Efficacy of Adjuvanted *Streptococcus agalactiae* Vaccine by Montanide ISA 763 A VG in Nile Tilapia (*Oreochromis niloticus* Linn.)

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ABSTRACT

This research investigated the efficacy of adjuvant (Montanide ISA 763 A VG) at concentrations of 0, 5, 10, 20 and 50% in formalin-killed *Streptococcus agalactiae* vaccine in Nile tilapia (*Oreochromis niloticus* Linn.). The results showed that adjuvant at 20 and 50% induced significantly higher protection (survival and RPS value) than other groups after three months of experimental trial ($P < 0.05$). However, only the antibody titer of 20% adjuvant was significantly higher than the control group ($P < 0.05$). The second experiment aimed to find the efficacy of a single vaccination with 10 and 20% adjuvant compared with non-adjuvant vaccine with a booster dose. The results showed that after the booster vaccination without adjuvant, the antibody titer was significantly higher than other treatments ($P < 0.05$). However, in terms of protection (survival and RPS), the vaccines with both 10 and 20% adjuvant showed similar efficacy to the booster vaccination until the third month of the trial ($P > 0.05$), and were significantly different from the vaccine without adjuvant (non-booster group) and from the control (non-vaccination) ($P < 0.05$). Thus, adjuvant vaccine was as effective as booster vaccination for the first three months of the four-month trial. Further study with the aim to extend protection with a single dose of adjuvant vaccine is being conducted.

Keywords: Adjuvant, Antibody, Booster, Nile tilapia, *Streptococcus agalactiae*, Vaccine

INTRODUCTION

Recently, global demand for tilapia has been increasing due to its high nutritive qualities and palatability, while the production from aquaculture industry are limited due to many factors such as diseases and environmental deterioration. It is quite obvious that infectious diseases are threatening the aquaculture industry worldwide. One of the main infectious diseases in the tilapia aquaculture industry is streptococcosis caused by *Streptococcus* spp., with *S. agalactiae* as the most common etiological agent for global tilapia culture (Evans *et al*., 2006). It is an important pathogen that can affect humans and animals as well as aquatic species, and is commonly found in freshwater, brackish water and seawater (Suanyuk *et al*., 2008), and some strains may present a zoonotic or anthroponotic hazard (Delannoy *et al*., 2013). The clinical signs of infected fish include abnormal swimming behavior, pop-eyes and hemorrhages (Amal and Zamai-Saad, 2011). Regarding the serotype of *S. agalactiae*, serotype Ia and III appeared to be the most commonly found especially in Asian countries such as Thailand (Suanyuk *et al*., 2008; Kayansamruaj *et al*., 2014; Dangwetngam *et al*., 2016; Kannika *et al*., 2017).

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Many preventive approaches have been introduced, mainly involved with immunomodulation of the target fish. Vaccination may be the most appropriate method for pathogen control currently available to the aquaculture sector. Although live attenuated vaccines displayed good protection from *S. agalactiae* infection in tilapia (Li *et al*., 2015), they have not been approved for use in aquaculture for safety reasons, while inactivated vaccines are generally safer and also highly immunogenic. Currently, the use of adjuvant is often necessary to enhance vaccine efficacy and ensure disease protection throughout the crop period (Tafalla *et al*., 2013).

There are two main functions of adjuvant. The first is to act as a delivery system (signal 1), either as oil in water or water in oil emulsion, depending on the type of antigen, route of administration and expected protection period. The second function is immunostimulation, in which the adjuvant mostly acts upon the innate immune response (signal 2) (Mutwiri *et al*., 2007; Tafalla *et al*., 2013). There were two experiments in this study. The first trial aimed to determine the vaccine efficacy of different concentrations of non-mineral oil adjuvant (Montanide ISA 763 A VG). This metabolizable oil is claimed to improve the efficacy and stability of vaccine and to reduce side effects in vaccinated fish, and is commonly used in the aquaculture industry (Ravelo *et al*., 2006; Tafalla *et al*., 2013). The second trial aimed to determine the efficacy of vaccine with low concentrations of adjuvant compared with non-adjuvant booster vaccination. The purpose was to reduce the need for booster vaccination, which can cause trauma and stress in fish and increase the cost and labor load when conducted in the field, and also to reduce the side effects in fish such as internal adhesions caused by sticky adjuvant.

