, total protein, alternative complement activity, blood respiratory burst activity, total Immunoglobulin, the expression of immune related genes (IL6: interleukin 6, and IgM) and antibody titer in sera from rainbow trout after immunization. Besides, fish were tested with live *S. iniae* and *L. garvieae* after vaccination to assess the viability of the vaccination.

2. Materials and methods

2.1. Preparation of bacteria

The bacterial strains of *Streptococcus iniae* and *Lactococcus garvieae* were gotten from contaminated fish of Dehdasht, Iran (these bacteria were utilized after PCR and sequencing) and developed in Trypticase Soy Broth (TSB; Difco) medium at 27 °C for 48 h. Formalin-killed *S. iniae* and *L. garvieae* (FKB) were prepared by Eko et al. [28] and Chu [29]. Briefly, formalin was added to a 48 h culture of the bacterium and after 3 h incubation at 35 °C, the suspensions were kept overnight at 4 °C. At that point, the cells were centrifugated (6500 g; for 30 min at 4 °C). After centrifugation, the suspensions were washed 3 times with phosphate buffered saline (PBS, pH: 7.4) and resuspended in PBS to get the final concentration of 1×10^{10} cells/ml. Additionally, an aliquot of each readied FKB was hatched on TSB agar at 27 °C for checking sterility. All FKB samples were put in storage at 4 °C until use.

2.2. Ionotropic gelation method

Alginate/chitosan nanoparticles were set up in a twostep strategy dependent on the ionotropic pre-gelation of polyanion with calcium chloride followed by polycationic crosslinking via a protocol earlier explained [30], modified by perfect pre-gelation stoichiometric proportion and time of drug association [31]. This instruction has been improved to better effect by our research team. Therefore, 7.5 ml of 18 mM calcium chloride solution was dropped for 60 min under 800 rpm into a measuring beaker containing 117.5 ml of a 0.63% (w/w) alginate solution. 5 ml of bacterial suspension (at concentration of 2.4×10^9 cells) was blended with the alginate solution before calcium chloride addition to give an alginate pre-gel. Then, 25 ml of chitosan solution (concentration: 0.07%, w/w) was poured dropwise into the pre-gel over 90 min. Nanoparticles were held with an extra mixing for 30 min to enhance curing, and separated by centrifugation (3000 g/45 min) at 4 °C. After preparation of the product, it was sprayed on pellets (Skretting, Italy, diameter = 1 mm).

2.3. Samples preparation for the scanning electron microscope

Scanning electron microscope (SEM) is utilized to evaluate the morphology and size distribution of dried microspheres. The freeze-dried microspheres were redistributed in distilled water and spilled into an aluminum stubs. Then, the solution was dried via air flow under encompassing conditions. In the long run, microspheres were covered with gold particles before dissecting by scanning electron microscope.

2.4. Preparing of samples for an optical microscope

After preparing *ionotropic* gelation, one drop of the item was exposed to an optical microscope (with 40 × 100 and 100 × 100 magnification) to assess the different quantities of bacteria and the thready structure of emulsification.

2.5. Experimental design

Fish were dispersed into 500 l tanks in four experimental treatments with three replicates. The treatments were: Group A) (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In groups A and B, fish were fed with vaccine for 14 days and afterward fed basic diet for 60 days. Like groups A and B, fish of group C were also supplemented 14 days with trial diets and afterward fed control diet. During the examination, fish were fed two times each day at a rate of 3% of body weight. Likewise, initial mean weights of fish were 10 ± 0.6 g, which achieve 50 ± 0.4 g after 60 days experiment.

2.6. Challenge experiment

The challenge test was done on day 60 post-vaccination by exposing fish of each exploratory group to doses of *S. iniae* (5×10^6 cfu/ml) and *L. garvieae* (4.7×10^5 cfu/ml) independently. The mortality rate was recorded during 10 days after challenge.

2.7. Immunological assays

In the present study, the defined immune factors were estimated on day 0, 20, 40, and 60 after the start of the investigation. The blood examining was applied utilizing an insulin syringe from caudal peduncle. Afterward, the blood samples were centrifuged (13,700 g for 10 min) to segregate the serum. Plasma samples were kept quickly at -80 °C until biochemical or immunological examines.

2.7.1. Complement activity

Alternative complement activity (ACH) was examined dependent on the hemolysis of rabbit red blood cells (RRBC) as indicated by the strategy for Yano [32] with a few adjustments. Briefly, sera were first diluted with Veronal buffer and 1% RRBC suspension was appended to each sample. After 24 h brooding at 4 °C, the samples were centrifuged at 3500 × *g* for 5 min and, 150 µl of supernatants were exchanged to the plates with flat bottom and the optical density of wells was perused at 540 nm utilizing an ELISA reader (Accu Reader, Taiwan).

2.7.2. Lysozyme activity

Serum lysozyme activity (U/ml) was estimated by turbidmetric examine strategy as indicated by Esteban et al. [33]. The strategy depended on lysis of a lysozyme-sensitive Gram-positive bacterium, *Micrococcus lysodeikticus* (Sigma, USA). Briefly, in the plate with flat bottom sera were added to a 0.15 mg/ml of *Micrococcus lysodeikticus* in 0.1 M acetate buffer (pH = 5) and the optical density of wells was perused after 3 min at room temperature (RT) at 540 nm utilizing an ELISA reader (Accu Reader, Taiwan). The reduce in absorbance of 0.001 min^{-1} was evaluated as one unit of Lysozyme activity.

2.7.3. Bactericidal activity

The bactericidal activity was assessed by Budino et al. [34] with a few alterations. Briefly, 50 µl of 10^6 cfu/ml of the previously mentioned bacteria was added to 25 µl of serum sample and 25 µl of sterile PBS. After 6 h hatching at RT, 50 µl MTT (dimethylthiazol-diphenyl tetrazolium bromid) (Sigma, M5655) was poured to suspension. At that point, the suspension was brooded at room temperature for 15 min. At last, the suitable bacteria (Formazan positive cells) were measured spectrophotometrically at 600 nm by an ELISA reader (Accu Reader, Taiwan).

2.7.4. Blood respiratory burst activity

The blood respiratory burst activity was tested dependent on the technique for Secombes [35]. For this goal, 100 µl of blood sample was first poured to 100 µl 2% NBT (Nitro Blue tetrazolium, Sigma) and hatched for 30 min at room temperature. Afterward, 100 µl of this mixture was added to 2000 µl dimethylformamide and after that centrifuged at 3000 rpm for 10 min. At last, the absorbance of the supernatant was perused by a spectrophotometer (Shimadzu, Japan) at 620 nm.

2.7.5. Total protein content and IgM

Total protein (mg/ml) was estimated by the convention recommended by Lowry et al. [36]. The measurement of IgM was estimated by Siwicki and Anderson [37] strategy. The process of sedimentation of immunoglobulin content was carried out by utilizing a 12% solution of polyethylene glycol (Sigma, USA).

