Immunostimulation by starch hydrogel-based oral vaccine using formalin-killed cells against edwardsiellosis in Japanese eel, *Anguilla japonica*

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ABSTRACT

Edwardsiellosis outbreaks cause significant losses in Japanese eel aquaculture. The causative agent, *Edwardsiella tarda*, is an intracellular pathogen, and the use of antibiotics has a limited effectiveness. As Japanese eels are sensitive to stress, injection vaccines are not recommended for treatment; immersion methods are less stressful, but not cost-effective. Alternatively, oral vaccination methods are more promising. The aim of this study was to develop a starch hydrogel-based oral (SHO) vaccine against edwardsiellosis in Japanese eel, using formalin-killed cells. To assess the protective effect, we compared SHO vaccine with the conventional formalin-killed cell (FKC) vaccine. A bacterial agglutination test showed that agglutination titers in SHO-vaccinated group were higher than in the FKC-vaccinated group. Japanese eel survival rate (%) was monitored after challenge by *E. tarda* at four weeks post-vaccination. Survival rates in the FKC group (60%, first trial; 70%, second trial) were lower than in SHO groups. Percentage survival rates in three SHO groups (first and second trials, respectively) were as follows: 70% and 80% in the group vaccinated once per day for one day; and 80% and 90% in both groups vaccinated for four and eight days. Additionally, a boost SHO vaccination at 46 days prompted a similar or even higher protection against edwardsiellosis than after the initial vaccination. Both FKC and SHO vaccination upregulated levels of pro-inflammatory cytokines (interleukin (IL)-6, tumor necrosis factor (TNF)-α), and host defense cytokine (interferon (IFN)-α) in all immunized groups of fish when compared with the control. These results reveal the immunostimulation effect of SHO vaccine in Japanese eel, emphasizing its potential as an oral vaccine in aquaculture.

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1. Introduction

The Japanese eel (*Anguilla japonica*) is known for its complicated life cycle and spawning migration [1]. The silver eels spawn far offshore in the ocean; after hatching, the lectocephali return to freshwater growth habitats, before migrating back to the ocean spawning area after achieving maturity. The species is also one of the most important cultured fish in East Asia, especially Japan, Korea, and China, because of its high market value and the increasing demand for consumption [2]. Eel aquaculture is among the foremost aquaculture industries in Korea, where eel production is increasing annually; production was 5217 ton (t) in 2013 and 10,589 t in 2018 [3]. In 2018, it accounted for 30% of the total Korean freshwater fisheries production, and for 67.9% of the total in monetary value [3].

Edwardsiella tarda, a gram-negative bacterium of the family *Enterobacteriaceae*, is the causative agent of edwardsiellosis [4]. A wide range of fish species are subject to infection, including economically important species, such as Japanese eel and olive flounder (*Paralichthys olivaceus*) [5,6]. *E. tarda* is one of the main bacterial pathogens in Japanese eel aquaculture [7] and causes significant loss, with outbreaks of edwardsiellosis being reported frequently [8].

To date, the use of antibiotics has been the principal means for controlling edwardsiellosis [9]. However, antibiotic therapy is losing its credibility in edwardsiellosis control because of its limited effectiveness. This is because *E. tarda* is an intracellular pathogen, and so antibiotic therapy is not as effective as the treatment for

extracellular pathogens [7]. Additionally, multiple antibiotic-resistant strains of *E. tarda* have been reported to be widely present in eel aquaculture environments [10]. The increasing prevalence of antibiotic resistance has made the development of an effective vaccine a high priority in controlling edwardsiellosis.

This study set out to develop a starch hydrogel-based oral (SHO) vaccine using formalin-killed cells, for use against edwardsiellosis in Japanese eel. The main aim was to assess the immunostimulating activity of the SHO vaccine and examine its efficacy in protecting Japanese eel from edwardsiellosis by comparing it with the formalin-killed cell (FKC) vaccine.

2. Materials and methods

2.1. Bacterial strain and growth condition

E. tarda SU53, which is pathogenic to fish [11], was isolated from cultured Japanese eel in Shizuoka Prefecture, Japan in 1980 [11,12]. The bacterial strain was cultured in tryptic soy broth (TSB) or tryptic soy agar (TSA) at 25 °C.

