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Title Page

Generation of a Novel *Streptococcus agalactiae* Ghost Vaccine and Examination of Its Immunogenicity against Virulent Challenge in Tilapia

Qishuo Wang[a,b], Xuepeng Wang[a,c]*, Xuemei Wang[d], Ruijuan Feng[e], Qian Luo[a], Jinjing Huang[a]

a Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention & Shandong Provincial Engineering Technology Research Center of Animal Disease Control and Prevention, Shandong Agricultural University, Taian, 271018, P.R. China

b Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, P.R. China

c Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 272000, P.R. China

d Shandong Yisheng Livestock Veterinary Science institute, 264000, Yantai, P.R. China

e Jiangsu Tianshen Co., Ltd., 224000, Huai'an, P.R. China

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First author: Qishuo Wang

* Corresponding Author: Xuepeng Wang

Tel.: + 86-538-8242478;

Fax: + 86-538-8241419.

E-mail addresses: xpwang@sdau.edu.cn (X.P. Wang)

Abstract

Streptococcus agalactiae (*S. agalactiae*) is a gram-positive pathogen that causes a wide range of infections in fish and other animals including humans. Bacterial ghosts (BGs) are nonliving, empty cell envelopes and are well represented as novel vaccine candidates. In this study, we examined the immunogenicity and protective efficacy of *S. agalactiae* ghosts (SAG) against a virulent challenge in tilapia. Nonliving SAG was generated by a culture with Penicillin and Streptolysin, and then treated with the MIC of sodium hydroxide. The formation of a transmembrane lysis tunnel structure in SAG was visualized by electron microscopy. To investigate the SAG as a vaccine candidate, fish were divided into three groups, A (SAG immunized), B [Formalin-inactivated *S. agalactiae* (FSA) immunized] and C (phosphate-buffered saline, PBS-immunized control). The IgM antibody responses were significantly stronger in the SAG-immunized group than in FSA-immunized group, which was higher than in the non-immunized control group ($P<0.05$). Moreover, phagocytic activity (percent phagocytes, PP) was significantly higher ($p<0.05$) in the SAG-immunized group than in FSA-immunized group, which was higher than in the non-immunized control group ($P<0.05$). In addition, non-specific immune immunity, such as lysozyme and superoxide dismutase activities, in the SAG-immunized fish showed significantly higher activities than FSA-immunized fish and the control group fish ($P<0.05$). Also, fish immunized with SAG and FSA showed significantly higher ($p<0.05$) gene expression of IL-1β, TNF-α, IFN-γ and TGF-β in the head kidney and spleen than fish treated with PBS during the whole observed period. In addition, fish immunized with SAG showed significantly higher gene expression of L-1β, TNF-α, and TGF-β in the spleen than in the FSA- immunized fish. Although there was no significant ($P>0.05$) difference of survival rate (SR) or relative percent survival (RPS) between SAG and FSA immunized groups, they were all significantly more protected against the *S. agalactiae* challenge (SR: 86.67%, RPS: 76.395) and (SR: 80.00%, RPS: 67.50%) respectively, compared to the PBS-treated group (SR: 33.33%). These results suggest that immunization with SAG induces immune responses and provides protection against a virulent *S. agalactiae* challenge.

Keywords: *Streptococcus agalactiae*; Bacterial ghosts; Humoral immunity; Cellular immunity; RPS

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1. Introduction

Although Tilapia (*Oreochromis niloticus*) are endemic to Africa, their tremendous aquaculture potential has led to nearly worldwide distribution within the past 30 years [1]. With the rapid development of tilapia aquaculture, a series of disease problems have been caused by a number of bacterial pathogens, most significantly by *Streptococcus agalactiae* (*S. agalactiae*). This has led to high world-wide morbidity and mortality and huge economic losses [2,3]. *S. agalactiae* is a gram-positive bacterial strain that is commonly found in seawater and fresh water fish [2,4]. This bacterium is widely distributed in aquatic environments and is infectious to a variety of species including fish, reptiles, amphibians, birds, mammals and humans [5]. However, they mostly infect fish, such as tilapia, grouper, golden pomfret, barcoo grunter, and zebrafish [4]. This organism's versatility with respect to the broad-range of hosts highlight the importance of developing strategies for the protection of both animals and humans from *S. agalactiae* infections. In recent years, chemotherapy has been used effectively in controlling fish and other animal infections. However, there is significant concern regarding food safety following chemotherapeutic interventions in addition to the danger of selecting for antibiotic-resistant *S. agalactiae* isolates which have been reported worldwide [6]. These concerns have prompted the development of novel vaccination strategies for the control of *S. agalactiae* infections.

Over the last decade, vaccination has become an important prevention strategy against numerous infectious agents affecting humans and farm animals including fish [7]. Although the development of *S. agalactiae* vaccines has been attempted, their efficacy against challenges has been inconsistent [8-10]. The commercial vaccines presently available consist of heat- or formalin-inactivated vaccines, subunit vaccines and attenuated formulations, etc. However, these strategies are accompanied by some drawbacks such as attenuated vaccine virulence reversion, subunit vaccines need of an immune adjuvant, and inactivated vaccines affect on the physio-chemical/structural properties of surface antigens, thereby negatively affecting the development of protective immunity [12].

Bacterial ghosts (BGs) are empty cell envelopes that are produced, for example, by the controlled expression of the *phi*X174 lysis gene *E* in gram-negative bacteria. Expression of lysis gene *E* leads to the formation of trans-membrane tunnels which

consequently leads to the loss of cytoplasmic contents [12]. However, the major drawback of the protein E-induced inactivation method is that it is restricted to gram-negative bacteria only [13]. Interestingly, *Streptococcus. iniae* ghosts produced by E gene-mediated lysis were suggested as a potential vaccine candidate [14]. However, a number of studies have demonstrated that the lysis efficiency of genetically inactivated BGs was 99.9% [12], suggesting a potential risk of their use as a vaccine. Alternatively, the new approach used to generate BGs induction with NaOH was faster than the protein E-mediated lysis system [15].