2.8. Enzyme-linked immunosorbent assay (ELISA) antibody titers

An Enzyme-linked immunosorbent assay (ELISA) was performed utilizing sera samples from test groups to measure antibody titers against *L. garvieae* and *S. iniae* in accord with Shelby et al. [38]. The antibody test was done independently for each tested bacteria.

Briefly, ELISA plates (Nunc, Denmark) were independently covered with 50 µl sonicated *S. iniae* or *L. garvieae* (100 µg/ml) antigen at a 1:50 dilution in coating buffer (carbonate bicarbonate with pH = 9.6) for 18 h at 4 °C. By using phosphate-buffered saline (PBS) including 0.05% of Tween-20 (PBS-T), plates were washed and after that impeded with 2.5% skim milk (High media, India) which mixing with phosphate-buffered saline + Tween-20 (PBS-T) for 1 h at 25 °C.

Following blocking, plates were again washed with PBS-T (3 times). The serum samples of rainbow trout (100 µl *S. iniae* or *L. garvieae*) were then poured at a 1:25 dilution in PBS-T-S (phosphate-buffered saline +0.05% Tween-20 (PBS-T) containing 0.1% skim milk). The plates were kept at 25 °C for 90 min with shaking and then washed again as above. After washing, 100 µl of mouse monoclonal anti-rainbow trout immunoglobulin (prepared by prof. Seyfi in our faculty) diluted (1:7500) in PBS-T-S was added to wells of ELISA plates. The plates were shaken for 60 min and then washed with PBS-T for three times. Afterward, 50 µl of goat anti-mouse IgG HRP conjugate (Sigma-Aldrich) diluted (1:2500) in PBS-T-S was added to wells and incubated for 60 min. After washing with PBS-T, 50 µl TMB (3,3′, 5,5; -tetramethylbenzidine-H2O2) chromogen solution was added to wells and allowed to react at 25 °C for 10 min. Finally, the reaction was stopped with 50 µl 2 N H2SO4 and serum antibody titers read at 450 nm using a ELISA reader (Accu Reader, Taiwan).

2.9. Gene expression analysis of IgM and IL-6

2.9.1. Total RNA extraction and cDNA synthesis

Entire RNA substance of head kidney was extracted by the RNX™ Isolation Reagent (SinaClone Bioscience, Iran) in view of the manufacturer's guidelines. The quantification of RNA was done by spectrophotometry (Eppendorf, Germany) utilizing routine methods. RNA isolates with 260/280 nm absorption ratio > 1.8 were considered to synthesis of cDNA. Reverse transcription was performed with the YTA cDNA synthesis kit utilizing 10 µl of RNA and random hexamer dependent on the protocol of the manufacture (Yektatajhiz, Iran).

2.9.2. Primer design for qPCR

The primers of qPCR were structured dependent on gene sequence of IL6 and IgM in GenBank of NCBI database utilizing Gene Runner (version 6.) software. The sequence of designed primers has been displayed in Table 1. In order to standardization of expression levels, EF1α gene was utilized as reference gene.

2.9.3. Quantitative real-time PCR (qPCR)

To assess changes in the expression level of IgM and IL-6, real-time PCR was utilized using a Lightcycler® Detection System (Roche, USA). For reference gene the elongation factor 1 α (EF1α) was utilized. In Table 1 the sequences of primers applied in the present examination are appeared. Processes were performed in a 12.5 µl mixture containing 6.25 µl qPCRTM Green Master Kit for SYBR Green I®: 0.25 µl of per primer (200 nM), 3 µl cDNA (100 ng), and 2.25 µl water without nuclease (Yektatajhiz, Iran). The PCR convention utilized comprised of 5 min denaturation at 94 °C pursued by 45 cycles of 94 °C for 15 s, 60 °C for 30 s. Two individual reactions without cDNA or with RNA were carried out in parallel as controls. The levels of relative gene expression

Table 1
Sequences of oligonucleotide primers used for real-time PCR.

Gene	Accession number	qPCR primers Forward/Reverse	Amplicon (bp)
IgM	S63348	TACAAGAGGGAGACCGGAGGA CTTCCTGATTGAATCTGGCTAGTGGT	220
IL-6	NM_001124657	CCTTGCGGAACCAACAGTTTG CCTCAGCAACCTTCATCTGGTC	187
EF1α	XM_020500543	CAAGGATATCCGTCGTGGCA ACAGCGAAACGACCAAGAGG	205

were calculated utilizing the comparative threshold cycle ($2-^{\Delta\Delta CT}$) strategy and Lightcycler 96® software. Approval of test to watch that the primer for the IgM and IL-6 had comparable enhancement efficiencies was executed as portrayed beforehand [39]. The analyses of all qPCR were applied according to procedure of Bustin et al. [40].

2.10. Data analysis

The SPSS software V.22 was used for data analysis. The normality of data was evaluated by Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) was employed to compare the means. Duncan complementary test were used for determining the significant differences among the groups. A p-value of < 0.05 was accepted as significant. Comparison of differences in survival among the groups were analyzed using the Kaplan–Meier survival analysis test (Log-rank value) (SPSS 22.0 version).

3. Results

3.1. Coating efficiency

The analysis of chitosan-alginate coated vaccines with SEM (Fig. 1) and optical microscope (Fig. 2) confirmed the high efficacy of structure of filaments (thready structure) and appropriate entrapment of these two inactivated bacteria (*S. iniae* and *L. garvieae*).

3.2. Innate immunity components

3.2.1. Complement activity, total protein and IgM

In all experimental groups, the serum alternative complement activity (Fig. 3, $P > 0.05$), total protein (Fig. 4, $P > 0.05$), and *IgM* (Fig. 5, $P > 0.05$), showed no significant differences during the 60 days experiment.

3.2.2. Lysozyme activity

The serum lysozyme activity of group A showed significant differences on days 20,40 and 60 of the experiment in comparison with control groups(C and D)(Fig. 6, $P < 0.05$). Other groups had no significant differences during the 60 days of experiment. Also between each sampling time a significant differences was observed between group A and control groups (C and D) on day 20,40 and 60 while, on day 40 a significant differences was observed between group A and all of other groups (B, C and D) (Fig. 6, $P < 0.05$).

3.2.3. Bactericidal activity

A significant differences in serum bactericidal activity was found on days 20,40 and 60 post-vaccination of group A compare to control groups (C and D) (Fig. 7, $P < 0.05$). There were significant differences in serum bactericidal activity of groups A and B on day 20 of experiment in each sampling time in comparison with groups C and D (Fig. 7, $P < 0.05$) and on days 40 and 60 these differences (about each sampling time) was found just in group A (Fig. 7, $P < 0.05$).