2.2. Fish

Five hundred and sixty healthy Japanese eels (average length, 28.0 cm; average weight, 82.4 ± 7.9 g) were procured from a fish farm in Gimpo-si, South Korea, which had no previous occurrence of edwardsiellosis. In order to confirm that the fish were free of *E. tarda* infection, 10 individuals were randomly collected prior to the experiments and subjected to polymerase chain reaction (PCR), as previously described [11]. Five hundred and fifty fish were then acclimatized to laboratory conditions in 5 t tanks at 27.5 ± 0.5 °C for one week. The fish were fed commercial dried pellets (Yooan Feed Co., LTD, Korea) once per day, at a quantity corresponding to 3% of the fish body weight. Approximately 50% of the water in the tanks was exchanged weekly. A total of 100 of the acclimatized Japanese eels were subject to an experimental challenge to determine the median lethal dose (LD_{50}) of *E. tarda* SU53; 400 fish were designated for use in the vaccination experiment. All animal care and experimental protocols were performed according to the guidelines of the Animal Ethical Committee at Seoul National University.

2.3. pH measurements in Japanese eel stomachs and intestines

At 1 h post-feeding, five Japanese eels were randomly chosen and then euthanized using MS-222 (Sigma-Aldrich, MO, USA). The gastrointestinal tracts of the fish were incised, and the pH of the stomach and intestine contents was measured using a pH meter (Thermo Scientific, MA, USA).

2.4. Vaccine preparation

The SHO vaccine was prepared as previously described [13], with modifications. Briefly, 1 ml of the *E. tarda* SU53 bacterial suspension (2.0×10^{10} colony-forming units; CFU) was inactivated by adding formalin (0.4% [v/v]), washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 1 ml of sterile PBS.

Starch (1.33 g; Fluka, Buchs, Switzerland) was dissolved in 35 ml of distilled water (DW) by stirring at 70 °C. Ammonium persulfate (100 mg; Fluka, Buchs, Switzerland) was added to the suspension, which was then stirred for 10 min. Acrylic acid (1.5 g; Merck, Darmstadt, Germany) and 1.5 g of 2-hydroxyethyl methacrylate (Merck, Darmstadt, Germany) were added to the solution by stirring. Separately, 100 mg of N, N'-methylenebisacrylamide was mixed into 5 ml of DW and poured into the starch solution; the mixture was then cooled to 60 °C. The antigen (FKC) was added to the mixture, which was then continuously stirred. After 1 h, the reaction product was allowed to cool to the ambient temperature for 30 min, and neutralized to pH 7.5 by adding 1 M sodium hydroxide solution. The hydrogel was poured to 200 ml of excess nonsolvent ethanol and dehydrated for 6 h. Then, the filtered hydrogel was lyophilized for 6 h and stored at −20 °C for further use.

2.5. Morphological analysis of SHO vaccine

The morphological analysis was performed using a field emission scanning electron microscope (FESEM; Sigma, Carl Zeiss, UK). Three specimens were prepared and mounted onto stubs: starch prior to hydrogel formation; synthesized hydrogels based on starch containing no antigen (FKC, *E. tarda* SU53); and SHO vaccine. Before scanning, the specimens were sputter-coated with gold for 180 s using a vacuum coater (EM ACE 200; Leica, Austria).

2.6. Vaccination

In order to manufacture SHO vaccine, the powdered hydrogel was thoroughly mixed with ground pellets, and 1 ml of PBS was then added to the mixture. A quantity of SHO vaccine was prepared, and the antigen content of each SHO vaccine was adjusted to 10^8 CFU per fish.

Prior to the immunization experiments, five Japanese eels were randomly chosen and anesthetized using MS-222. SHO vaccine was orally administered to these fish, which were maintained at 27.5 ± 0.5 °C. One week post-vaccination, the fish were euthanized with MS-222 and blood was drawn from the caudal vein to determine if they had been immunized by the SHO vaccine.

Four hundred acclimatized Japanese eels were divided into five groups (each group, *n* = 80) in 1 t tanks at 27.5 ± 0.5 °C. The five groups were: the control, in which fish were orally supplied pellets that had been PBS-impregnated only once; the FKC group, which was orally supplied ground pellets mixed with FKC once per day for four days; and three SHO groups (SHO 1, SHO 4, and SHO 8), which were orally supplied SHO once a day for one, four, and eight days, respectively. The total antigen content in a single administration of each vaccine (FKC or SHO) was adjusted to 10^8 CFU per fish.