MATERIALS AND METHODS

Bacteria

S. agalactiae serotype Ia (KKN 3/1 [L]) and *S. agalactiae* serotype III (UBN 6/2 [K]) were obtained from stocks maintained at -80 °C in the Aquatic Animal Health Management Laboratory, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Thailand. Bacterial glycerol stocks were confirmed for serotyping before being used by multiplex PCR assay of capsular polysaccharide (cps) gene cluster as described by Imperi *et al*. (2010).

Trial 1 Efficacy of adjuvant at different concentrations in *S. agalactiae* vaccine

Fish

Healthy male Nile tilapia were obtained from a commercial good aquaculture practice (GAP) farm with initial average weight of 34.78 ± 4.78 g. They were acclimatized for two weeks in 1.5 ton concrete tanks supplied with flow-through dechlorinated tap water and continuous aeration. Fish were fed daily with commercial diet at 3–5% fish body weight. Water quality was monitored weekly during the experiment. Measured parameters included dissolved oxygen (6.5–7.0 $mg \bullet L^{-1}$), pH (7.5–8.3), total ammonia nitrogen (0.25–3.0 $mg \bullet L^{-1}$), total nitrite (less than 0.03 $mg \bullet L^{-1}$) and water temperature (28.5–31.7 °C).

Preparation of vaccine

Each serotype of *S. agalactiae* was grown in BHI broth at 37 °C for 24 h, the length of culture time which showed higher vaccine efficacy than others (18 and 36 h) in our previous study (results not presented in this publication). Bacterial cells were inactivated by addition of 1% ($v \bullet v^{-1}$) formalin (formaldehyde 38% $w \bullet v^{-1}$) and kept at 4 °C overnight. Cell viability was checked by spreading 100 µl of aqueous vaccine on PCA agar and incubating at 37 °C overnight. Vaccine was used only when no viable cells were found. Vaccine was centrifuged at 3,700 rpm for 7 min, then inactive bacteria pellets were re-suspended in sterile saline 0.85% NaCl to obtain a final concentration of 1 x 10^9 $CFU \bullet ml^{-1}$ measured by optical density (OD) of 0.67 at 600 nm using a spectrophotometer. Finally, the vaccines of both serotypes were combined at equivalent doses (1:1) before use in the trials.

Concentrations of adjuvant (Montanide ISA 763 A VG) at 0, 5, 10, 20 and 50% were thoroughly mixed with combined vaccine by homogenizer following SEPPIC procedure (https://www.seppic.com). Briefly, the beaker containing adjuvant was agitated gently at 7,600 rpm. The combined vaccine was slowly added into the adjuvant, then agitation speed was increased to 11,600 rpm for 3 min.

Vaccination

Two hundred and forty fish/treatment (80 fish per replicate for 3 replicates) were intraperitoneally injected as follows for the six treatments: 0.1 ml sterile 0.85% NaCl (control), combined vaccine without adjuvant (0% adjuvant), and combined vaccine with adjuvant at 5, 10, 20 and 50%. Vaccinated fish were reared in 1.5 ton concrete tanks for three months without a booster dose.

Antibody titer

Blood samples were collected from 12 fish of each group in order to measure the antibody titer before vaccination and at 7, 14, 21, 28, 60 and 90 days post-vaccination. The blood was obtained from the caudal vein using 1 ml syringe without anticoagulant and left to clot for 1 h at room temperature, then centrifuged at 2,500 rpm for 15 min at 25 °C. Serum was taken and analyzed for antibody titer by agglutination assay (two-fold dilution of serum) following the method of Klesius *et al*. (2000). Briefly, each well of a 96 round well plate was filled with 50 µl sterile 0.85% NaCl from well two to the last well, then 50 µl of serum sample was added to the first two wells of each row, mixed and then 50 µl of diluted serum was serially diluted into the remaining wells. Next, 50 µl of *S. agalactiae* (antigen) was added to each well and mixed. The plate was covered and incubated at 37 °C for 24 h. The presence of white compact cells (white button) was considered a negative reaction, while the absence of white button was considered a positive reaction. The antibody titer of each serum sample was defined as the highest serum dilution that showed a positive reaction.