The bacterial envelope of *S. agalactiae* is composed of peptidoglycan, teichoic acid, and proteins. Several studies have suggested that *S. agalactiae* envelope components are potential vaccine candidates in animal models [8-10]. Recently, immunization with peptidoglycan of gram-positive bacteria such as *S. aureus* has been found to induce protective immunity to a lethal challenge in experimental animals [16]. Several reported that *S. agalactiae* vaccine or subunit protein factors can be used as a vaccine and be able to induce both humoral and cell-mediated immunity [8-10]. Altogether, these whole-cell envelope components of *S. agalactiae* represent an attractive vaccine candidate.

In the present study, we developed a new method to generate novel *S. agalactiae* ghosts (SAG) by using Penicillin and Streptolysin with the MIC of NaOH. Additionally, we demonstrated that immunization with SAG vaccine via the intraperitoneal route could induce both humoral and cellular immune responses in tilapia. Furthermore, these immune responses provided protective immunity to a challenge with virulent *S. agalactiae*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. agalactiae (16S ribosomal RNA gene, GenBank accession number KU311702) was isolated in 2012 from different moribund Nile tilapia during a streptococcus outbreak on a farm in China. This isolate was grown on brain heart infusion (BHI, HuanKai, China) plates for 24 h at 28 °C. Then, a single clone of *S. agalactiae* was cultured BHI broth at 28 °C in a shaking incubator at 200 rpm. Growth and lysis rates were measured spectrophotometrically by determination of optical density at 600 nm (OD_{600}nm). When required, penicillin and Streptolysin were added to the culture at the final concentration at

50μg/ml and 25μg/ml, respectively. *Staphylococcus aureus* was cultured in tryptic soy broth (TSB; Difco) at 37°C in a shaking incubator at 200 rpm.

2.2. Determination of MIC

Determination of the MIC of NaOH for *S. agalactiae* was performed by the 2-fold broth dilution method as described previously [15], with some modifications. Briefly, a virulent culture of *S. agalactiae* was grown in BHI and adjusted to 1×10^8CFU/ml. The initial concentration of NaOH was 60 mg/ml. Two-fold dilutions of NaOH were added to samples of the virulent bacterial culture, and they were incubated at 28°C for 24 h. After incubation, the turbidity of each individual tube was assessed visually, and the MIC was determined as the lowest concentration of NaOH that completely killed the bacterial growth. Further, to determine viability, the culture that showed no visible bacterial growth was verified by spreading 100 μL of the culture onto BHI agar plates and incubating them at 28°C for 36 h. The MIC was determined in three independent experiments.

2.3. Production of vaccine and Identification

SAG were produced by using the Penicillin and Streptolysin with the MIC of NaOH as described previously [15], with some modifications. In brief, *S. agalactiae* was inoculated into 300 mL BHI broth and incubated at 28ºC for 8 h, then Penicillin and Streptolysin was added at the final concentration at 50μg/ml and 25μg/ml and cultured until 24h, the biomass of 24-h-old *S. agalactiae* cells was collected by centrifugation (10,000×g for 10 min at 4°C) and washed three times with phosphate-buffered saline (PBS). One milliliter of the MIC of NaOH was added to 2 mL of the bacterial suspension (1×10^8 CFU/ml) and incubated at 28°C for 60 min. To determine the lysis rate, samples of cells treated with the MIC of NaOH and control cells were collected at 15-min intervals (15, 30, 45, and 60 min) after treatment and spread onto BHI plates. After incubation at 28°C for 24 h, viable colonies were enumerated, and results were expressed in numbers of CFU/ml. At the end of the lysis process, SAG were harvested by centrifugation (10,000×g for 10 min at 4°C) and washed three times with PBS. The final pellet was suspended in sterile PBS and stored at 4°C until further use.

Morphological features of SAG and *S. agalactiae* were examined by scanning electron microscopy (Hitachi S-2400) and transmission electron microscopy (7650;

Hitachi) as previously described [12].

For formalin-inactivated *S. agalactiae* (FSA) preparation, formalin was added to 24 h cultures of the bacterium to a final concentration of 0.5%. After a 24 h incubation, cells were washed three times with PBS and resuspended in 10 mL PBS and the suspensions were plated on BHI agar plates to confirm inactivation. FSA was stored at 4ºC until use.

2.5. Immunization protocol and challenge infection

The tilapias were purchased from Taian Tilapia Aquaculture Farm. The fish were supplied with flow-through dechlorinated city water heated between 25℃ and 28 ℃ at a rate of 0.5 L/min. Fish were divided into three immunization groups (A-C, n=60 for each group), and fish from groups A and B fish were immunized on d0 and d14 with either SAG or FSA. Group C fish were immunized with PBS (negative control) at similar times. For group A immunizations, the dose of SAG (corresponding to $1x10^7$ dead BGs) in 50 µL of PBS were vaccinated intraperitoneally and group B fish were immunized with FSA ($1x10^7$ CFU dead bacterial) in the same fashion. Three fish were lightly anesthetized with methoxyflurane and serum was collected from the tail vein at d7, d14, d21, d28 and d35 for the analysis of anti-*S. agalactiae* antibody responses. After, the fish were dissected for kidney and spleen collection which be used for qPCR test.