2.7. Blood sample collection and serum agglutination test

Three fish were randomly selected from each of the five groups every week for six weeks: sampling was performed from one week after the first vaccination in every group except SHO 8, in which sampling was performed from two weeks after the first vaccination. Blood samples, which were collected from the caudal vein using a 1 ml syringe following euthanasia with MS-222, were transferred to centrifuge tubes (Eppendorf, Hamburg, Germany). Serum was collected after centrifugation at 6500*g* at 4 °C for 10 min. The serum was heat-treated (44 °C, 20 min) to inactivate complement activity. The serum agglutination experiment was performed using a 96-well U-bottom plate (Sigma-Aldrich, St. Louis, MO, USA). The serum was serially diluted two-fold in PBS, and the same volume of heat-killed *E. tarda* SU53 (ca. 10^7 CFU/ml) was then added to each well. Serum containing antibody was used as a positive control. Plates were incubated overnight at 25 °C. Agglutination activity was determined according to the lowest dilution with no agglutination, and the resulting value was considered the reciprocal of that dilution rate.

2.8. Assessment of effect of boost vaccine

The second immunization (boost vaccination) was performed only once at 46 days after the first vaccination, when the serum agglutinating antibody titer was found to decrease. Twenty fish were randomly selected from each of the five groups at 46 days post-first vaccination. The fish in the control group were orally supplied PBS- impregnated pellets; the FKC group fish were orally supplied ground pellets mixed with FKC; and fish in SHO 1, SHO 4, and SHO 8 were orally supplied SHO. The fish from each group were then separately transferred to 200 L fiberglass-reinforced plastic aquaria at 27.5 ± 0.5 °C. At seven and eight weeks after the first vaccination, three fish were randomly selected from each of the five groups and blood sampling was performed. The serum agglutination experiment was performed as described above.

2.9. Experimental challenge test

E. tarda SU53 in its early-exponential phase was used for the challenge test, and was serially diluted 10-fold with PBS. To determine the LD_{50} concentration of the strain, duplicate fish groups (n = 10, per group) were administered 100 µl of the bacterial suspension by intraperitoneal (i.p.) injection. The final injection doses ranged from 2.0×10^4 to 2.0×10^7 CFU/fish. Fish in the control group were injected with 100 µl of sterile PBS. After injection, fish were monitored for 15 days. Dead fish were sampled every day; the bacteria were isolated from their kidneys and identified using PCR, as previously described [11].

Injection (i.p.) challenge tests were performed four weeks after the first vaccination. Duplicate fish groups (n = 10, per group) were administered i.p. injections containing 100 µl of the LD_{50} concentration of the strain. The challenged fish were maintained at 27.5 ± 0.5 °C in 100 L fiberglass-reinforced plastic aquaria supplied with well-aerated flowing water. Clinical signs of disease and cumulative mortalities were monitored twice a day for 15 days after injection. Bacterial samples were obtained from the kidneys of dead fish, then cultured on TSA at 25 °C. Isolates were identified using PCR, as described above.

2.10. RNA extraction and reverse transcription

After blood sample collection, three fish from each group were selected for RNA extraction. Total RNA was extracted from the head kidney and liver using TRIzol Reagent (CWBio, Beijing, China). RNA concentration and purity were quantified by spectrophotometry, which showed 260:280 ratios between 1.6 and 1.8; RNA quality was verified using electrophoresis on 1% agarose gels supplemented with 0.5 µg/mL ethidium bromide. In order to eliminate DNA contamination, total RNA samples were treated with DNAse I (Promega, Madison, WI, USA) according to the manufacturer's instructions. Extracted RNA was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit (TaKaRa Bio, Otsu, Japan), following the manufacturer's protocol. The resulting cDNA was stored at −80 °C until use.

2.11. Real-time quantitative PCR analyses of gene expression

The expression of genes involved in the immune response such as interferon (IFN)-α [GenBank accession number: KT156977], interleukin (IL)-6 [DQ866150], and tumor necrosis factor (TNF)-α [AJ401677] was monitored using a Rotor-Gene Q real-time quantitative PCR (RT-qPCR) Detection System (QIAGEN; Hilden, Germany) as previously described [14,15]. All qPCR reactions were performed using SYBR Premix Ex Taq™ Perfect Real-Time Kits (TaKaRa Bio, Otsu, Japan), following standard protocols. Gene expression was normalized using the house-keeping gene for

Table 1
Primers used for RNA expression analysis by qRT-PCR.

Target gene	Primer sequence	Reference
TNF-α	F[a]: CGCTGACACAGTGCAGTGGA R[b]: TCCCCGATGGAGTCCGAATA	[15]
IL-6	F: TTTCAGAAGCCCGTGGAAGAGA R: TCTTTGACCAGCCCTATCAGCA	[15]
IFN-α	F: GTGAGCGGCGAATCCTTGT R: TTTGTCATCCACCTGTGCTTTG	[14]
β-actin	F: ATCGTGCGTGACATCAAGGA R: GCTCGTTGCCGATGGTGAT	[14]

[a] F, forward.
[b] R, reverse.