Disease protection

Sixty fish/treatment (20 fish/replicate) were challenged every month for three months with *S. agalactiae* (serotype III) by intraperitoneal injection (IP) containing 1 x 10^8 CFU•ml^{-1} at 0.2 ml/fish. Injected fish were kept in 250 L plastic tanks (200 L of water). Cumulative mortality was observed for 21 days and relative percent survival (RPS) was determined. Confirmation of the cause of mortality was performed by bacterial isolation from dead fish. RPS was calculated according to the following formula:

$$RPS\ (\%) = [1 - (\text{cumulative mortality of the vaccine fish/cumulative mortality of the non-vaccine fish})] \times 100$$

Trial 2 Efficacy of booster vaccination without adjuvant and vaccine with adjuvant (without booster)

Fish

Male Nile tilapia (36.00 ± 1.50 g) were used in the trial with 120 fish/replicate/tank for three replicates (1,800 fish in total). Fish were acclimatized and held under the same conditions as the previous trial. Water quality was periodically monitored during the experiment, with parameters measured as follows: dissolved oxygen 5.8–6.5 mg•L^{-1}, pH 7.5–8.3, total ammonia nitrogen 0.25–3.0 mg•L^{-1}, total nitrite less than 0.03 mg•L^{-1} and water temperature 24.5–30.5 °C.

Vaccination

Three hundred and sixty fish/treatment were IP injected as follows for five treatments: sterile 0.85% NaCl (control), combined vaccine (same vaccine as trial 1) without adjuvant but with booster, combined vaccine without adjuvant and without booster, and combined vaccine with adjuvant at 10 and 20%, respectively (without booster). The booster vaccination was given four weeks after the first vaccination. This experiment was conducted for four months.

Antibody titer

Blood samples were collected from 12 fish of each group for measuring antibody titer before vaccination and at 7, 14, 21, 28, 35, 42, 49, 56, 90 and 120 days post-vaccination. Antibody titers were analyzed by agglutination assay (two-fold dilution of serum) with three antigens: *S. agalactiae* serotype Ia, serotype III and combined antigen (Ia and III).

Disease protection

After vaccination, 60 fish/treatment (20 fish/replicate) were challenged every month for four months by IP injection with *S. agalactiae* (serotype III) at a concentration of 1 x 10^8 CFU •ml^{-1} at 0.2 ml/fish. Injected fish were kept at the same conditions as the previous trial. Cumulative mortality was observed for 21 days. Cause of mortality was confirmed by bacterial isolation. RPS was calculated as previously described.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's New Multiple Range Test (DMRT). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Bacterial confirmation

Serotype of *S. agalactiae* was determined by *cps* Multiplex PCR with serotype Ia: *cpsL* (688 bp) + *cpsG* (272 bp) and serotpye III: *cpsL* (688bp) + *cpsG* (325bp) (Figure 1).

Trial 1 Efficacy of adjuvant in *S. agalactiae* vaccine

Antibody titer

Figure 2 showed fluctuation in antibody titer of vaccinated groups. At week two, vaccinated fish without adjuvant (FKC) showed the highest antibody titer but without significant difference from fish vaccinated with 5 and 20% adjuvant ($P > 0.05$). After two weeks, antibody titer for vaccinated fish without adjuvant rapidly decreased. Meanwhile, the antibody titers of vaccinated fish with adjuvant decreased slightly. At week 8, only vaccinated fish with 20% adjuvant showed a significant difference from other treatments ($P < 0.05$). In summary, the antibody response did not show a conclusive pattern in correspondence to adjuvant concentrations. It appeared from our study that adjuvant did not affect the antibody level but did have a positive effect by prolonging the antibody presence in fish serum.