3.2.4. Blood respiratory burst activity

Fish immunized by chitosan-alginate coated vaccine (Group A) showed more blood respiratory burst activity on days 20,40 post-vaccination compared to control groups(C and D) (Fig. 8, $P < 0.05$). About this factor, there were significant changes between group B and control groups (C and D) on day 20 ($P < 0.05$). But on day 40, no significant differences were observed between group B and control groups (C and D) (Fig. 8, $P > 0.05$).

3.3. Expression of immune-related genes in kidney

3.3.1. IL-6

The relative expression of IL-6 in group A was affected significantly by oral vaccine immunization. Expression of this gene, was significantly higher in group A compared to all of other groups (B, C and D) on days 20 and 60 of experiment (Fig. 9 A, $P < 0.05$). Also the highest relative gene expression of IL-6 was observed on day 20 in group A. Moreover, about the each sampling time in the days 20 and 60 of trial, the expression of IL-6 was significantly higher in group A compared to groups B, C and D (Fig. 9 A, $P < 0.05$).

3.3.2. IgM

Similar results were obtained about the relative expression of IgM. So, the expression of this gene was significantly higher in group A compared to groups B, C and D on days 20 and 60 of trial (Fig. 9 B, $P < 0.05$). But, in contrast with IL-6 of group B, the expression of IgM of group B, was significantly higher compared to groups C and D on

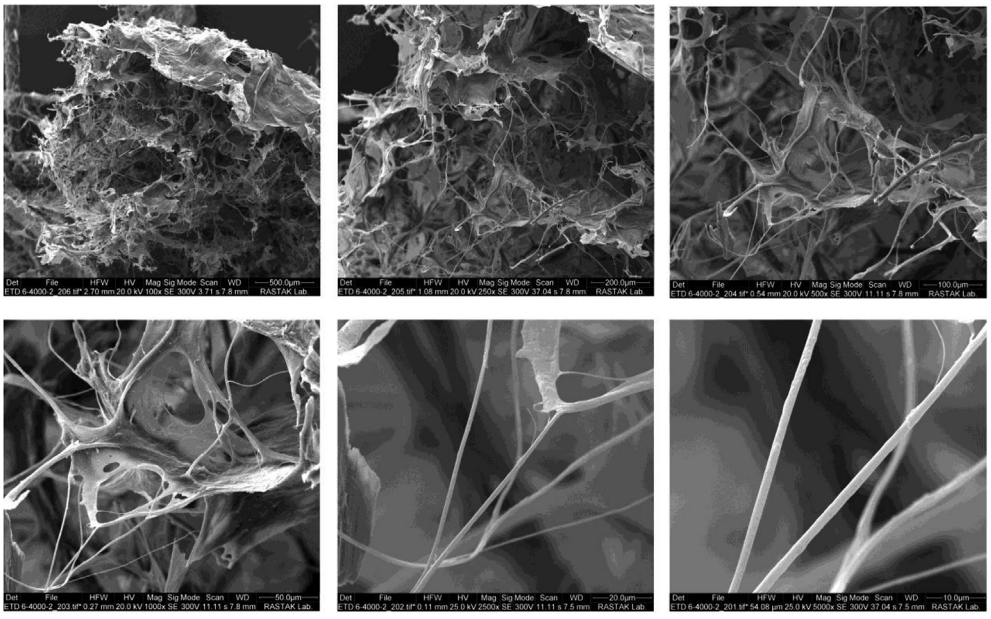
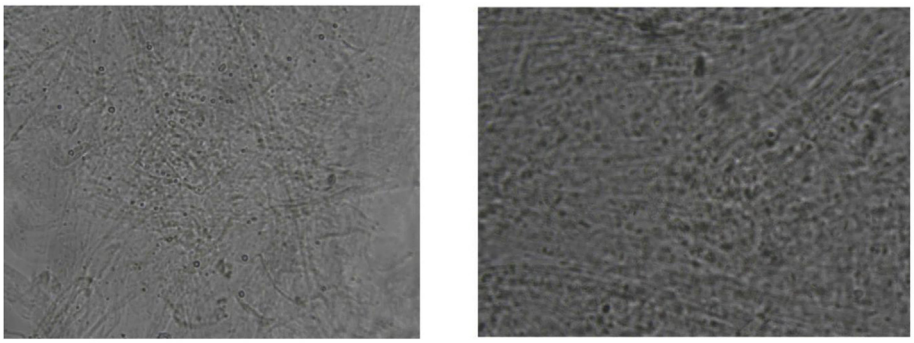


Fig. 1. SEM images of ionotropic gelation procedure from Low magnification to high magnification (100x, 250x, 500x, 1000x, 2500x, 5000x) with high quality of the thready structure of the images.



A: An optical image (Magnification: 40×100) B: An optical image (Magnification: 100×100)

Fig. 2. An optical microscope image of ionotropic emulsion–gelation method with inactivated bacteria (Image A: with magnification of 40 × 100 and image B with magnification of 100 × 100).

days 20 and 60 of examination (Fig. 9 B, $P < 0.05$). Also, about the each sampling time, on days 20 and 60 of experiment, the relative expression of immune related genes of IgM was higher in fish immunized by chitosan-alginate coated vaccine (Group A) compared to groups B, C and D.

3.4. Antibody titer

3.4.1. Antibody titer against L. garvieae

The serum ELISA antibody titer against *L. garvieae,* increased significantly on days 20 and 40 of experiment in fish immunized by chitosan-alginate coated vaccine (Group A) compared to control groups (C and D) (Fig. 10 A, $P < 0.05$). In each sampling time, the antibody titer against *L. garvieae* showed significant differences between group A compared to groups B, C and D on days 20 and 40 of post-vaccination (Fig. 10 A, $P < 0.05$).

3.4.2. Antibody titer against S. iniae

The serum ELISA antibody titer against *S. iniae* was significantly higher on days 40 and 60 of experiment in fish immunized by chitosan-alginate coated vaccine (Group A) compared to groups B, C and D (Fig. 10 B, $P < 0.05$). In each sampling time, the antibody titer against *S. iniae* was significantly higher on days 40 and 60 of experiment between vaccinated fish by ionic gelation process (group A) and other groups (B,C and D) (Fig. 10 B, $P < 0.05$).

3.5. Bacterial challenge

After bacterial challenge, survival percentage of 76.67 ± 5.77% (challenged with *S. iniae*) and 66.67 ± 5.77% (challenged with *L. garvieae*) were observed respectively in groups immunized by chitosan-alginate coated vaccine (Group A), which were higher than survival of other experimental groups ($P < 0.05$) (Table 2).