β-actin. Table 1 indicates the PCR primer sequences used for qPCR. The reaction mixture included 10 µl SYBR Premix Ex Taq™, 1 µl of the forward and reverse primers (10 mM), and 1 µl cDNA. Ultrapure water was then added to the reaction to bring it to a final total volume of 20 µl. The reaction conditions and cycle index were conducted at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 30 s. After the amplification phase, a melting curve analysis was conducted to account for the possibility of non-specific amplification or primer-dimer formation. A standard curve was created from serial dilutions of sample cDNA and drawn by plotting the natural log of the threshold cycle (Ct) against the number of molecules. The standard curve of each gene was run in triplicate to determine that the coefficient of determination (R^2) of all standard curves was >0.99, and amplification efficiencies were between 90% and 110%. Data for the FKC group and SHO groups were compared with those obtained from the control group. The relative expression of the target genes was analyzed using the standard $\triangle\triangle C_T$ method. In all cases, each sample was processed in triplicate.

2.12. Statistical analysis

Analysis of variance (ANOVA) tests were used to analyze the data. A Tukey's test was used to analyze differences between experimental groups. OriginPro software (version 8.5; OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis. The significance level was fixed at $P < 0.05$.

3. Results

3.1. Characteristics of SHO vaccine

Before vaccine preparation, the starch was distributed uniformly (Fig. 1A). The starch successfully formed a lumped hydrogel by water absorption and expansion (Fig. 1B). With the addition of an antigen (FKC), FKCs were coated in the starch hydrogel, and SHO vaccine was manufactured (Fig. 1C).

3.2. pH measurements in Japanese eel stomachs and intestines

pH measurements of five Japanese eels revealed that the pH of the stomach was between 2 and 2.4 (average pH: 2.14) and the pH of the intestine was between 7.9 and 8.3 (average pH: 8.06).

3.3. Adaptive immune responses

Prior to immunization experiments, no detectable antibody was found in any groups. In vaccinated groups, FKC or SHO vaccination resulted in an increase of agglutination titer at two weeks post-vaccination (wpv) (Fig. 2). The highest value of the agglutination titer was observed at 3 wpv, and decreased thereafter until the
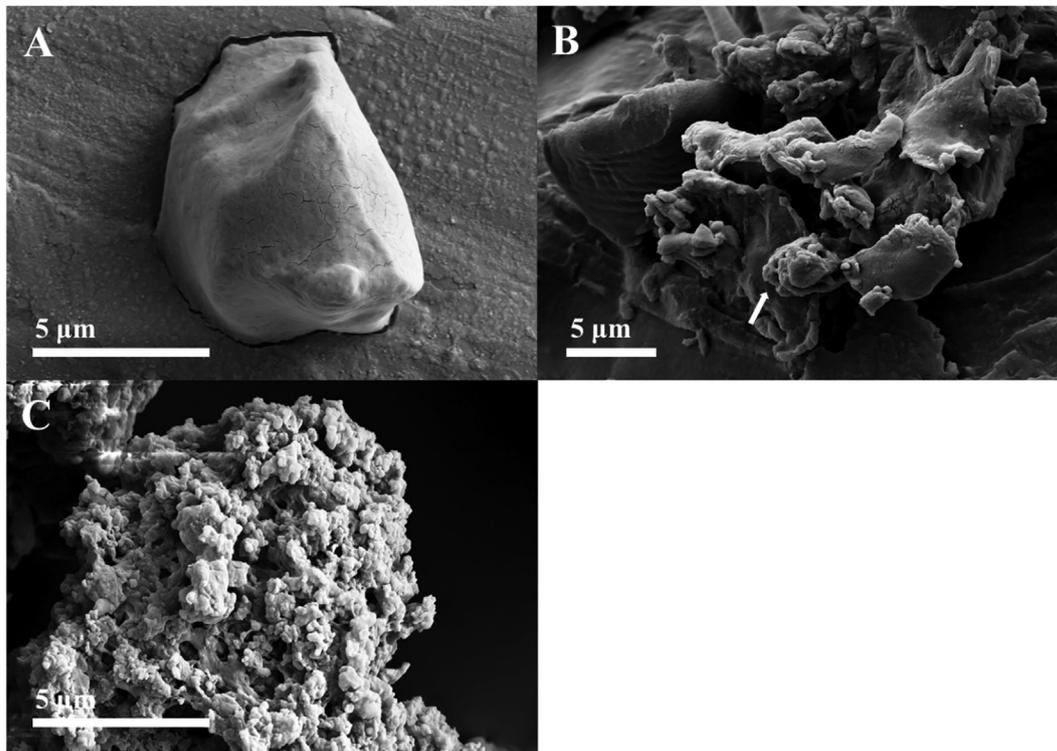
Fig. 1. Morphological analysis of starch hydrogel-based oral (SHO) vaccine. Field emission scanning electron microscopic (FESEM) specimen features: starch prior to forming hydrogel (A); synthesized hydrogel based on starch containing no antigen (an arrow defining porous structure) (B); SHO vaccine: starch-based hydrogel containing formalin-killed cells (*E. tarda* SU53) (C). The scale bar is 5 μm.
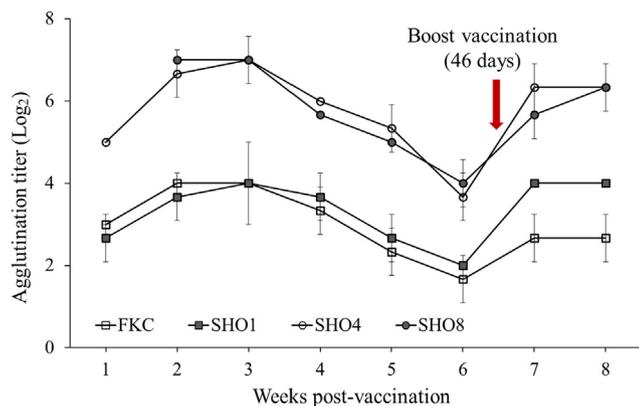