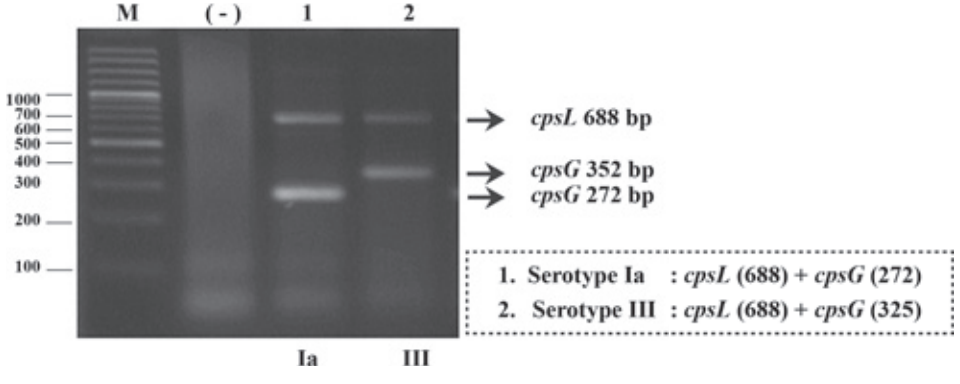

Figure 1. Gel electrophoresis of multiplex PCR amplification products of *cps* gene clusters of *S. agalactiae*. The analysis of product size allowed the determination of *S. agalactiae* serotype. Serotype Ia: *cpsL* (688 bp) + *cpsG* (272 bp), Serotpye III: *cpsL* (688bp) + *cpsG* (325bp), M: molecular marker 100 bp plus DNA Ladder (Thermo Fisher Scientific) and (-): negative control.

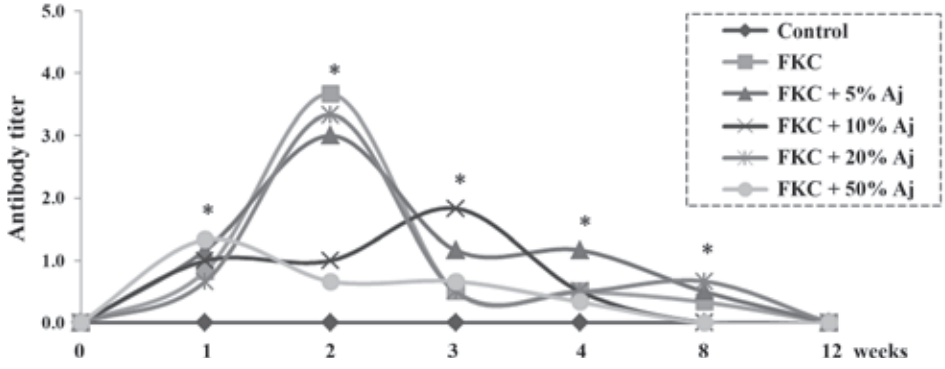

Figure 2. Average antibody titer in Nile tilapia after immunization with combined vaccine (serotype Ia and III) with different concentrations of adjuvant.
*Indicates significant difference among treatments in each week ($P < 0.05$).

