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Immunogenicity of the LrrG protein encapsulated in PLGA microparticles in Nile tilapia (*Oreochromis niloticus*) vaccinated against *Streptococcus agalactiae*

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ABSTRACT

LrrG protein is one of the conserved surface proteins of *Streptococcus agalactiae*. It has been found exists in all kinds serotypes of *S. agalactiae* strains. Preliminary experimentation showed that LrrG protein of *S. agalactiae* isolated from tilapia protected tilapia from *S. agalactiae* infection. In order to investigate the immune protective effects of LrrG protein of *S. agalactiae* encapsulated by poly-(D, L-lactic-*co*-glycolic) acid (PLGA) on tilapia, PLGA microparticles containing LrrG protein were prepared by double emulsion-solvent evaporation. The average diameter of PLGA-LrrG protein microparticle was 4.5 μm, the encapsulation efficiency was 38.54%, the drug loading was 1.98%, and the cumulative rate of drug-release over 28 days was 78.97%. Healthy tilapias were immunized with PLGA-LrrG microparticles by intraperitoneal injection or oral administration. The results show that the relative percent survival (RPS) of vaccinated groups from both intraperitoneal injection and oral administration were significantly higher than that of the control groups. Although in the same dose group, the RPS from intraperitoneal injection was generally higher than that from oral administration, one microgram per gram (1 μg/g) PLGA-LrrG microparticle also showed good immune protection and RPS (77.54%) in oral administration. These results suggest that PLGA-LrrG microparticles can be used to protect tilapia from *S. agalactiae* infection and it may have practical value as an orally administered genetically engineered vaccine against streptococcosis in tilapia.

1. Introduction

Continual outbreaks of streptococcal diseases in tilapia culture have severely affected the sustainable development of the Chinese tilapia industry in recent years. The prevention and control of streptococcosis has become a major focus and challenge in the tilapia industry. *Streptococcus agalactiae* is one of the major causes of streptococcosis in tilapia (Fang et al., 2016). *S. agalactiae* is a gram-positive bacterium with spherical or oval morphology and paired- or short-chained arrangement. It exhibits negative catalase and oxidase activities, positive Christie, Atkins, and Munch-Petersen (CAMP) test results, and exhibits beta hemolysis (Liu et al., 2013). The Lancefield serotype classification system, in which streptococci are divided into ten serotypes (Ia, Ib, and II–IX) based on antigenic differences of capsular polysaccharides, classifies *S. agalactiae* as Group B streptococcus (Slotved et al., 2007). *S. agalactiae*, which induces streptococcosis, includes three serotypes, including Ia, Ib, and III, of which Ia is the most common causative pathogen of streptococcosis in tilapia in China (Li et al., 2013; Zhang et al., 2015). Vaccination is considered the most effective means of preventing and controlling streptococcosis. However, there is a dearth of effective cross-protective vaccines for different *S. agalactiae* serotypes (Chen et al., 2012).

Leucine-rich repeat protein (LrrG protein) is one of the most conserved surface proteins of *S. agalactiae* serotypes and exhibits an abundance of the leucine-rich repeat (LRR) protein structural motifs (Seepersaud et al., 2005). For this reason, LrrG has been considered an ideal candidate target for vaccine antigen and immunoassay development. Yu et al. (2007) showed that the LrrG protein has good immunogenicity in mice and is one of the important surface proteins in human *S. agalactiae*. In an earlier work, Chen et al. (2014) immunized tilapia with naked LrrG recombinant protein of *S. agalactiae* isolated from tilapia. The results showed that immunized tilapias were resistant to *S. agalactiae* infection, with a relative immune protection rate of 69.28%.

In 1979, Preis and Langer used poly-(lactic-*co*-glycolic acid) (PLGA) microspheres in a single-step immunization with tetanus toxoid. This