On d28 post primary immunization fish from all groups were further divided into six subgroups (1-6, n=5 for each subgroup) and were intraperitoneally challenged with 2.0×10^7 CFU/fish (100 µL). Fish which died were necropsied and liver samples homogenized and plated onto a BHI agar plate.

2.6. Antibody response assessment

The presence of specific immunoglobulin M antibodies against *S. agalactiae* following immunization was determined by an enzyme-linked immunosorbent assay (ELISA) with the same method as our earlier research [17]. Briefly, the sera from immunized and control fish were titrated through a ten-fold dilution series. Added to the plates, then incubated at 37°C for 1 h and washed 3 times with PBS-T, then probed with 100 µL self-made rabbit anti-tilapia-IgM antibody incubated at 37°C for 1 h, and washed 3 times with PBS-T, horseradish peroxidase-conjugated goat anti-rabbit IgG was added for 1 h at 37°C. Plates were washed four times with PBS-T and binding visualized by adding TMB (Tiangen, Beijing, China) according to the manufacturer's instructions (100

μL/well). The plates were incubated at room temperature for 20 min and the reaction stopped with 100 μL of 2 M H_2SO_4 and the absorbance read at 450 nm.

2.7. Phagocytic activity

S. aureus was inoculated into common broth agar slants for 24 h, inactivated by 0.5% formaldehyde for 24 h, washed with sterile saline 3 times, adjusted to 1.0×10^8 CFU/ml and stored in 4℃. The phagocytosis was used as the phagocytic activity of leukocytes, which was determined using the same method as our earlier research [18]. We took 100 μL of anticoagulant and added 100 μL of *S. aureus*. Then shook it and put it into the water at 28℃ for 60min, shaking once every 10 min. After this, the mixture was drawn with a pipette on the slides, dried and fixed with methanol for 10 min, and then Giemsa stained for 1 h. Finally, slides were washed and dried to observe with oil microscope. The phagocytic percentage (PP) was calculated according to the following equation (1).

$$PP = \frac{\text{The number of cells involved in phagocytosis in one hundred phagocytes}}{100}\times100\% \quad (1)$$

2.8. Non-specific immune parameters assay

Acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD), and lysozyme (LZM) activity were determined at d7, d14, d21, d28 and d35 using the detection kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocols. One ACP and AKP activity unit was expressed as the production of 1 mg phenol by reaction between every 100 ml of serum and substance in 30 min.

2.9. Determination of immune-related genes expression in tilapia

Total RNA of kidney and spleen was extracted as described above using total RNA isolation system. The primers for cytokines (IL-1β, IFN-γ, TNF-α and TGF-β) used are shown in Table 1. The qRT-PCR was carried out in an ABI 7300 real-time detection system by using an SYBR ExScript qRT-PCR kit (as described above) [19]. Each assay was performed with β-actin as a control. The $2^{-\Delta\Delta CT}$ method was used to analyze the mRNA level. All data were given in terms of relative quantities of mRNA expressed as the means plus or minus standard errors from three separate experiments [20].

2.10. Statistical analysis.

Statistical significance was determined by ANOVA analysis. Differences were

considered significant at $P<0.01$ or $P<0.05$.

3. Results

3.1. Production and characterization of bacterial ghosts

To produce SAG, we first determined the MIC of NaOH for *S. agalactiae* strain KU311702 by the 2-fold broth dilution method. The MIC of NaOH for *S. agalactiae* was 7.0 mg/ml, and this specific concentration was used to produce SAG. Electron microscopic analysis of NaOH-lysed *S. agalactiae* cells revealed no gross alterations in cellular morphology compared to unlysed cells (Fig. 1 A, C) except for the lysis pore (Fig. 1B, D). The morphology of the cell, including all cell surface structures, were unaffected by lysis. Pores ranging from 100 to 300 nm in diameter were observed in SAG by scanning electron and transmission microscopy (Fig. 1B, D). The loss of cytoplasmic material and structural integrity were observed in SAG by transmission electron microscopy (Fig. 1D).

3.2. SAG and FSA immunizations and *S. agalactiae* challenge

After the first and the second intraperitoneal immunizations with SAG, FSA or PBS, the fish behaved normally and did not exhibit any signs of illness. Following a challenge infection with *S. agalactiae* fish were monitored daily for 7 days post-challenge. Disease manifestations appeared between days 2-4 post-challenge and included reduced activity, lethargy, anorexia, convulsions and death. All fish were euthanized 14 days post-challenge.

Survival rates of all groups after post-challenge a week are shown in Table 2. SAG-immunized fish (group A) showed the highest survival rates (26/30, 86.67% survival rate, RS) and FSA-immunized fish (group B0 (24/30, 80% RS) were significantly better protected than PBS-treated controls (10/30, 33.33% survival). The relative percent survival (RPS) of groups A and B was 76.39% and 67.50%, respectively, which were significantly higher than PBS-treated controls. However there was no significant ($P>0.05$) difference of SR or RPS between groups A and B. During challenge trials, dead fish showed typical clinical symptoms of *S. agalactiae* infection, such as hemorrhage and proptosis.

3.3. Antibody response analysis

Serum IgM antibodies were induced in fish after immunization and a challenge, as

shown in Fig. 2. Specific anti-*S. agalactiae* IgM antibodies were detected in the serum of fish immunized with either SAG or FSA. No antibody reactivity could be detected in the serum collected from the control fish. Fish immunized twice with either SAG or FSA on days 0 and 14 (groups A and B) had significantly higher antibody levels than the control from days 7-35. Fish immunized with SAG showed significantly higher IgM titers than fish immunized with either FSA, and the FSA group showed significantly higher IgM titers than the PBS-treated group ($p<0.05$), and fish immunized with SAG showed significantly higher IgM titers than fish immunized with FSA after the 21st day.