Fig. 2. Serum agglutination titers of Japanese eels after oral administration of the formalin-killed cell (FKC) and starch hydrogel-based oral (SHO) vaccines. Fish were selected from each of five groups orally administrated according to: pellets impregnated with PBS (control); ground pellets mixed with FKC once per day for four days (FKC); and SHO once per day for one, four, and eight days (SHO 1, SHO 4, and SHO 8, respectively). A boost vaccination was performed once at 46 days after the first vaccination with PBS, FKC, or SHO. Bars represent the mean ± standard deviation (*n* = 3). No antibodies were detected in the control group.

second vaccination was performed at 46 days post vaccination. The agglutination titer dramatically increased after the second vaccination, reaching the highest value at 7 wpv in every vaccinated group except SHO 8, in which it reached 8 wpv. SHO vaccination produced a higher antibody titer than FKC vaccination. FKC and SHO 1 resulted in relatively lower titers, although SHO 1 produced a higher titer than FKC after the second vaccination; SHO 4 and SHO 8 produced higher titers. Fish in SHO 4 and SHO 8 showed a similar pattern of change in antibody titers, producing the same value as the highest titer at 3 wpv, and after the second vaccination at 8 wpv.

3.4. Post-vaccination survival assessment

To determine the 15-day LD_{50} concentration of *E. tarda* SU53, the experimental challenge test was repeated twice (data not shown). In the control group and the group administered the lowest concentration (2.0×10^4 CFU/fish), no mortality was observed during experimental infections of fish. In other administered groups, mortality occurred from three days post challenge (dpc), and continued up to 9 dpc. After 9 dpc, challenged fish survived for the rest of the experimental period and were asymptomatic. Survival rates of fish challenged with 2.0×10^5 CFU/fish (70%, 1st trial; 70%, 2nd trial), 2.0×10^6 CFU/fish (40%; 50%), and 2.0×10^7 CFU/fish (0%; 0%) were proportional to the final injection doses. The LD_{50} concentration was calculated using the method described by Reed and Muench [16], and was found to be 2.0×10^6 CFU/fish. All dead fish exhibited typical clinical signs of edwardsiellosis. Bacteria isolated from these fish were cultured on TSA plates, and confirmed as *E. tarda* by PCR, as described above.