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procedure was the first of its kind to be approved by the World Health Organization (WHO) (Preis and Langer, 1979). PLGA is a lactic acid-glycolic acid copolymer formed by the irregular polymerization of lactic acid and glycolic acid. It is non-toxic and non-stimulating, biodegradable, biocompatible, and it exhibits slow-release properties, for which reasons it was approved by the U.S. Food and Drug Administration as an alternative to biological tissues and as a drug carrier (Makadia and Siegel, 2011; Sonam et al., 2013). PLGA microspheres have several advantages that make them useful drug carriers. They can encapsulate vaccines, peptides, proteins, and small-molecule drugs and have broader routes of administration, require fewer administrations and lower doses, foster longer drug action to several months, and foster better control of drug release than other methods (Jeffery et al., 1993; Makadia and Siegel, 2011). They can also improve drug stability, reduce toxicity and stimulation, and maintain protein immunogenicity (Derman et al., 2015). This makes PLGA microspheres attractive carriers in vaccine applications. In large-scale industrial fisheries, a number of unique factors are important to evaluating the efficacy of immunization. Oral immunization involves less damage to the fish, less labor, and less time than other methods and is thus thought to be the best fit for aquatic animal immunization. Many studies have indicated that PLGA application technology, which involves an adjuvant encapsulating immunogens in oral vaccine preparations, is relatively safe and efficient (Dubey et al., 2016; Sarti et al., 2011). Here, we studied the efficacy of PLGA application as an oral vaccine in the prevention and control of important diseases of aquatic animal and its convenience of administration and ability to improve treatment efficacy.

The present study builds on our previous reports, which evaluated purified LrrG soluble recombinant protein as an antigen and PLGA as adjuvants in the preparation of PLGA-LrrG protein microspheres. Here, we injected or fed microspheres to tilapia to immunize them and evaluated the immunoprotective effect of the PLGA microsphere vaccine. The present study provides a foundation for the development of highly effective oral vaccines against *S. agalactiae* infection in tilapia.

2. Materials and methods

2.1. Experimental strains and animals

The virulent *S. agalactiae* strain LZ1F originating from tilapia was isolated from *Oreochromis niloticus* (GIFT strain), identified, and preserved at the Breeding and Disease Research Laboratory, National Tilapia Industrial Technology System, China. Experimental *O. niloticus* (average bodyweight of 6.0 ± 1.2 g) were provided by the Gaoyao Aquatic Germplasm Engineering Base of the Pearl River Fisheries Research Institute of the Chinese Academy of Fishery Sciences, Guangdong Province, China. All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee at the research facility.

2.2. LrrG protein and PLGA

LrrG protein was purified and preserved in our laboratory. It exhibited a protein molecular weight (Mw) of 108.9 kDa and a concentration of 3.40 mg/mL (Chen et al., 2014). Poly-(D, L-lactide-*co*-glycolide) (LA: GA = 75:25, Mw = 50,000–75,000 Da) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Polyvinyl alcohol (PVA-1788, Mw = 88,000 Da) was purchased from Amresco (Solon, OH, U.S.).

2.3. PLGA-LrrG protein microsphere preparation and detection of encapsulation efficiency and drug loading capacity

The preparation of PLGA-LrrG protein microspheres was primarily based on the methods described in Gao et al. with slight modifications (Gao et al., 2015). The procedures were as follows: 100 mg of PLGA was dissolved in 4 mL dichloromethane, followed by addition of 1 mL LrrG protein after the PLGA was completely dissolved. The mixture was vortexed with high-speed oscillation for 5 min using a vortex mixer to form the primary emulsion. The emulsion was then added to 1 mg/mL PVA aqueous solution (containing 5% NaCl, W/V) and subjected to 8 min of high-speed vortex oscillation to produce water-in-oil-in-water (W/O/W) double emulsions. The double emulsions were added to 80 mL 5% NaCl solution, followed by mixing for 5 to 6 h using a magnetic stirrer (JB-1, Shanghai Lei Magnetic Instrument Factory, China) to volatilize methylene chloride followed by centrifugation of the microsphere suspension at 5000 rpm and 4 °C for 5 min to collect the microspheres. After three washes in distilled water, the microspheres were freeze-dried for 24 h (LGJ-12 Standard laboratory freeze dryer, Beijing SongYuan Huaxing Technology Develop. Co., Ltd., Beijing, China) and preserved at 4 °C.

Appropriate amounts of freeze-dried PLGA-LrrG microspheres were rehydrated in water, followed by observation with light microscopy and scanning electron microscope (Quanta 200 SEM, Holland). The diameters of 50 random microspheres were measured to find the distribution of the particle sizes and calculate mean sizes. Ten milligrams of PLGA-LrrG protein microsphere was weighed and added to 2 mL of a 0.1 mol/L NaOH solution (containing 5% SDS, W/V) and oscillated at 37 °C until the microspheres were completely dissolved. The bicinchoninic acid (BCA) assay (Shanghai Biocolors BioScience and Technology Co., Ltd., Shanghai, China) was used to quantify the protein concentration in the supernatant after addition of LrrG protein and to determine protein concentration in the dissolved microsphere solution in order to calculate the encapsulation efficiency and drug loading capacity of the microspheres (as calculated below).