4. Discussion

Chitosan-alginate particles have been so far utilized for covering of oral vaccination in fish, however, no reports are accessible with respect to the utilization of this covering for orally vaccination of rainbow trout against *L. garvieae* and *S. iniae.* In the present examination, for the first time, an oral immunization by chitosan-alginate (the modified ionotropic method) was utilized to immunize rainbow trout against *L. garvieae* and *S. iniae*. Until now, different microparticles have been utilized for encapsulation of the oral vaccines in salmonidae. These are coatings, for example, alginate [20], sodium alginate [41], Eudragit L30D-55 [42], poly lactide-co-glycolide (PLGA) polymers [41,43] and ECAMs [44]. In the investigation of Joosten et al. [20], more antibody titers were seen when fish were immunized orally with pellets covered with alginate microparticles, yet they did not report fish mortality after test. Comparable outcomes were gotten when the rainbow trout were nourished an oral vaccine covered with poly lactide-co-glycolide acid (PLGA) [43]. Once more, there were no reports in regards to the fish mortality in the investigation of Lavelle et al. [43]. After test with *L. garvieae*, a survival level of 53–63% was seen in rainbow trout vaccinated orally with vaccine covered with sodium alginate and lactide-co-glycolide (PLGA) microparticles [41]. In the present examination, for the first time this improved and modified alginate/chitosan mixture was utilized for generation of an oral vaccine against *S. iniae* and *L. garvieae.* In this research, survival rates of 76.67 ± 5.77% and 66.67 ± 5.77% were acquired in groups vaccinated with pellets covered with alginate/chitosan (Group A) after challenge with *S. iniae* and *L. garvieae* respectively. These survival rates were fundamentally higher

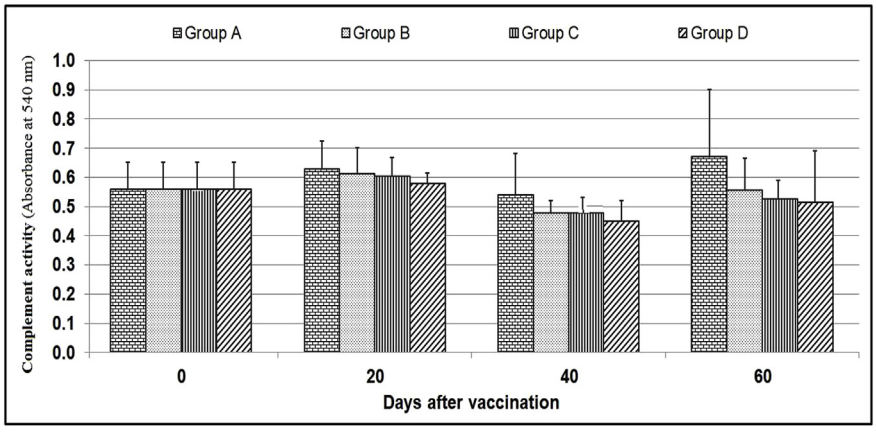


Fig. 3. Serum complement activity (the mean OD values) of rainbow trout fed different experimental diets. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment ($P < 0.05$).

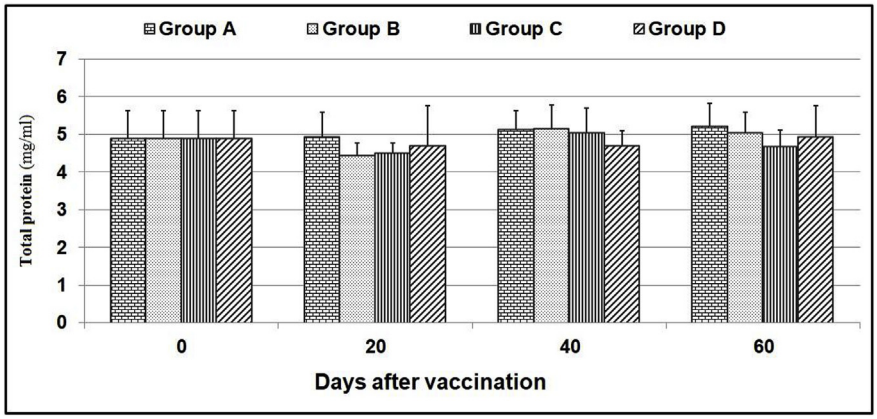


Fig. 4. Serum total protein of rainbow trout fed different experimental diets. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment ($P < 0.05$).

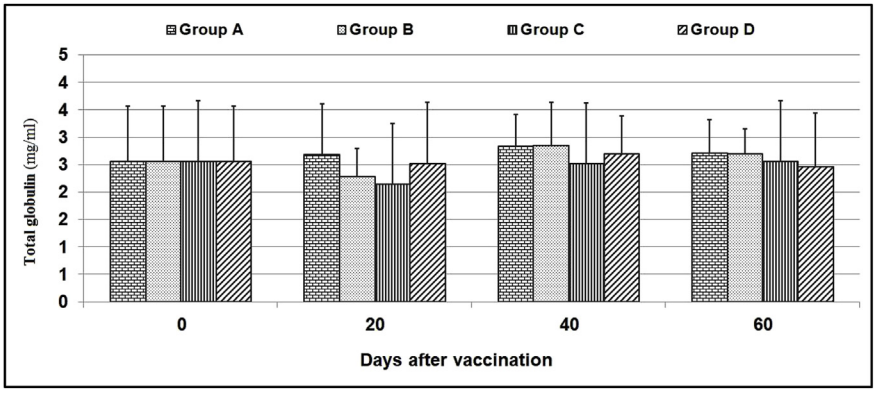


Fig. 5. Serum total immunoglobulin of rainbow trout fed different experimental diets. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment ($P < 0.05$).

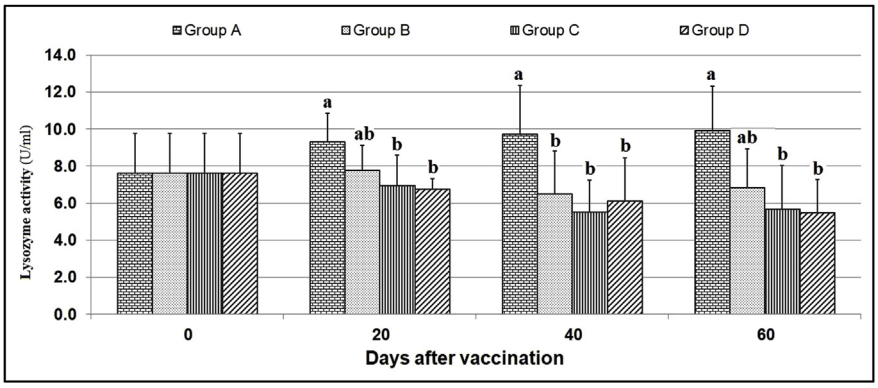


Fig. 6. Serum lysozyme activity of rainbow trout fed different experimental diets. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment ($P < 0.05$).