The protective effects of both the FKC and SHO vaccines against edwardsiellosis are shown in Fig. 3. Mortality after challenge in all vaccinated groups was lower than that in the control group at 4 wpv. In all groups, the progression of mortality after challenge was consistent with the result of the experimental challenge performed to determine the LD_{50} concentration of *E. tarda* SU53: mortality was recorded from 3 dpc to 9 dpc, and thereafter no further mortality was observed. The survival rates of fish in the FKC group were 60% and 70% in the first and second trials, respectively. The survival rate of SHO-immunized fish was higher than the FKC-immunized group. Survival rates of the SHO vaccination groups were as follows: 70%, first trial, and 80%, second trial in SHO 1; 80% and 90% in SHO 4; and 80% and 90% in SHO 8. There was no significant difference in the survival rate between SHO 4 and SHO 8, which both had the highest and same level of protection.
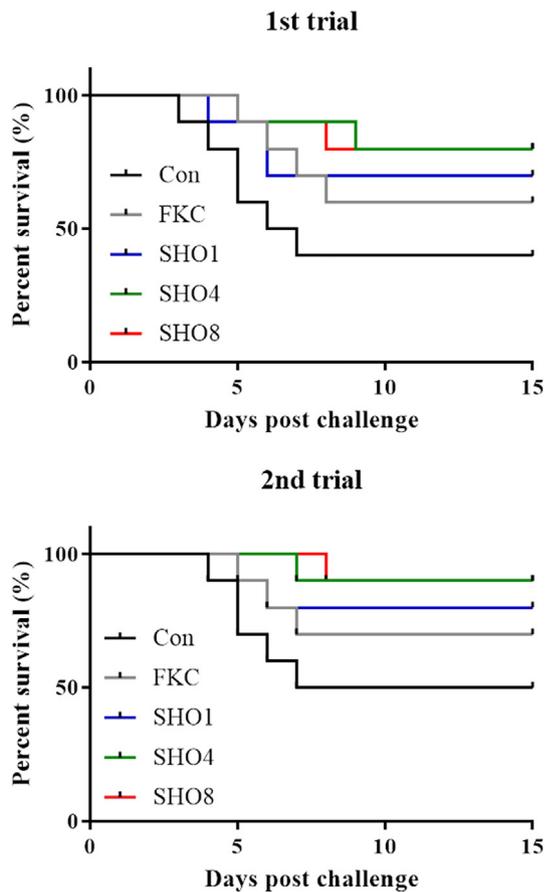
Fig. 3. Kaplan-Meier survival curve of challenge test on Japanese eels. The injection (i.p.) challenge was performed at four weeks after the first vaccination and the percentage survivals in the five groups (control, FKC, SHO 1, SHO 4, and SHO 8) are illustrated (*n* = 10). The challenge experiment was performed in duplicate; the results are shown as the first and second trial, respectively. Control, orally supplied pellets that had been PBS-impregnated only once; formalin-killed cell vaccine (FKC), orally supplied ground pellets mixed with FKC once per day for four days; starch hydrogel-based oral vaccines (SHO) 1, SHO 4, and SHO 8, orally supplied once per day for one, four, and eight days, respectively.

3.5. Effect of vaccination on cytokine gene expression

Fig. 4 shows the cytokine gene expression in the head kidney or liver of Japanese eels in each group after vaccination. In all immunized groups, vaccination increased the level of cytokine genes when compared with the control. SHO vaccination produced a higher level of cytokine gene expression than FKC vaccination; its expression level was higher in SHO 4 and SHO 8 compared with SHO 1 (Fig. 4). Cytokine gene expression in SHO 4 and SHO 8 increased at 3 wpv, decreased at 4 wpv, and increased again at 8 wpv, with the highest level observed in SHO 8 at 8 wpv. The difference in cytokine gene expression over time was not significant in SHO 1 or the FKC group. However, IL-6 expression tended to increase in these two groups from 3 wpv to 8 wpv. IFN-α expression in SHO 1 and the FKC group varied in the same way as in SHO 4 and SHO 8, increasing at 3 wpv, decreasing at 4 wpv, and increasing again at 8 wpv. TNF-α expression showed an increasing tendency in the FKC group, but in SHO 1 decreased between 3 and 4 wpv, before increasing by 8 wpv.