Encapsulation efficiency (%)
= (protein content of microsphere dissolved in solution
/LrrG protein content used in microsphere preparation) × 100%

Drug loading capacity (%)
= (protein content of microspheres dissolved in solution
/microsphere weight) × 100%

Bovine serum albumin (BSA) was used as the standard protein to prepare standard curves for the BCA protein quantification assay and to identify the concentration of soluble protein in the PLGA-LrrG protein microsphere solutions.

2.4. Calculation of in vitro release of PLGA-LrrG protein microspheres

Ten micrograms of microspheres were weighed and added to 4 mL PBS (pH 7.4) buffer to prepare a slow-release medium that was oscillated with 150 rpm at 30 °C and then centrifuged for 1 h, 3 h, 5 h, 8 h, 18 h, 1 d, 2 d, 3 d, 5 d, 7 d, 9 d, 13 d, 19 d, and 28 d. Two milliliters of supernatant were then used to quantify protein concentration using the BCA assay. The suspension was supplemented with 2 mL PBS each time the supernatant was removed. The analysis was performed in triplicate (Yeh et al., 2002). The cumulative release of microspheres was quantified using the following equation:

$$\text{The nth cumulative release content}(\%) = \frac{C_n \times 4 + \sum_{i=1}^{n-1} Ci \times 2}{W \times X}$$

Here, C_n refers to the protein concentration of slow-release medium measured at the nth point in time; Ci refers to the protein concentration of slow-release medium measured at the ith point in time; W refers to the total weight of the injected microspheres; and X refers to microsphere loading content.

2.5. Immunoprotection assays of tilapia PLGA-LrrG protein microspheres

Healthy GIFT tilapias of the same size (bodyweight of 6.0 ± 1.2 g)

were fed for a week and then randomly divided into 30 groups (65 fish each group) for later experiments. Each fish was fed a quantity equal to 3% of its body weight twice daily and maintained with a 24 h oxygen supply and a 30% water change every two days. The temperature of the aquarium was maintained at 30 ± 2 °C. The sampled fish were examined to confirm the absence of Group B *Streptococcus* (GBS) infection before the start of the experiment.

The protein microspheres were diluted into three vaccination doses based on the PLGA-LrrG protein microsphere loading capacity and vaccination dose: 0.5 μg/g (protein content/fish bodyweight) (P1 group), 1 μg/g (P2 group), and 2 μg/g (P3 group) concentrations. The vaccinations were used in a single-step administration through either intraperitoneal injection or oral/intragastric administration into the experimental tilapia (100 μL/fish). Oral/intragastric administration was done by intubation. Fish were intraperitoneally or orally vaccinated only one-time after an adaptation period and then normal feeding was administered thereafter. Fish in the control groups were treated instead with PBS (CK1 group) and empty PLGA microspheres (CK2 group). Each group had three parallel repetitions. After immunization, three fish were randomly selected from each group each week and sera were collected from blood samples in the following steps: a 1 mL sterile syringe was used to collect venous blood samples from the fish's tails and each sample was placed in a 1.5 mL centrifuge tube. These tubes were then incubated at room temperature for 1–2 h and stored at 4 °C. The blood samples were then centrifuged at 3000 rpm and 4 °C for 15 min to collect sera from the supernatants. These serum samples were preserved at − 80 °C for later experiments.

The medium lethal dose experiment was carried out at the same time as the vaccination trial. All the animals used in the study are healthy GIFT tilapias which have the similar size and source. The median lethal dose of *S. agalactiae* LZ1F was determined by preparing four bacterial concentrations (1×10^5 CFU/mL, 1×10^6 CFU/mL, 1×10^7 CFU/mL, and 1×10^8 CFU/mL). The diluted *S. agalactiae* LZ1F inocula (100 μL) were then injected intraperitoneally into each fish (a total of 10 fish per concentration) and these fish were monitored for 14-day mortality. The Bliss method was used to calculate the median lethal dose of *S. agalactiae* LZ1F (LD_{50}) (Bliss, 1938).