There have been very few studies about the effect of different concentrations of adjuvant in fish vaccine by injection. Most studies used the high concentrations recommended by the manufacturer of 50-70%. These dosages could cause negative side-effects such as internal adhesion in vaccinated fish. Noia *et al*. (2014) reported the use of 50% oil-based adjuvants (Montanide ISA 763 and Freund's complete adjuvant [FCA]) resulted in severe internal adhesions (scale of 4-5) in the turbot *Scophthalmus maximus*. Meanwhile, adhesions caused by aluminum hydroxide and saponin-based adjuvant (Matrix-Q) were very light with adhesion rating scale of 1-3 and 0-1, respectively. They also found that to minimize the cell vaccine mass (CVM) that leads to adhesions, the adjuvant vaccine should be injected by pointing the needle towards the anterior part of the peritoneal cavity. A long-term study by Bowden *et al*. (2003) with Atlantic halibut (*Hippoglossus hippoglossus* L.) showed the injection of human gamma globulin potentiated with 50% FCA did induce a significant development of internal adhesion when compared with control (phosphate buffered saline [PBS]), while 50% Montanide ISA 711 showed slight adhesion with non-significance with PBS at 8 of 12 sampling months. Thus, the side effects of adjuvant will be related to type and concentration. Furthermore, previous studies using Montanide ISA 763, a metabolizable oil, displayed the insignificant side effects of fish vaccines (Ravelo *et al*., 2006; Tafalla *et al*., 2013). Thus, we expected that using Montanide at low concentrations in fish vaccine could show positive effects of both fewer adhesions and high protection. Interestingly, there have been some studies on the efficacy of various concentrations of adjuvant in fish by oral vaccine application. Recently, Aminudin *et al*. (2018) studied feed-based, killed vaccine of *S. agalactiae* in red tilapia containing palm oil adjuvant (POA) at 0, 3, 5 and 10% compared with 0, 3, 5 and 7% concentrations of Freund's incomplete adjuvant (FIA). Antibody titer, analyzed by ELISA technique, tended to be higher with increased concentrations of both adjuvants. The highest survival rate and RPS were found in 10% POA which was similarly reported for 10% FIA vaccine against streptococcosis in the same fish (Ismail *et al*., 2016). Both studies used oral vaccination, so the function of adjuvant might be in coating the antigen and feed so that no internal adhesions occurred. Regardless of the route of adjuvant vaccine administration, different concentrations of adjuvant could affect the protection level in fish.

Disease protection

Survival rates (Table 1, Figure 3A) showed that in each month after challenge, all vaccinated groups showed higher protection than the control group ($P < 0.05$). Moreover, different concentrations of adjuvant showed no difference in disease protection at the first month ($P > 0.05$). However, high dosages of adjuvant (20 and 50%) affected vaccine efficacy by prolonging protection, as reflected by significant differences from vaccine without adjuvant at two and three months ($P < 0.05$), though they were not significant from vaccines with 5 and 10% adjuvant ($P > 0.05$).

Relative percent survival (RPS) (Table 2, Figure 3B) showed non-significance among groups for two months ($P > 0.05$), while at the third month, only high dosages of adjuvant (20 and 50%) were significantly different from vaccine without adjuvant ($P < 0.05$).

Based on the results of this trial, we selected 10 and 20% adjuvant for the second trial, since the aim of this experiment was to determine the efficacy of low concentrations of adjuvant that showed good efficacy and minimum side effects, along with low cost of administration.

Trial 2 Efficiency of booster vaccination but without adjuvant and vaccine with adjuvant (without booster)

Antibody titer

Antibody titers of combined vaccine which were analyzed with different antigens (serotype Ia, serotype III and combined antigen) showed different results (Figure 4 [A, B and C]). Meanwhile, the antibody titer analyzed by serotype III antigen (Figure 4B) showed a similar pattern to antibody titer analyzed with combined antigen (Figure 4C). When serotype III and combined serotypes were used as antigen to determine antibody, we found that after the first vaccination (weeks 1–4), antibody titer was not significantly different among vaccinated fish ($P > 0.05$), except that at week three there were significant differences between vaccinated fish without adjuvant (FKC) and vaccinated fish with adjuvant (FKC + 10, 20% Aj) ($P < 0.05$). FKC groups showed rapid decrease from week two, while FKC + Aj showed slight decreases and were quite stable at weeks 2–3. After the second vaccination, FKC-booster fish without adjuvant developed a peak titer at week 5 (one week after the booster) and then titer decreased. No antibody was detected from week 8 onwards ($P > 0.05$) (Figure 4B and 4C).

Table 1. Percent survival of vaccinated Nile tilapia after challenge with *S. agalactiae*.