4. Discussion

To date, several vaccines have been reported to be effective against edwardsiellosis [8,17,18]. These vaccines are administered
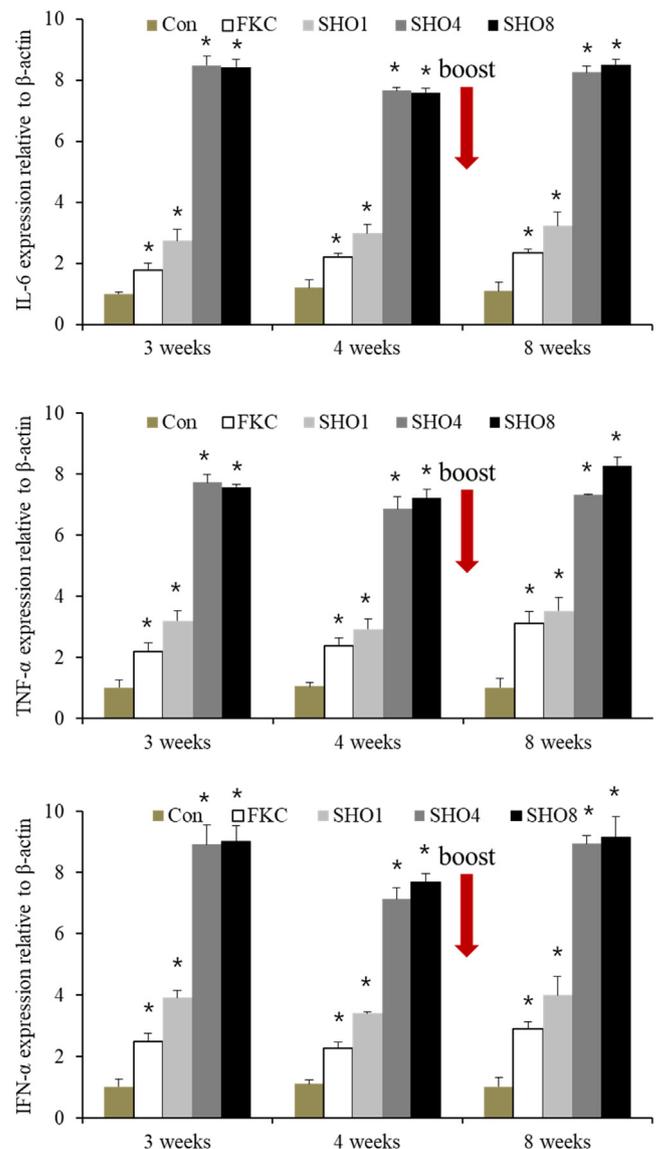

Fig. 4. Relative mRNA expression of pro-inflammatory cytokines (interleukin (IL)-6, tumor necrosis factor (TNF)-α) in the head kidney, and host defense cytokine (interferon (IFN)-α) in the liver of Japanese eels after vaccination with either formalin-killed cell (FKC) or starch hydrogel-based oral (SHO) vaccines. There were three SHO groups: SHO 1, SHO 4, and SHO 8, which orally supplied with SHO once per day for one, four, and eight days, respectively. A boost vaccination was performed 46 days after the first vaccination (red arrows). Bars represent the mean ± standard deviation (*n* = 3). Significant differences when compared with the control (Con) are indicated by an asterisk (*p* < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by injection, which is generally considered more effective than oral and immersion routes. However, injection vaccines have critical disadvantages [19]. Vaccine injection requires a professionally trained vaccinator, causing additional costs, including labor. It is also stressful to fish, causing immediate appetite reduction after injection [20,21], and is therefore economically damaging. For these reasons, many Japanese eel aquaculturists reject the use of injected vaccines. The preliminary experiment by i.p. injection of vaccine had not improved the survival of fish: vaccine-injected fish lost appetite and eventually died being lethargic. It was considered that an injectable vaccine cannot be an ideal vaccine candidate in stress-sensitive fish species such as Japanese eel. Another approach, vaccination by immersion, has been proposed. However, although this method is less stressful for fish than injection, it

requires a large quantity of vaccine [22]. In the current study, we endeavored to develop an oral vaccine, administered through feeding, because oral vaccines do not have the same disadvantages as the injection and immersion vaccination methods [19]. However, oral vaccination does have a disadvantage, in that its efficacy is inconsistent [19]. We speculated that multiple administration of oral vaccine may minimize this inconsistency; determining the optimal administration schedule for oral vaccine is therefore the primary requisite in disease prevention.

In the initial phase of our study, we attempted to form hydrogels because of their porous structure [13]. The pores enable water to permeate and interact with external stimuli, which can facilitate antigen release from the hydrogel-based complex. The starch-based compound was superabsorbent [23], and could therefore function as an antigen reservoir. The low efficacy of oral vaccines is usually caused by a number of vaccination-administered antigens being denatured in the stomach. The quantity of antigens released from the starch hydrogel-based compound is dependent on the pH of the environment: the antigen quantity decreases and increases at low and high pH values, respectively [13,23]. SHO was therefore expected to be a suitable candidate for oral vaccine in Japanese eels, because the species was shown to have a low pH (ca. 2) in the stomach and high pH (ca. 8) in the intestine. In order to develop an oral vaccine, we therefore focused on the starch hydrogel-based technique given its major potential as an oral vaccine in aquaculture.