Four weeks after the single-step immunization, the GBS median lethal dose determined above (LD_{50}) was used to intraperitoneally challenge all immunized fish (100 μL/fish). The number of deaths in each group was continuously monitored to calculate the relative percent survival (RPS) after two weeks according to the following equation: RPS = (1 − mortality of the experimental group / mortality of the control group) × 100%.

2.6. Analysis of serum antibody levels in tilapia

Tilapia sera collected during 1–4 weeks were used to evaluate antibody responses after immunization, but before GBS challenge. Sera collected between 5 and 6 weeks were used to evaluate antibody responses both after vaccination and challenge. All antibody responses were measured by ELISA using the steps described as follows: coating buffer was used to dilute the LrrG soluble protein to 10 ng/μL and 100 μL of diluted LrrG was added to each well of a 96-well plate and incubated overnight at 4 °C. After washing in PBST, blocking solution was added to each well of a 96-well plate and incubated at 37 °C for 2 h. After the wells were washed with PBST, the tilapia serum samples were added to the wells based on gradient dilution (the first well was diluted 1:100), and incubated at 37 °C for 1 h. After washing in PBST, 1:1000 HRP-labeled rabbit anti-tilapia IgM antibody (Nanjing Zoonbio Biotechnology Co., Ltd., Jiangsu Province, China) was added to each well and incubated at 37 °C for 1 h, then washed in PBST and developed with 3,3′,5,5′-tetramethylbenzidine (TMB) for 30 min. The reaction was stopped by addition of 2 mol/L H_2SO_4. The optical density of each sample was measured at 450 nm using a microplate reader (Sunrise, TECAN, Germany).

In addition, serum lysozyme activity was measured simultaneously using the blank control method (Lysozyme Activity Assay Kit, Nanjing Jiancheng Bioengineering Institute, Jiangsu Province, China) in which activity is measured as follows:

Lysozyme activity (U ml) = (detected absorbance − blank absorbance)/(standard absorbance − blank absorbance) × standard concentration (200 U/mL) × dilution ratio of sample before detection.

2.7. Statistical analysis

SPSS 20.0 and Microsoft Excel 2010 were used for data sorting and analysis. One-way ANOVA was used to determine the statistical significance of differences among sample groups. $P < 0.05$ was considered significant and $P < 0.01$ highly significant.

3. Results

3.1. Particle size, encapsulation efficiency, and drug loading capacity of PLGA-LrrG protein microspheres

PLGA-LrrG protein microspheres were spherical, smooth surface and lack of porosity (Fig. 1). All particle formulations had an average size between 2.1 and 7.3 μm, with > 90% of the particles in each batch being < 4.5 μm. The encapsulation efficiency and drug loading capacity of the PLGA-LrrG protein microspheres were 38.54% and 1.98%, respectively.

3.2. In vitro release curve of PLGA-LrrG protein microspheres

Time-series analyses showed PLGA-LrrG protein microspheres to

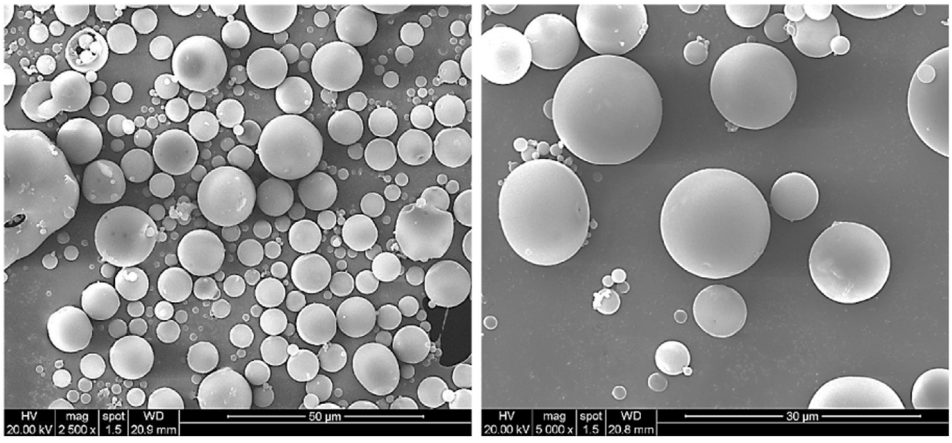

Fig. 1. Scanning electron micrographs of PLGA microspheres incorporating LrrG.