Treatment	Survival rate (%)		
	1st month	2nd month	3rd month
Control	40.00 ± 13.23[a]	30.00 ± 5.00[a]	20.00 ± 5.00[a]
FKC	65.00 ± 10.00[b]	43.33 ± 5.77[b]	35.00 ± 8.66[b]
FKC + 5% adjuvant	70.00 ± 5.00[b]	51.67 ± 10.41[bc]	40.00 ± 10.00[bc]
FKC + 10% adjuvant	61.67 ± 2.89[b]	56.67 ± 7.64[bc]	46.67 ± 2.89[bc]
FKC + 20% adjuvant	66.67 ± 7.64[b]	58.33 ± 5.77[c]	50.00 ± 10.00[c]
FKC + 50% adjuvant	73.33 ± 2.89[b]	58.33 ± 7.64[c]	51.67 ± 2.89[c]

Data represent mean value ± SD, significant differences indicated by different letters within the same month ($P < 0.05$).

Table 2. Relative percentage survival (RPS) of vaccinated Nile tilapia after challenge with *S. agalactiae*.

Treatment	RPS (%)		
	1st month	2nd month	3rd month
Control	-	-	-
FKC	41.67 ± 16.67[a]	19.05 ± 8.25[a]	18.75 ± 10.83[a]
FKC + 5% adjuvant	50.00 ± 8.33[a]	30.95 ± 14.87[a]	25.00 ± 12.50[ab]
FKC + 10% adjuvant	36.11 ± 4.81[a]	38.10 ± 10.91[a]	33.33 ± 3.61[ab]
FKC + 20% adjuvant	44.44 ± 12.73[a]	40.48 ± 8.25[a]	37.50 ± 12.50[b]
FKC + 50% adjuvant	55.56 ± 4.81[a]	40.48 ± 10.91[a]	39.58 ± 3.61[b]

Data represent mean value ± SD, significant differences indicated by different letters within the same month ($P < 0.05$).

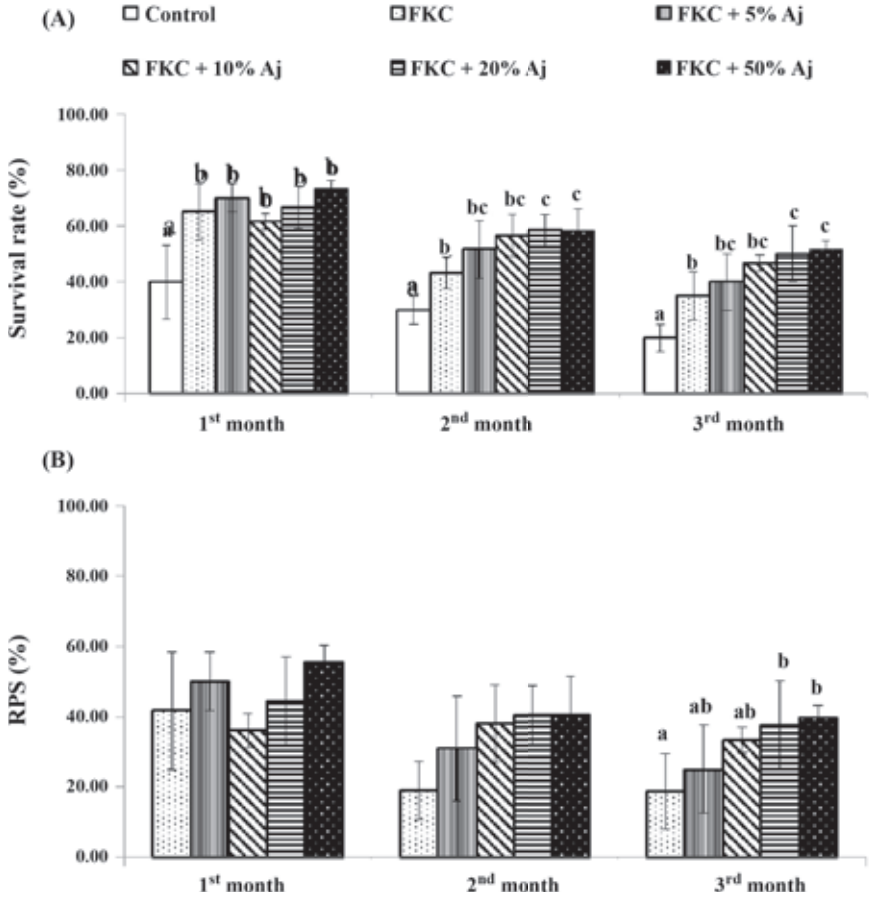

Figure 3. Percent survival rate (A) and RPS (B) of vaccinated Nile tilapia after challenge with *S. agalactiae*. Significant differences indicated by different letters within the same month ($P < 0.05$).