It has been reported that the Japanese eel antibody titer peaks at 4 wpv when the fish are i.p. injected with aquaculture vaccines [8]. A similar result has also been shown in an immunization model used in rainbow trout, in which the antibody titer peaked at 4 wpv [24]. However, the results of the injection challenge testes performed in our study did not show a maximum antibody titer at 4 wpv, in contrast with these previous reports [8,24]. Although we considered it desirable to perform bacterial challenge tests at different time points, such as 8 wpv, in order to demonstrate the higher immunization effect, 4 wpv was ultimately selected as suitable for providing a realistic assessment of the protective effect of the SHO vaccine. Indeed, the antibody titer at 4 wpv was lower than after the boost vaccination, indicating that maintaining this level is a feasible goal in Japanese eel aquaculture farms.

Our results revealed that the SHO vaccine conferred protective effects against edwardsiellosis in Japanese eels, resulting in a higher rate of survival than conventional FKC vaccination. Consistent with the result of the bacterial challenge test, trends suggestive of higher agglutination titers were observed in groups administered SHO, compared with the FKC group. However, slightly higher agglutination titers were observed in SHO 1 than in the FKC group at 1–3 wpv. We found that multiple SHO administrations produced better efficacy, but there was no significant difference in disease prevention between SHO 4 and SHO 8. It was therefore concluded that administering the vaccine four times maximizes the effect of the SHO vaccine. However, administering the vaccine more than four times is not cost-effective. Furthermore, ease-of-additional-vaccination is a strength of oral vaccination, which is an essential characteristic when managing long-term aquacultured species, including Japanese eel. We therefore tested the effect of providing a boost SHO vaccine in Japanese eels, and demonstrated that subsequent SHO vaccinations following the initial administration enhanced the degree of protection against edwardsiellosis. Additionally, it was proved that a single boost vaccination was enough to achieve a similar or even higher protection against edwardsiellosis than the protection afforded after the initial vaccination.

Pro-inflammatory cytokines, such as IL-6 and TNF-α, play an important role in antigen-specific immune responses in fish, especially against pathogenic bacteria, by causing immune inflammation [25]. In the current study, upregulation of IL-6 and TNF-α expression after SHO vaccination was observed in the head kidney of Japanese eels as previously described [15]. This indicated that the SHO vaccination induces a stimulatory action upon pro-inflammatory processes, and is a potent stimulator of cytokine secretion. Oral tolerance is, however, a hindrance in oral vaccine performance. Antigens administered by oral vaccination induce oral tolerance in fish guts, which weakens the immune responses to the antigens [19]. A desirable oral vaccine characteristic is therefore the ability to break down oral tolerance and stimulate immune inflammation, enabling strong immune responses to the antigen. The results of the IL-6 and TNF-α stimulation in our study demonstrated that SHO vaccine is an excellent oral vaccine for use with Japanese eels, without risk of oral tolerance.

IFNs are multifunctional cytokines, which are induced in response to pathogen infection, especially viral infection [26]. It has been reported that type-1 interferon (IFN-α) is important in host defense against both viral and bacterial infections in fish, such as the Japanese flounder [27], rock bream [28], and Japanese eel [14], although the functional role of IFN-α in fish in relation to bacterial infection is only recently discovered and not yet fully understood, in contrast with the good understanding of its role in mammals [14]. In our study, we observed the Japanese eel liver to have a significantly higher expression of IFN-α than any of its other organs [14], which is why we selected the liver for IFN-α expression analysis. Liver samples yielded a higher IFN-α expression than kidney samples (data not shown), which is in agreement with a previous report [14], in which upregulation of IFN-α expression in the liver revealed that its function is to participate in the antibacterial immune response [14]; we found a similar result.

Additive materials, such as chemical substances added during the manufacture of oral vaccine, may alter the flavor or smell of the feed, leading to appetite impairment in the fish. Although likely to occur generally during oral vaccine development, the SHO vaccine prepared in our study did not cause this issue. The last hurdle for commercialization of the SHO vaccine we developed is the need for further research. In particular, studies into its practical application in Japanese eel aquaculture farms, involving the selection of volunteer farms, are required in order to assess the efficacy of SHO vaccine in field aquaculture conditions.

CRediT authorship contribution statement

Jin Woo Jun: Data curation, Writing - original draft, Writing - review & editing. **Jeong Woo Kang:** Methodology, Investigation, Data curation. **Sib Sankar Giri:** Software, Visualization. **Saekil Yun:** Validation, Visualization. **Hyoun Joong Kim:** Methodology, Investigation. **Sang Guen Kim:** Software, Visualization. **Sang Wha Kim:** Methodology, Investigation. **Se Jin Han:** Methodology, Investigation. **Jun Kwon:** Methodology, Investigation. **Woo Taek Oh:** Investigation, Software. **Se Chang Park:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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