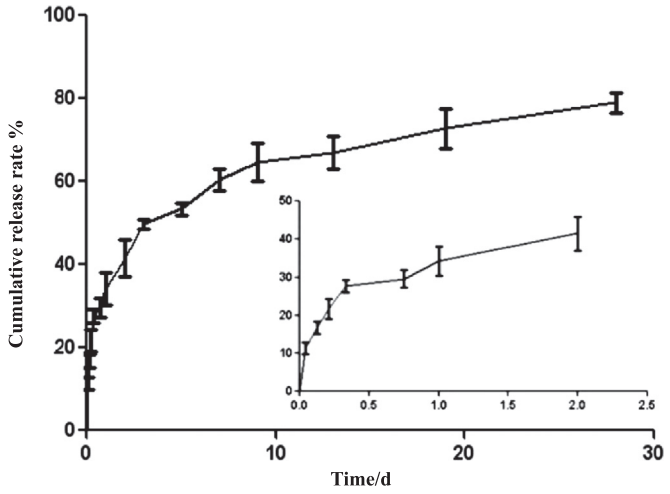

Fig. 2. *In vitro* release profile of the PLGA microparticles containing LrrG protein. The inset shows release during the first 2 d.

have slow-release properties. Encapsulated LrrG proteins were released in bursts and sustained stages. The microsphere-burst-release stage occurred in the first 8 h, accounting for 27.74% of the total release protein, followed by a 28 d sustained-release stage, with the cumulative release protein accounting for 78.97% of the total (Fig. 2).

3.3. Immunoprotective effect of PLGA-LrrG protein microspheres in tilapia

Four weeks after the single-step immunization, we used the GBS median lethal concentration LD_{50} (2.1×10^6 CFU/mL) to challenge the tilapia. Fish began to die two days after the GBS challenge. Table 1 shows the mortality of fish in each group and the RPS of the vaccinations in each group. Different protein microsphere experimental groups exhibited different immunoprotective effects. The RPS values of the injection groups were greater than that of the oral administration groups. Injection groups given 0.5 μg/g (protein content/fish bodyweight) and 1 μg/g PLGA-LrrG protein microspheres showed relatively high immunoprotective rates, 77.89% and 77.81%, respectively. Fish intraperitoneally injected with 2 μg/g PLGA-LrrG protein microspheres showed a 71.65% RPS. In addition, 1 μg/g PLGA-LrrG of orally administered protein microspheres (RPS: 77.54%) had a significantly higher immunoprotective rate than the fish given 0.5 μg/g (RPS: 50.97%) and 2 μg/g (RPS: 60.24%). Control groups given intraperitoneal injection and oral administration of empty microspheres (CK2) also exhibited minor immunoprotective effects, with the RPS values of 3.27% and 8.85%, respectively.

3.4. Analysis of serum antibody levels in tilapia

Indirect ELISA was used to measure the serum antibody levels in tilapia after immunization. ELISA results of the intraperitoneal injection and oral administration groups showed that fish immunized with PLGA-LrrG protein microspheres had significantly elevated levels of serum antibodies, and these serum antibody levels remained stable or declined at the third week after immunization(Fig. 3A–B). Serum antibody levels in different groups at the fourth week after GBS challenge were greatly increased, with the OD of serum antibody reaching 0.9133 in the 1 μg/g PLGA-LrrG protein microsphere oral administration group. The OD of serum antibody was as high as 1.0577 in the 2 μg/g PLGA-LrrG protein microsphere intraperitoneal group. Serum antibody levels in the protein microsphere experimental groups were significantly different from the PBS control groups ($P < 0.05$). No significant difference in serum antibody levels was found between the PBS and empty PLGA microsphere groups ($P > 0.05$).

3.5. Analysis of serum lysozyme activity in tilapia

The serum lysozyme activities in tilapia of the protein microsphere immunized groups were significantly ($P < 0.05$) or very significantly ($P < 0.01$) higher than in the control groups (Fig. 4). They showed gradual increasing trends in all groups after immunization. At the fourth week after GBS challenge, the lysozyme activities in the immunized groups greatly increased, with a maximum lysozyme activity of 347.59 U/mL in the 2 μg/g PLGA-LrrG protein microsphere intraperitoneal injection group (Fig. 4A). The lysozyme activity (306.04 U/mL) of the 1 μg/g PLGA-LrrG protein microsphere oral administration group was significantly higher than either the 0.5 μg/g or the 2 μg/g PLGA-LrrG protein microsphere oral administration groups (Fig. 4B). No significant difference in serum lysozyme activity was observed between the empty PLGA microsphere and PBS groups ($P > 0.05$).