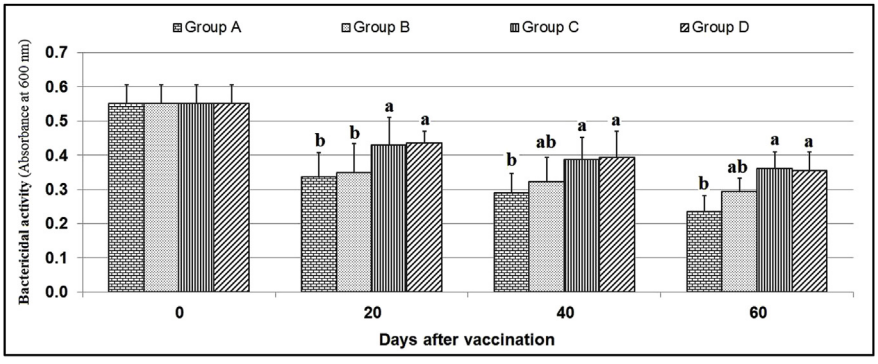


Fig. 7. Serum bactericidal activity of rainbow trout fed different experimental diets. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment ($P < 0.05$).

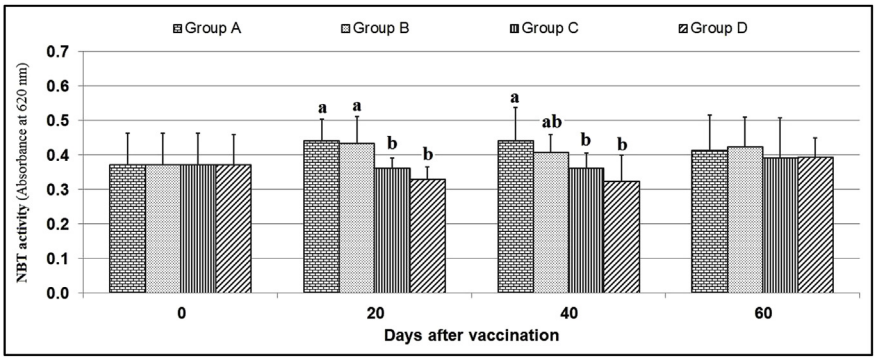


Fig. 8. Blood respiratory burst activity (NBT) of rainbow trout fed different experimental diets. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment ($P < 0.05$).

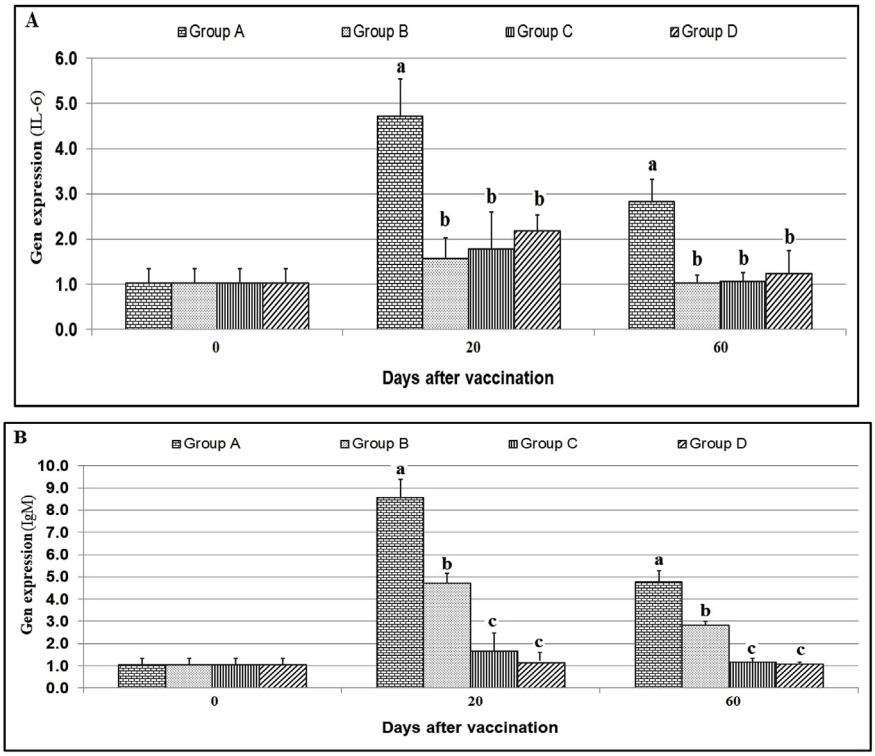


Fig. 9. The relative expression of IL6 (plot A) and IgM (plot B) genes in head kidney of rainbow trout fed different experimental diets. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment ($P < 0.05$).

in comparison with different groups. Besides, the vaccine covered pellets in the present examination brought about higher survival rate after test with *L. garvieae* in comparison with the consequences of Altun et al. [41], where fish had been vaccinated with oral immunization covered with SA and PLGA. Considering about these outcomes, it could be presumed that alginate/chitosan of the present study has more viability in covering and conveying the oral vaccine and ensuing assurance of fish against *S. iniae* and *L. garvieae* in comparison with different coatings, for example, SA and PLGA.

In this study, the evaluation of the immune reactions demonstrates that complement activity, total protein and IgM are not invigorated by the oral vaccine. However, lysozyme activity and bactericidal activity demonstrated a noteworthy increment on day 20, 40 and 60 of investigation in fish immunized by vaccine covered pellets with alginate/chitosan (group A) compared to control groups (C and D) and about the blood respiratory burst activity similar results were observed on days 20 and 40 post-vaccination. These results represent that our oral vaccine could protect the inactive antigen from acidic conditions of fish stomach and could present coated vaccine to the intestine of rainbow trout. In coincidence with our results, Yogeshwari et al. [45] observed significant increases in bactericidal activity of *common carp* with PLGA coated inactive *A. hydrophila*. In many studies [46–49] the effect of chitosan on the level of the blood respiratory burst activity of fish were observed which are consistent with the current research results. Moreover, according to our resulls, Maqsoud et al. [50] in the common carp and Aathi et al. [51], in the *Labeo rohita* demonstrated that oral administration of chitosan could increase the lysozyme activity.

In contrast to some parts of innate immunity (such as complement activity, total protein and IgM), the relative expression of immune-related genes including IL-6 and IgM qualities was higher in vaccinated fish, with the highest expression considered in those vaccinated by alginate/chitosan covered pellets (Group A). These outcomes unmistakably demonstrate that immune reactions to the vaccine are increasingly obvious in the level of gene expression than in different dimensions of intrinsic immunity. Moreover, both of these genes (IL-6 and IgM) were increased significantly on days 20 and 60 in vaccinated group (A) compared to control groups (C and D). Possibly, the coating of the vaccine with alginate/chitosan has been effective in maintaining healthy and intact inactivated antigen and delivering better and more antigens in the intestine, and consequently the production of these cytokines (IgM and IL-6) has increased compared to control treatments. Similar to our research, several scientists reported the increase in these two genes in their research [52–56].