3.4. Effects on the phagocytic activity of leukocytes

The phagocytic activity of leukocytes in the blood of fish improved significantly after immunization with SAG and FSA vaccines (Fig. 3). After 7 days of immunization, the PP in each group was significantly ($P<0.05$) higher than in the control group and maintained a higher level. In addition, the PP in each immunized group continued to increase with time. At the 28th day after immunization, the highest PP values of groups A and B were 51.33% and 45.17% respectively. To summarize, the results showed a statistical PP ($p<0.05$) increase in fish immunized with SAG and FSA, which was significantly higher ($p<0.05$) than fish treated with PBS during the whole period. In addition, the PP of fish immunized with SAG was significantly higher ($p<0.05$) than fish treated with FSA after the 21st day after first immunization.

3.5. Change of non-specific immune parameters assay

The AKP, ACP, LMZ and SOD activities were recorded and shown in Fig 4. ACP activity (ACPa) of fish in group A and B was significantly higher ($p<0.05$) than fish treated with PBS on average throughout the study and specifically on 7th day after first immunization. However, the ACPa of fish in groups A and B did not show significant differences during the whole period. AKP activity (AKPa) of serum in groups A and B was significantly higher ($p<0.05$) than fish treated with PBS during the whole period or on 7st day after first immunization. However, there was not significant differences of AKPa in groups A and B during the whole period. Lysozyme activity (LZMa) and the SOD activity (SODa) of vaccinated groups (A and B) was increased after being vaccinated with SAG or FSA，and both vaccine groups were significantly higher than group C after 14 days ($P<0.05$), and reached the highest level on the 28th day. Meanwhile,

the LZMa and SODa of group A was significantly increased compared to group B averaged over the whole experiment.

3.6. Expression profile of cytokine genes in tilapia

Expression of immune-related genes was examined by qRT-PCR analysis of the transcription of the genes encoding IL-1β, IFN-γ, TNF-α, and TGF-β in the head kidney and the spleen of fish (Fig. 5 and Fig. 6). Fish immunized with SAG and FSA showed significantly higher ($p<0.05$) gene expression of IL-1β, IFN-γ, TNF-α, and TGF-β in the head kidney than fish treated with PBS throughout the whole observed period, however there was not significant differences ($p>0.05$) between SAG and FSA immunized groups. Fish immunized with SAG and FSA showed significantly higher ($p<0.05$) gene expression of IL-1β, IFN-γ, TNF-α, and TGF-β in the spleen than fish treated with PBS in the whole observed period, in addition fish immunized with SAG showed significantly higher gene expression of L-1β, TNF-α, and TGF-β in the spleen than in the FSA-immunized fish.

4. Discussion

Bacterial ghosts are a new type of vaccine candidate. Because of its bacterial membrane containing intact membrane proteins and other immune-stimulatory components, these antigens can be discriminated by the host's related membrane receptor of dendritic cells and macrophages and be swallowed to stimulate the immune effects [17]. Although one of the gram-positive bacteria ghosts, *S. iniae*, was produced by E gene-mediated lysis [14], there is the risk using vaccines made with this method because the lysis of inactivated BGs was not complete [12]. It is well known that NaOH has the ability to create transmembrane lysis tunnels on the bacterial cell surface, degrade DNA, and turn bacteria into empty cell envelopes [15]. In addition, Penicillin works best on gram-positive bacteria by inhibiting peptidoglycan production, making the cell wall leaky and fragile [21], and the Streptolysin damages the protein membrane [22]. So we designed a new method to generate the gram-positive bacteria ghosts by using Penicillin, Streptolysin and NaOH. And the result of electron microscope (SEM and TEM) showed this method can induce transmembrane lysis tunnels in SAG. Furthermore, there was not any cellular morphology damage except for the lysis pore. These data suggest that this method would be sufficient to produce inactivated *S. agalactiae* bacteria. Generation of

BGs from *S. agalactiae* has not been reported previously. Therefore, we hypothesized this method might successfully be used to produce BGs from gram-positive bacteria.

Antibodies are commonly considered as one of the most important humoral immune responses to pathogen invasion in invertebrates. In this study, we found that the antibody responses were significantly stronger in the SAG-immunized group than in FSA-immunized group, which was higher than in the non-immunized control group. Most importantly, the RS and RPS of groups A and B were significantly higher than control group fish. Recently, it has also been reported that the *S. iniae* ghost vaccine stimulated higher serum bactericidal antibody levels than the formalin-killed vaccine and protected experimental animals from a subsequent challenge with *S. iniae* [14]. In addition, it has also been reported that the *S. aureus* ghost vaccine, administered subcutaneously, orally, and intravenously, could induce CD4+ T cells to respond to staphylococcal antigen during a virulence challenge [15]. An earlier report showed that peptidoglycan induces strong bactericidal antibodies capable of eradicating the bacterial pathogen [23]. All these results suggest that the SAG vaccine could be used as an effective vaccine candidate to induce humoral immune responses and protect fish from Streptococcus.

Phagocytosis is another important cellular immune reaction to pathogen invasion in invertebrates [24]. It has been reported that the phagocytosis of tilapia is the most important form of non-specific cellular immunity [25]. In the present study, the PP in each immunized group was significantly higher than in the control group after 7 days of immunization. In addition, the PP in each immunized group continued to increase with time. The highest PP values of groups A and B were 51.33% and 45.17% respectively on the 28th day after immunization. Furthermore, the PP of fish immunized with SAG was significantly higher than fish treated with FSA after the 21st day after first immunization. Compared with other inactivated vaccines, the main advantage of BG vaccines is the ability to preserve their surface antigenic components, which themselves can provide excellent natural intrinsic adjuvant properties [26]. More importantly, the empty cell envelope of SAGs contains pathogen associated molecular patterns (PAMPs) such as peptidoglycan, lipoteichoic acid, and lipoproteins. A number of studies have suggested that these PAMPs can be recognized by Toll-like receptors (TLRs), which induce an

innate immune response [27,28]. In particular, the leucocyte digesting pathogens can retain the relevant antigen information and transmit the information to the relevant lymphocytes, thereby inducing the humoral and cellular immunity in the host [29].