4. Discussion

Vaccination is one of the most effective means for the prevention of infectious diseases. The surface protein LrrG of *S. agalactiae* has demonstrated good immunogenicity and it has been shown to be an ideal antigen candidate for protein vaccines and it has drawn considerable focus from researchers studying the prevention of streptococcal infection (Seepersaud et al., 2005). LrrG protein is a conserved surface protein of *S. agalactiae* and research has shown that human *S. agalactiae* LrrG protein has good immunogenicity in mice (Yu et al., 2007). Purified LrrG soluble recombinant protein of tilapia *S. agalactiae* has been obtained in previous works, and it has been characterized as having a Mw of 108.9 kDa (Chen et al., 2014).

Studies have shown that single-step administration of the new generation of vaccines, including pure proteins and peptides, generally produces low immunogenicity (Behera et al., 2010). However, this can be improved by use of adjuvants. Adjuvants of traditional vaccines such as micro-particles, microbial products, and oil emulsions have been studied extensively (Gupta and Siber, 1995) but they have some deleterious side effects (Mutoloki et al., 2006). There is a need for safe and highly effective vaccine adjuvants. Polymer microspheres have great

Table 1
Immunoprotection of PLGA-LrrG microspheres against *S. agalactiae* infection in tilapia.

	Intraperitoneal injection					Oral administration				
	P1	P2	P3	CK1	CK2	P1	P2	P3	CK1	CK2
Immunization dose (μg g^{-1})	0.5	1	2	PBS	Empty PLGA microsphere	0.5	1	2	PBS	Empty PLGA microsphere
Number of fish challenged	126	136	131	65	72	135	131	111	76	78
Number of fish survived	114	123	115	37	42	108	119	93	45	49
Mortality (%)	9.52	9.56	12.22	43.08	41.67	20.00	9.16	16.22	40.79	37.18
RPS (%)	77.89	77.81	71.65	/	3.27	50.97	77.54	60.24	/	8.85

Note:P1 represents 0.5 μg/g (protein content/fish bodyweight), P2 represents 1 μg/g (protein content/fish bodyweight), P3 represents 2 μg/g (protein content/fish bodyweight), CK1 represents the control groups which treated instead with PBS, and CK2 represents the control groups which treated instead with empty PLGA microspheres.

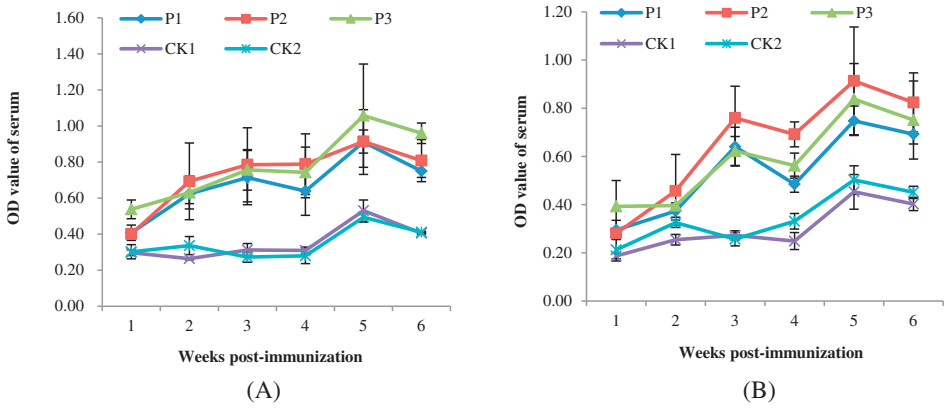

Fig. 3. Serum antibody level in tilapia immunized by (A) intraperitoneal injection or (B) oral administration. P1 represents 0.5 μg/g (protein content/fish bodyweight), P2 represents 1 μg/g (protein content/fish bodyweight), P3 represents 2 μg/g (protein content/fish bodyweight), CK1 represents the control groups which treated instead with PBS, and CK2 represents the control groups which treated instead with empty PLGA microspheres.