In the present study, the antibody titer against *L. garvieae* elevated

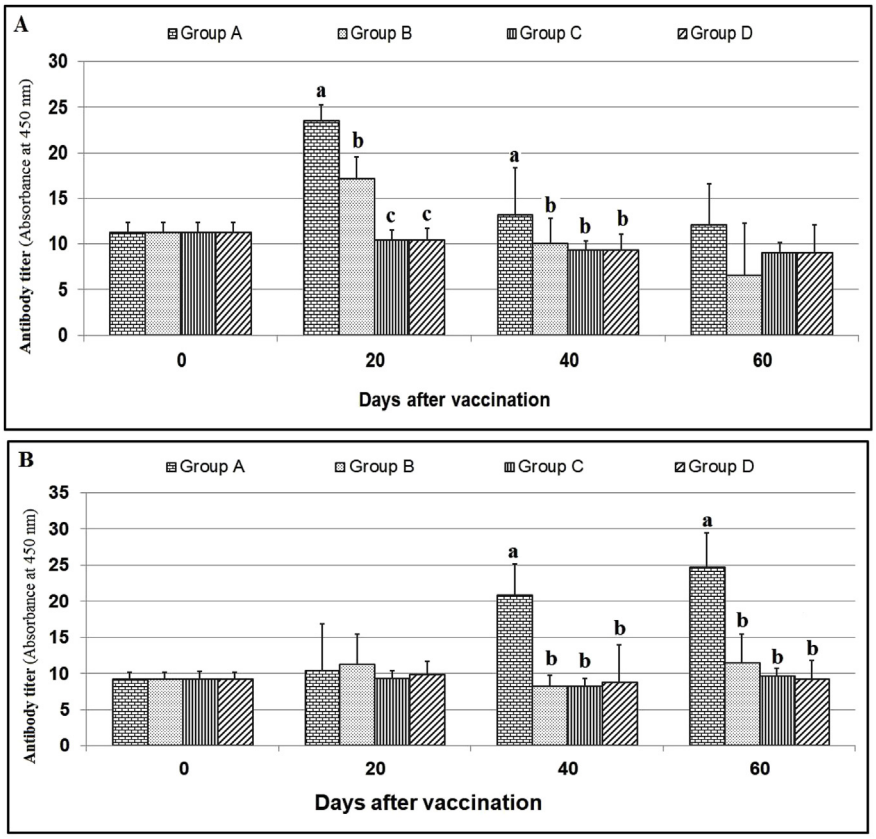


Fig. 10. Serum ELISA antibody titer of rainbow trout against *L. garvieae* (plot A) and *S. iniae* (plot B). Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). In each group, bar assigned with different letters denote separately the significant differences during 60 days experiment (P < 0.05).

Table 2
The total survival percent of rainbow trout after 10 days challenge with *Streptococcus iniae* and *Lactococcus garvieae* in experimental groups. Group A (n = 150): fish immunized by chitosan-alginate coated vaccine, Group B (n = 150): fish immunized by non-coated vaccine, Group C (n = 150): fish feed by chitosan-alginate coated pellets without vaccine and Group D (n = 150): fish feed by basic diet (non-coated and without vaccine). Means with different letters denote significant differences (P < 0.05).

Streptococcus iniae		
Experimental groups	Fish number	Total survival percent (%)
Group A	30	% 76.67 ± 5.77 [a]
Group B	30	% 50.00 ± 10.00 [b]
Group C	30	% 23.33 ± 5.77 [c]
Group D	30	% 21.67 ± 5.77 [c]
Lactococcus garvieae		
Experimental groups	Fish number	Total survival percent (%)
Group A	30	% 66.67 ± 5.77 [a]
Group B	30	% 50.00 ± 10.00 [b]
Group C	30	% 23.33 ± 5.77 [c]
Group D	30	% 23.33 ± 5.77 [c]

on days 20 and 40 of test in group A contrasted with groups C and D. For estimating each sampling time, on days 20 and 40 of trial, the significant differences were seen in immune response titer against *L. garvieae* between group A and all of other groups (B, C and D). Additionally, the antibody titer against *S. iniae* was higher in fish vaccinated by chitosan-alginate covered pellets (Group A) on days 40 and 60 contrasted with group B and control groups (C and D). The reaction of fish body's against both of these two antigens, demonstrating the more efficient incitement of adaptive immunity by oral vaccine (group A). Moreover, it can be concluded that mucosal immunity that has been produced by our oral vaccine coating, causes proper antibody titer and by stimulating cytokines, high protection has been created against *S. iniae* and *L. garvieae*. In concurrence with our results, numerous investigations have demonstrated the upgrade of the adaptive immunity of fishes through informing the increments in titer of antibody [18,41,44,57–60].

In conclusion, our results demonstrated that this work is a unique work on an oral bivalent streptococcus-lactococcus vaccine with high efficacy and acceptable immunogenicity. Moreover, mix of alginate and chitosan microparticles with modified and improved ionotropic method increased the viability of conveyance and immunization of practical oral vaccines, because higher survival rate, immune-related gene expression and antibody titer were seen in fish immunized by chitosan-alginate coated vaccine.

Acknowledgments

This research was a project that was financially assisted by a grant from the office of President, Vice-Presidency for Science and Technology, Iran National Science Foundation (code no. 94003765). The authors acknowledge the experts of our faculty laboratory and the expert of pharmacy school of Ahvaz Jundishapur University of Medical Sciences for kindly helping us in carrying out this work.

References

[1] A. Eldar, C. Ghittino, *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases, Dis. Aquat. Org. 36 (3) (1999) 227–231.
[2] D. Vendrell, J.L. Balcázar, I. Ruiz-Zarzuela, I. De Blas, O. Gironés, J.L. Múzquiz, *Lactococcus garvieae* in fish: a review, Comp. Immunol. Microbiol. Infect. Dis. 29 (4) (2006) 177–198.
[3] J. Prieta, Lactococcosis de la trucha arco iris, Med. Vet. 10 (1993) 367–373.
[4] J.L. Muzquiz, F.M. Royo, C. Ortega, I. De Blas, I. Ruiz, J.L. Alonso, Pathogenicity of streptococcosis in rainbow trout (*Oncorhynchus mykiss*): dependence on age of diseased fish, Bull. Eur. Assoc. Fish Pathol. 19 (1999) 114–119.
[5] D. Vendrell, J.L. Balcazar, I. Ruiz-Zarzuela, I. de Blas, O. Girones, J.L. Muzquiz,