Although the specific immune mechanism of fish is the deficiency, a large number of studies have proved that the non-specific immune system plays a more key role than the specific immune system in the fish's anti-infection. Therefore, to defend against pathogen invasion in fish, the non-specific immunity enzyme including ACP, AKP, LMZ and SOD are considered as the primary defense [30-33]. The result found that ACP, AKP, SOD, and LMZ activities were significantly increased in both immunized groups. Meanwhile, the LZMa and SODa of SAG-immunized group was significantly more increased than the FSA-immunized group. Specifically, recent studies suggest that a multicomponent vaccine rather than a single-component vaccine could trigger both humoral and cellular immunity and induce protective immunity against staphylococcal diseases [34,35]. Therefore, our novel strategy of using SAGs as a vaccine could be a new way to prevent *S. agalactiae* infections.

Cytokines are a group of cell-signaling molecules that act as a bridge linking the innate and adaptive immune systems. They are produced and secreted by T-lymphocytes and mononuclear phagocytes of the specific and non-specific immunity pathways [36]. In fish, immune cells produce cytokines to initiate the defense mechanism of the immune system against pathogens [37]. In this study, four immunological genes of tilapia were used to monitor the stages of fish immune response during the immunized period. The result showed that fish immunized with SAG and FSA showed significantly higher gene expression of IL-1β, IFN-γ, TNF-α, and TGF-β in the head kidney and the spleen than fish treated with PBS in the whole observed period, in addition fish immunized with SAG showed significantly higher gene expression of L-1β, TNF-α, and TGF-β in the spleen than in the FSA-immunized fish. These pro-inflammatory cytokines play a defensive role against pathogenic bacteria and are considered an essential component of the host defense mechanism.

In conclusion, non-living SAGs have been success-fully generated by using the new method. Interestingly, the present strategy may open the door to the production of BGs from gram-positive bacteria. We have shown that immunization with SAGs induced

significant humoral and cellular immune responses and provided strong protection against a virulent challenge in tilapia. Therefore, our present findings could be useful in the future development of vaccines against *S. agalactiae* infections in aquaculture.

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484 **Tables**

485

Table 1 Primers for testing the gene expression of cytokines in tilapia by Real time PCR

Primer name	Primer sequence (5'-3')
IL-1β-F	TGCACTGTCACTGACAGCCAA
IL-1β-R	ATGTTCAGGTGCACTATGCGG
IFN-γ-F	GAGCTGCAAATGGATGGATGA
IFN-γ-R	GGCGGTACAAGTGATTAGGGA
TNF-α-F	CTTCCCATAGACTCTGAGTAGCG
TNF-α-R	GAGGCCAACAAAATCATCATCCC
TGF-β-F	TGCGGCACCCAATCACACAAC
TGF-β-R	GTTAGCATAGTAACCCGTTGGC
Act-β-F	GCTACTCCTTCACCACCACAG
Act-β-R	CGTCAGGCAGCTCGTAACTC

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Table 2 Survival rates following vaccination and *S. agalactiae* challenge[1]

Groups	Subgroups (n=5)[2]						Survival rate (%)[3]	Relative percent survival (%)[3]
	1	2	3	4	5	6		
A	1	0	1	0	1	1	86.67[a]	76.39[a]
B	1	0	2	1	1	1	80.00[a]	67.50[a]
C	4	3	3	5	2	3	33.33[b]	/[b]

[1] Fish were infected intraperitoneally.
[2] Number of dead fish/subgroup post-challenge.
[3] Significant differences from the control group are indicated by lowercase letter ($P<0.05$).