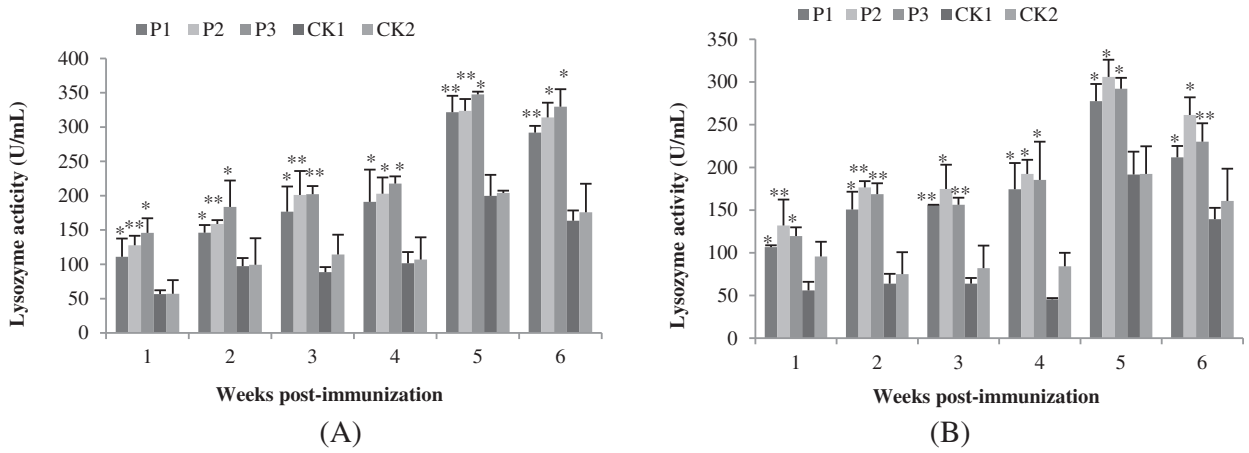

Fig. 4. Lysozyme activity in tilapia immunized by (A) intraperitoneal injection or (B) oral administration. P1 represents 0.5 μg/g (protein content/fish bodyweight), P2 represents 1 μg/g (protein content/fish bodyweight), P3 represents 2 μg/g (protein content/fish bodyweight), CK1 represents the control groups which treated instead with PBS, and CK2 represents the control groups which treated instead with empty PLGA microspheres. * $P < 0.05$, ** $P < 0.01$ relative to the control group.

potential to become a new generation of biocompatible, cost-effective adjuvants due to their ability to effectively encapsulate antigens and mediate slow release of those antigens (Behera et al., 2010). Unlike other carrier-type adjuvants, polymer microspheres are not toxic and can cause *in vivo* humoral immunity and cellular immunity (Men et al., 1995). Mammal experiments have confirmed that polymer microsphere adjuvant reduces the number of immunizations and antigen dosages needed and also improves immune response in mammal bodies (Eldridge et al., 1990; Lawlor et al., 2016; O'Hagan et al., 1991). PLGA in particular has been widely used in the sustained release of proteins, peptides, and DNA vaccines due to its good histocompatibility, non-toxicity, and biodegradability (O'Donnell et al., 1996; Pandey et al., 2016; Zhang et al., 2016). In addition, PLGA protects the *in vivo* hydrolysis of soluble antigens (Peacock et al., 2005). Moreover, its hydrolysis showed no correlation with *in vivo* enzyme activity. Hydrolysate lactic acid and glycolic acid are harmless *in vivo*. They enter the tricarboxylic acid cycle and are eventually excreted from the body as water and carbon dioxide (O'Hagan et al., 1991; Jiang et al., 2005). Experiments have confirmed that PLGA microsphere adjuvant increases immunity to a greater extent than other adjuvants do (Cleland et al., 1997; O'Hagan et al., 1995; Singh et al., 1995) and it can prolong the release of immunogens in animals effectively (McKeever et al., 2002). Tilapia-related PLGA vaccine experiments by Gao et al. (2015) showed that although the relative immunoprotection rate of the inactivated vaccine against *S. agalactiae* (94.53%) was significantly higher than the rate achieved through oral administration of the whole bacterial PLGA microsphere (57.63%), the antibody levels in the fish that were given the vaccine orally is maintained at relatively high levels compared to antibody levels of the vaccinated group 14 days post-treatment. These results highlight the slow-release properties of PLGA and the advantages of longer-term prevention.