Evaluation in rainbow trout (*Oncorhynchus mykiss*) of Ichtiovac-Lg, a vaccine against *Lactococcus garvieae*, Proceedings of the Sixth International Symposium on Fish Immunology, Nordic Society for Fish Immunology, Turku, Finland, 2004.
[6] M.R. Weinstein, M. Litt, D.A. Kertesz, P. Wyper, D. Rose, M. Coulter, et al., Invasive infections due to a fish pathogen, *Streptococcus iniae*, N. Engl. J. Med. 337 (9) (1997) 589–594.
[7] D. Lahav, M. Eyngor, A. Hurvitz, C. Ghittino, A. Lublin, A. Eldar, *Streptococcus iniae* type II infections in rainbow trout *Oncorhynchus mykiss*, Dis. Aquat. Org. 62 (1–2) (2004) 177–180.
[8] T.P. Evelyn, A historical review of fish vaccinology, Dev. Biol. Stand. 90 (1997) 3–12.
[9] S. Vervarcke, F. Ollevier, R. Kinget, A. Michoel, Oral vaccination of African catfish with *Vibrio anguillarum* O2: effect on antigen uptake and immune response by absorption enhancers in lag time coated pellets, Fish Shellfish Immunol. 16 (3) (2004) 407–414.
[10] N.A. Ballesteros, S.R. Saint-Jean, S.I. Perez-Prieto, Food pellets as an effective delivery method for a DNA vaccine against infectious pancreatic necrosis virus in rainbow trout (*Oncorhynchus mykiss, Walbaum*), Fish Shellfish Immunol. 37 (2) (2014) 220–228.
[11] A.E. Ellis, General principles of fish vaccination, in: A.E. Ellis (Ed.), Fish Vaccination, Academic press, London, 1988, pp. 1–31.
[12] A. Obach, F.B. Laurencin, Vaccination of rainbow trout *Oncorhynchus mykiss* against the visceral form of coldwater disease, Dis. Aquat. Org. 12 (1) (1991) 13–15.
[13] M.H. Rahman, M. Ototake, Y. Iida, Y. Yokomizo, T. Nakanishi, Efficacy of oil-adjuvanted vaccine for coldwater disease in ayu *Plecoglossus altivelis*, Fish Pathol. 35 (4) (2000) 199–203.
[14] B.R. LaFrentz, S.E. LaPatra, G.R. Jones, J.L. Congleton, B. Sun, K.D. Cain, Characterization of serum and mucosal antibody responses and relative per cent survival in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following immunization and challenge with *Flavobacterium psychrophilum*, J. Fish Dis. 25 (12) (2002) 703–713.
[15] S. Hart, A.B. Wrathmell, J.E. Harris, T.H. Grayson, Gut immunology in fish: a review, Dev. Comp. Immunol. 12 (3) (1988) 453–480.
[16] A. Lillehaug, Oral immunization of rainbow trout, *Salmo gairdneri Richardson*, against vibriosis with vaccines protected against digestive degradation, J. Fish Dis. 12 (6) (1989) 579–584.
[17] E.J. Dunn, A. Polk, D.J. Scarrett, G. Olivier, S. Lall, M.F. Goosen, Vaccines in aquaculture: the search for an efficient delivery system, Aquacult. Eng. 9 (1) (1990) 23–32.
[18] G. Wong, S.L. Kaattari, J.M. Christensen, Effectiveness of an oral enteric coated vibrio vaccine for use in salmonid fish, Immunol. Investig. 21 (4) (1992) 353–364.
[19] A.E. Ellis, Recent development in oral vaccine delivery systems, Fish Pathol. 30 (4) (1995) 293–300.
[20] P.H.M. Joosten, E.W. Tiemersma, A. Threels, C. Dhieux-Caumartin, J.H.W.M. Rombout, Oral vaccination of fish against *Vibrio anguillarum* using alginate microparticles, Fish Shellfish Immunol. 7 (7) (1997) 471–485.
[21] K.P. Plant, S.E. LaPatra, Advances in fish vaccine delivery, Dev. Comp. Immunol. 35 (12) (2011) 1256–1262.
[22] M.J. Barea, M.J. Jenkins, Y.S. Lee, P. Johnson, R.H. Bridson, Encapsulation of liposomes within pH responsive microspheres for oral colonic drug delivery, Int. J. Biom. 2012 (2012) 458712.
[23] M. George, T.E. Abraham, Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan—a review, J. Control. Release 114 (1) (2006) 1–14.
[24] D. Elieh-Ali-Komi, M.R. Hamblin, Chitin and chitosan: production and application of versatile biomedical nanomaterials, Int. J. Adv. Res. 4 (3) (2016) 411–427.
[25] L. Illum, I. Jabbal-Gill, M. Hinchcliffe, A.N. Fisher, S.S. Davis, Chitosan as a novel nasal delivery system for vaccines, Adv. Drug Deliv. Rev. 51 (1–3) (2001) 81–96.
[26] M.N.R. Kumar, A review of chitin and chitosan applications, React. Funct. Polym. 46 (1) (2000) 1–27.
[27] M. Rinaudo, Chitin and chitosan: properties and applications, Prog. Polym. Sci. 31 (7) (2006) 603–632.
[28] F.O. Eko, M.P. Szostak, G. Wanner, W. Lubitz, Production of *Vibrio cholerae* ghosts (VCG) by expression of a cloned phage lysis gene: potential for vaccine development, Vaccine 12 (13) (1994) 1231–1237.
[29] W.H. Chu, Adjuvant effect of propolis on immunisation by inactivated *Aeromonas hydrophila* in carp (*Carassius auratus gibelio*), Fish Shellfish Immunol. 21 (1) (2006) 113–117.
[30] M. Rajaonarivony, C. Vauthier, G. Couarraze, F. Puisieux, P. Couvreur, Development of a new drug carrier made from alginate, J. Pharm. Sci. 82 (9) (1993) 912–917.
[31] B. Sarmento, A. Ribeiro, F. Veiga, R. Neufeld, D. Ferreira, Insulin-loaded alginate/chitosan nanoparticles produced by ionotropic pre-gelation, Revista Portuguesa Farmá cia LII (2) (2005) 139–140.
[32] T. Yano, Assay of hemolytic complement activity, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, S.C. Hattari, A.F. Rowley (Eds.), Techniques in Fish Immunology, SOS Publications, New Jersey, 1992, pp. 131–141.
[33] M.A. Esteban, A. Cuesta, J. Ortuno, J. Meseguer, Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata L.*) innate immune system, Fish Shellfish Immunol. 11 (4) (2001) 303–315.
[34] B. Budiño, R.M. Cal, M.C. Piazzon, J. Lamas, The activity of several components of the innate immune system in diploid and triploid turbot, Comp. Biochem. Physiol. Mol. Integr. Physiol. 145 (1) (2006) 108–113.
[35] C.J. Secombes, Isolation of salmoid macrophages and analysis of their killing activity, Techniques in Fish Immunology 1 (1990) 137–155.
[36] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1) (1951) 265–275.