489

Figures

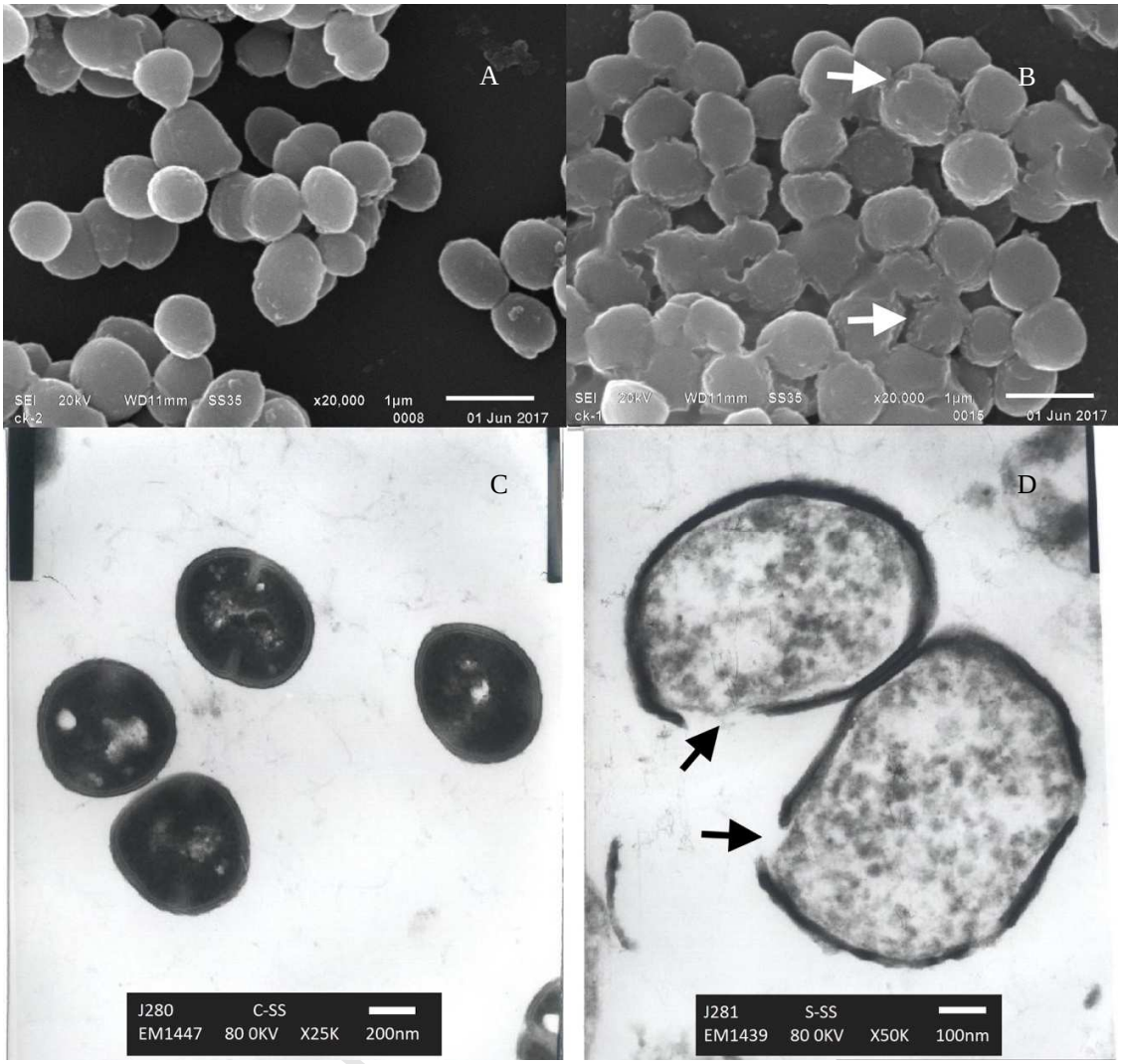
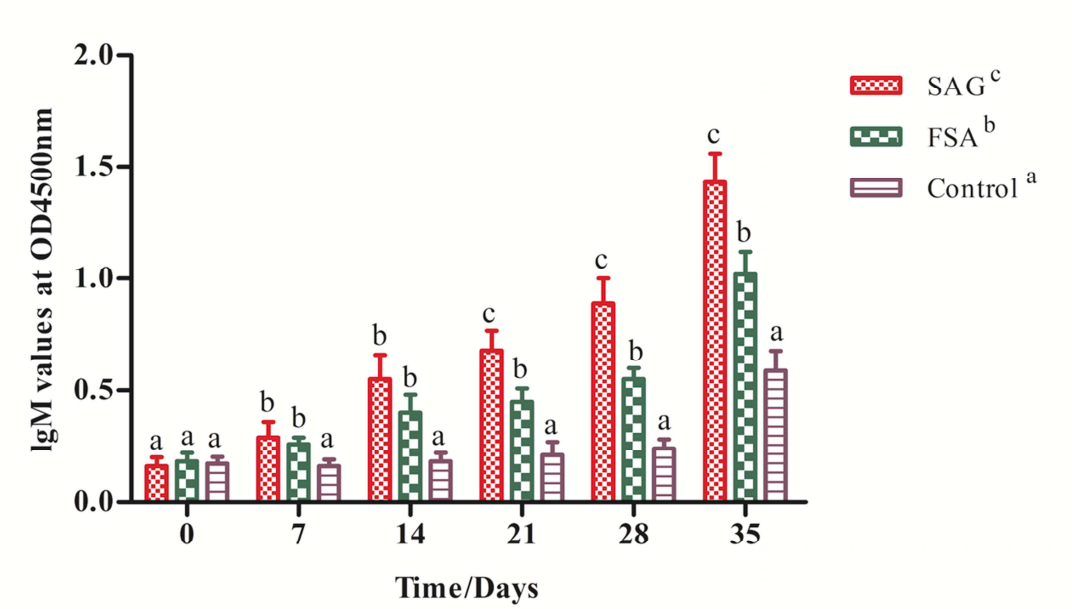


Fig. 1. Evaluation of *S. agalactiae* and *S. agalactiae* ghosts by SEM (A and B) and TEM (C and D). A) Naive *S. agalactiae* examined by SEM. B) *S. agalactiae* ghosts examined by SEM. C) Naive *S. agalactiae* examined by TEM. D) Loss of cytoplasmic material of *S. agalactiae* ghosts.