The ideal PLGA vaccine microsphere should be of suitable size for oral administration with high encapsulation efficiency and drug loading capacity in order to minimize the dosage needed for immunization (Behera et al., 2010). Tabata et al. showed that orally administered microspheres smaller than 10 μm particle size can be absorbed by intestinal associated lymphoid tissue and microspheres smaller than 5 μm can also be delivered to the spleen and other lymphoid tissues (Eldridge et al., 1990; Tabata et al., 1996). The PLGA-LrrG protein microspheres generated *via* double emulsion solvent evaporation techniques in this study had an average particle diameter of 4.5 μm, which was found to facilitate microsphere digestion and absorption in the intestine. Importantly, these PLGA-LrrG protein microspheres are also suitable for oral immunization. Behera et al. (2010) and Panyam et al. (2003) prepared PLGA protein vaccines with an encapsulation rate of approximately 25%. PLGA-LrrG protein microspheres in the present study had an encapsulation rate and drug loading capacity of 38.54% and 1.98%, respectively, which were better than values reported in above studies. However,some other peptide PLGA nanoparticle vaccine in human and mice with an encapsulation rate was approximately 50–74% (Chong et al., 2005; Manish et al., 2013), which suggest the encapsulation rate of PLGA-LrrG protein microspheres could be further improved. In addition, while these microspheres are not readily internalized by cells, they are retained in the tissue, thus providing prolonged antigen release (Agarwal and Mallapragada, 2008). Therefore, the released antigen can transfect the cells at the delivery site with the

protein product acting locally or distributed systematically. Our *in vitro* slow-release curve indicated that the cumulative release rate of 28-day LrrG protein reached 78.97%, suggesting that most protein antigens were released slowly. This provides the basis for the use of slow-release protein in the vaccination of tilapia and suggests the possibility of continuous immunization.

Lysozyme is one of the most important non-specific immune substances in peripheral blood. It plays an important role in mitigating bacterial infection in fish. Lysozyme activity partially determines the magnitude of *in vivo* non-specific immune response. Antibodies are specifically generated in the body after antigen stimulation, depending on the specific immune response. In this study, the serum antibody levels and lysozyme activities of tilapia after intraperitoneal injection or oral administration of PLGA-LrrG protein microspheres were significantly higher than those of the control groups. However, groups vaccinated by injection using PLGA-LrrG protein microspheres had higher serum antibody levels and lysozyme activities than the orally vaccinated groups. No significant differences in serum antibody levels or lysozyme activity were observed in groups exposed to empty PLGA microsphere and PBS. These results are consistent with the relative percent survivals in different experimental groups after GBS challenge. After GBS challenge, fish given different doses (0.5 μg/g, 1 μg/g, and 2 μg/g) of PLGA-LrrG protein microsphere injections had similar relative percent survivals (77.89%, 77.81%, and 71.65%, respectively). These values were, however, better than in the corresponding groups given the same dosages of the PLGA-LrrG protein microsphere orally (50.97%, 77.54%, and 60.24%, respectively). Oral administration of 1 μg/g PLGA-LrrG protein microspheres led to higher relative percent survival (77.54%) than in the corresponding oral administration groups, with 0.5 μg/g (50.97%) and 2 μg/g (60.24%) doses and immunization with naked LrrG recombinant protein (2 μg/g, relative percent survival of 69.28%) (Chen et al., 2014). These results indicated that a 1 μg/g PLGA-LrrG protein antigen dosage was suitable for oral administration and would protect tilapia from *S. agalactiae* infection. These results also suggest that the oral immune tolerance may exist in PLGA-protein vaccine to some extent as shown in other recombinant vaccines (Mowat, 2003; Chehade and Mayer, 2005; Moyle, 2017), and on the other hand, PLGA could reduce the antigen dosages needed and improve immune response as shown in mammal (Eldridge et al., 1990; Lawlor et al., 2016; O'Hagan et al., 1991).

These results collectively suggest that PLGA-LrrG protein microsphere vaccination is useful in the establishment of immunoprotection against *S. agalactiae* infection in tilapia, although there is still a potential for improvement.

Conflicts of interest

The authors have no conflicts of interest to declare.

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