[37] A.K. Siwicki, D.P. Anderson, Nonspecific Defense Mechanisms Assay in Fish: II. Potential Killing Activity of Neutrophils and Macrophages, Lysozyme Activity in Serum and Organs and Total Immunoglobulin Level in Serum, Fish Dis Diag Preven Meth Olsztyn, Poland, 1993, pp. 105–112.
[38] R.A. Shelby, C.A. Shoemaker, P.H. Klesius, Development of and ELISA to measure the humoral immune response of hybrid striped bass *Morone chrysops M. saxatilis* to *Streptococcus iniae*, Aquacult. Res. 35 (2004) 997–1001.
[39] M.R. Tabandeh, H. Jafari, S.A. Hosseini, M. Hashemitabar, Ginsenoside Rb1 stimulates adiponectin signaling in C2C12 muscle cells through up-regulation of AdipoR1 and AdipoR2 proteins, Pharm. Biol. 53 (1) (2015) 125–132.
[40] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (4) (2009) 611–622.
[41] S. Altun, A. Kubilay, S. Ekici, B.I. Didinen, Ö. Diler, Oral vaccination against Lactococcosis in rainbow trout (*Oncorhynchus mykiss*) using sodium alginate and poly (lactide-co-glycolide) carrier, Journal of the Faculty of Veterinary Medicine, University of Kafkas, Kars (Turkey) 16 (2010) 211–217.
[42] M. Halimi, M. Alishahi, M.R. Abbaspour, M. Ghorbanpoor, M.R. Tabandeh, Efficacy of a Eudragit L30D-55 encapsulated oral vaccine containing inactivated bacteria (*Lactococcus garvieae/Streptococcus iniae*) in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 81 (2018) 430–437.
[43] E.C. Lavelle, P.G. Jenkins, J.E. Harris, Oral immunization of rainbow trout with antigen microencapsulated in poly (DL-lactide-co-glycolide) microparticles, Vaccine 15 (10) (1997) 1070–1078.
[44] J.D. Piganelli, J.A. Zhang, J.M. Christensen, S.L. Kaattari, Enteric coated microspheres as an oral method for antigen delivery to salmonids, Fish Shellfish Immunol. 4 (3) (1994) 179–188.
[45] G. Yogeshwari, C. Jagruthi, J. Ramasamy, Poly D, L-lactide-co-glycolic acid (PLGA)-encapsulated CpG-oligonucleotide (ODN) on immune response in *Cyprinus carpio* against *Aeromonas hydrophila*, J. Aquac. Res. Dev. 6 (4) (2015) 327–337.
[46] A.N. Nakhla, A.J. Szalai, J.H. Banoub, K.M.W. Keough, Serum anti-LPS antibody production by rainbow trout (*Oncorhynchus mykiss*) in response to the administration of free and liposomally-incorporated LPS from *Aeromonas salmonicida*, Fish Shellfish Immunol. 7 (1997) 387–401.
[47] A. Gopalakannan, V. Arul, Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds, Aquaculture 255 (2006) 179–187.
[48] S. Lin, Y. Pan, L. Luo, L. Li, Effects of dietary β-1, 3-glucan, chitosan or raffinose on thegrowth, innate immunity and resistance of koi(*Cyprinus carpio koi*), Fish Shellfish Immunol. 31 (2011) 788–794.
[49] S. Lin, S. Mao, Y. Guan, L. Luo, L. Luo, Y. Pan, Effects of dietary chitosan oligosaccharides and Bacillus coagulans on the growth, innate immunity and resistance of koi (*Cyprinus carpiokoi*), Aquaculture 342–343 (2012) 36–41.
[50] S. Maqsood, P. Singh, M.H. Samoon, A.K. Balange, Effect of dietary chitosan on non-specific immune response and growth of *Cyprinus carpio* challenged with *Aeromonas hydrophila*, Int. Aquat. Res. 2 (2010) 77–85.
[51] K. Aathi, V. Ramasubramanian, V. Uthayakumar, S. Munirasu, Effect of supplemented diet on survival, growth, hematological, biochemical and immunological responses of Indian major carp *Labeo rohita*, Int. Res. J. Pharm. 4 (5) (2013) 141–147.
[52] M.K. Raida, K. Buchmann, Temperature-dependent expression of immune-relevant genes in rainbow trout following *Yersinia ruckeri* vaccination, Dis. Aquat. Org. 77 (1) (2007) 41–52.
[53] M. Cui, Q. Zhang, Z. Yao, Z. Zhang, H. Zhang, Y. Wang, Immunoglobulin M gene expression analysis of orange-spotted grouper, *Epinephelus coioides*, following heat shock and *Vibrio alginolyticus* challenge, Fish Shellfish Immunol. 29 (6) (2010) 1060–1065.
[54] E. Wang, K. Wang, D. Chen, J. Wang, Y. He, B. Long, L. Yang, Q. Yang, Y. Geng, X. Huang, P. Ouyang, Evaluation and selection of appropriate reference genes for real-time quantitative pcr analysis of gene expression in nile tilapia (*Oreochromis niloticus*) during vaccination and infection, Int. J. Mol. Sci. 16 (5) (2015) 9998–10015.
[55] S. Deshmukh, P.W. Kania, J.K. Chettri, J. Skov, A.M. Bojesen, I. Dalsgaard, K. Buchmann, Insight from molecular, pathological, and immunohistochemical studies on cellular and humoral mechanisms responsible for vaccine-induced protection of rainbow trout against *Yersinia ruckeri*, Clin. Vaccine Immunol. 20 (10) (2013) 1623–1641.
[56] T. Wang, C.J. Secombes, Identification and expression analysis of two fish-specific IL-6 cytokine family members, the ciliary neurotrophic factor (CNTF)-like and M17 genes, in rainbow trout *Oncorhynchus mykiss*, Mol. Immunol. 46 (11) (2009) 2290–2298.
[57] T. Irie, S. Watarai, T. Iwasaki, H. Kodama, Protection against experimental *Aeromonas salmonicida* infection in carp by oral immunisation with bacterial antigen entrapped liposomes, Fish Shellfish Immunol. 18 (3) (2005) 235–242.
[58] S.R. Kwon, E.H. Lee, Y.K. Nam, S.K. Kim, K.H. Kim, Efficacy of oral immunization with *Edwardsiella tarda* ghosts against edwardsiellosis in olive flounder (*Paralichthys olivaceus*), Aquaculture 269 (1) (2007) 84–88.
[59] F.P. Tu, W.H. Chu, X.Y. Zhuang, C.P. Lu, Effect of oral immunization with *Aeromonas hydrophila* ghosts on protection against experimental fish infection, Lett. Appl. Microbiol. 50 (1) (2010) 13–17.
[60] J.A. Tobar, S. Jerez, M. Caruffo, C. Bravo, F. Contreras, S.A. Bucarey, M. Harel, Oral vaccination of Atlantic salmon (*Salmo salar*) against salmonid rickettsial septicaemia, Vaccine 29 (12) (2011) 2336–2340.