498



499
500 Fig. 2. Anti-*S. agalactiae* titers in fish following immunization with either SAG, FSA or PBS and
501 intraperitoneally challenge with *S. agalactiae*. Data are means for six assays and presented as the
502 means ± SD. Alphabet a, b and c: indicate significant difference of antibody levels at $P<0.05$.
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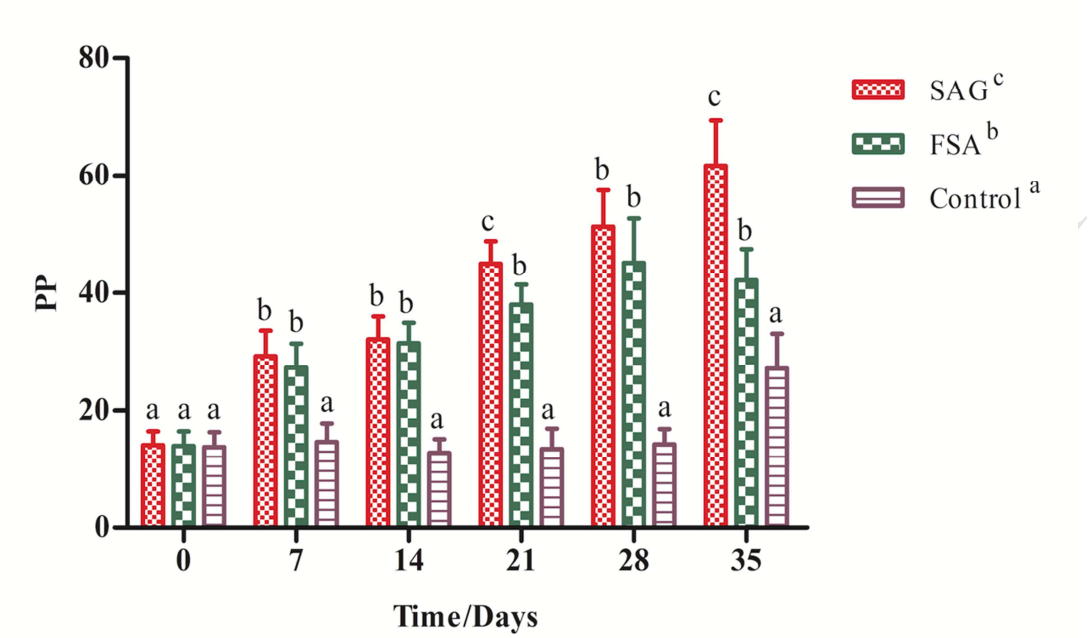

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509 Fig. 3. The phagocytic activity of leukocytes in tilapia post-immunization with SAG, FSA or PBS and
510 intraperitoneally challenge with *S. agalactiae*. Values are representative of at least six independent
511 experiments and are expressed as Mean ± Standard Error. Alphabet a, b and c: indicate significant
512 difference of phagocytic activity at $P<0.05$.
513

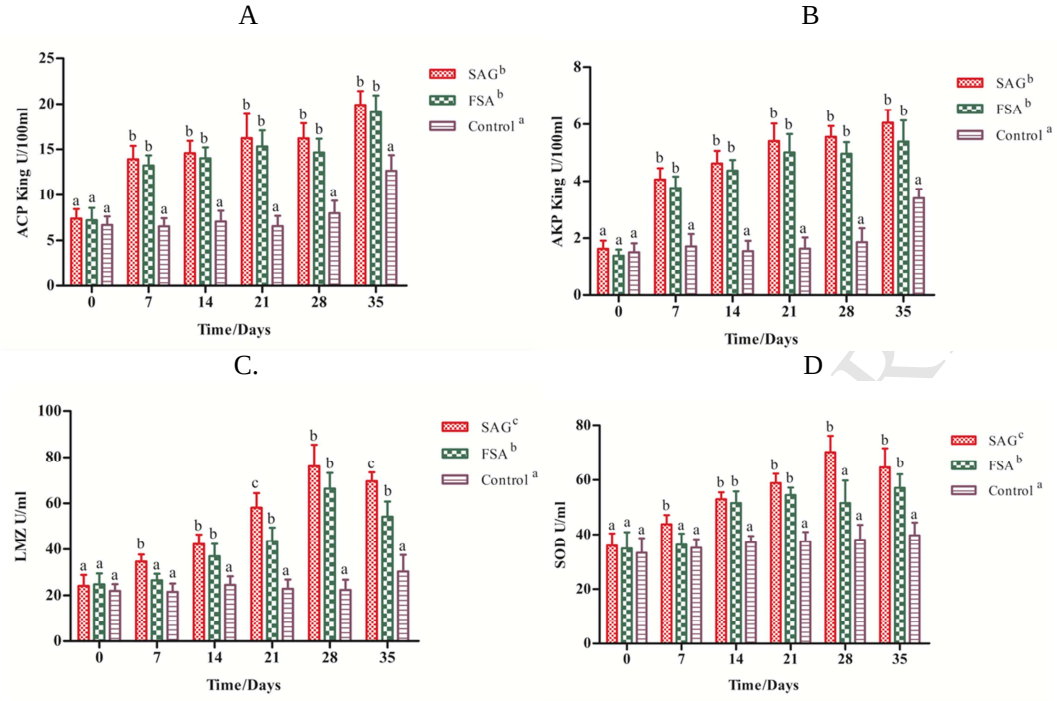
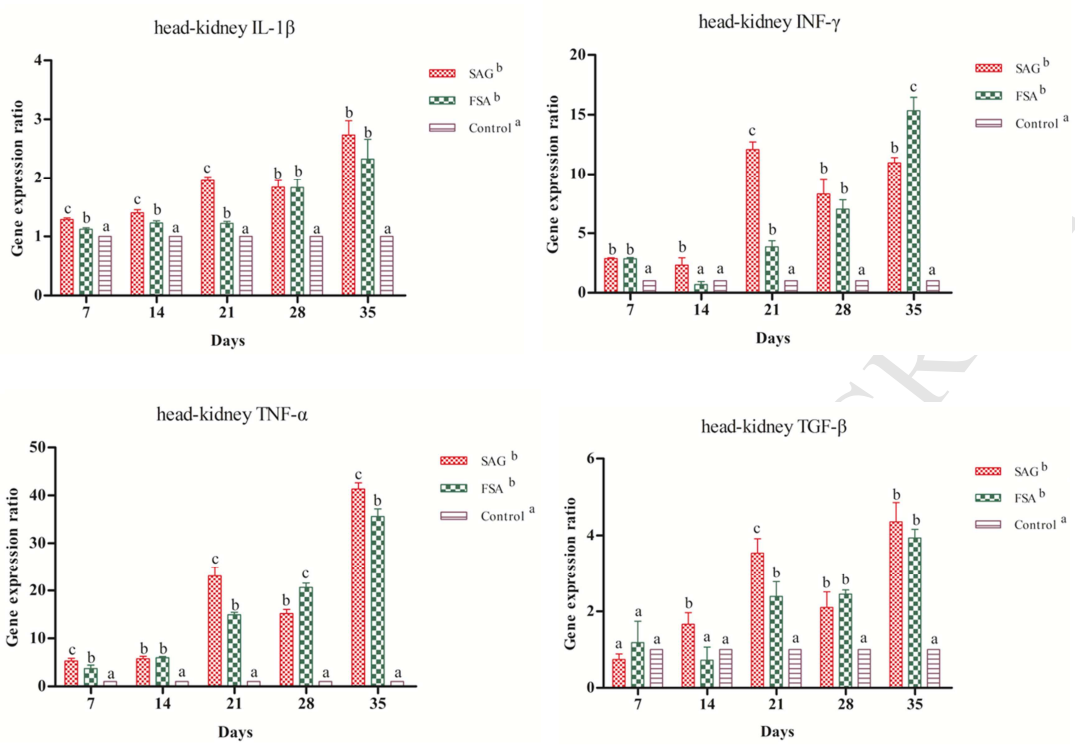


Fig. 4. Change of non-specific immune parameters of tilapia post-immunization with different concentrate SAG, FSA or PBS and intraperitoneally challenge with *S. agalactiae*. (A) acid phosphatase (ACP) activity, (B) alkaline phosphatase (AKP) activity, (C) lysozyme (LZM) activity and (D) superoxide dismutase (SOD) activity. Data are means for six assays and presented as the means ± SD. Alphabet a, b, c and d: indicate significant difference of antibody levels at $P<0.05$.

528



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533 Fig. 5. The gene expression of cytokine-related genes in the head-kidney of tilapia which were
534 immunized with SAG, FSA or PBS and intraperitoneally challenged with *S. agalactiae*. Alphabet a, b
535 and c: Values indicate significant difference of the gene expression in tilapia ($P<0.05$).

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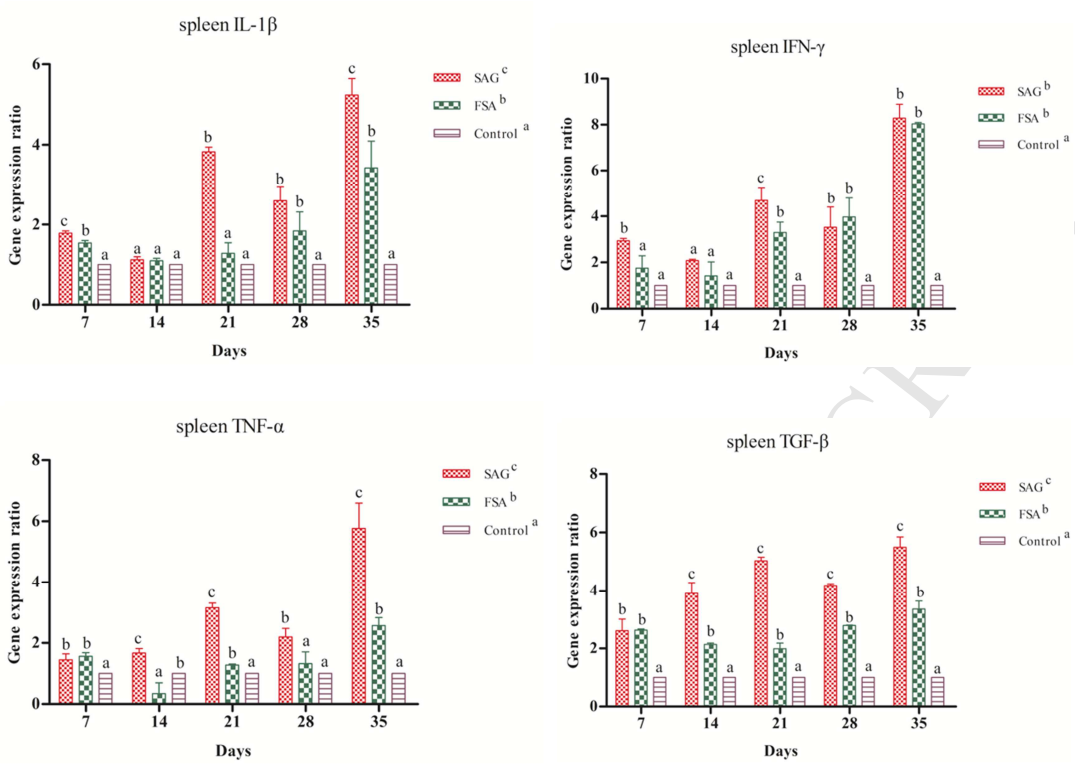

539
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543 Fig. 6. The gene expression of cytokine-related genes in the spleen of tilapia which were immunized
544 with SAG, FSA or PBS and intraperitoneally challenged with *S. agalactiae*. Alphabet a, b and c:
545 Values indicate significant difference of the gene expression in tilapia ($P<0.05$).
546

Highlights

- SAG was generated by using Penicillin and Streptolysin with the MIC of NaOH.
- SAG could stimulate cellular and humoral immunity in *Oreochromis niloticus*.
- SAG can protect *Oreochromis niloticus* from Streptococcus.