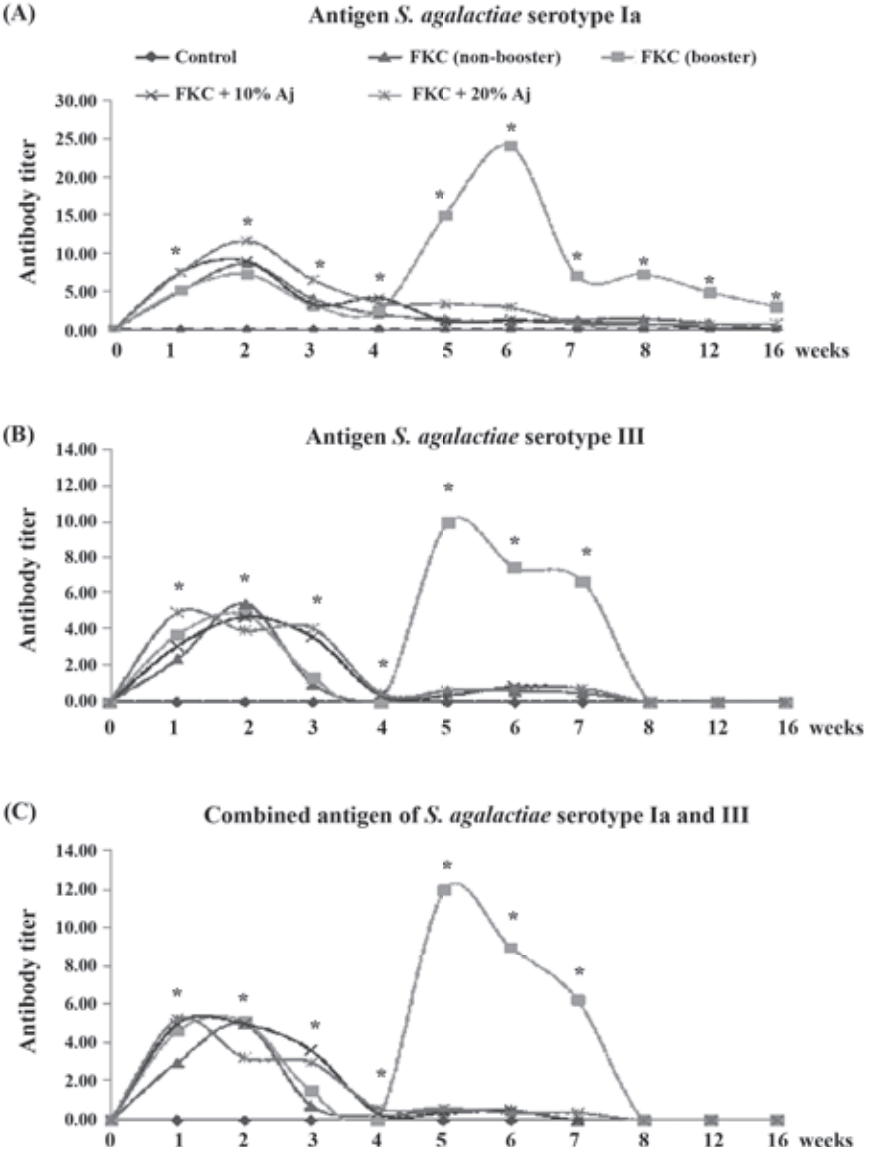

Figure 4. Average antibody titer after immunization with combined vaccine (serotype Ia and III). Second vaccination of FKC booster group was performed at week 4. Antibody analysis was performed by direct agglutination using *S. agalactiae* serotype Ia antigen (A), serotype III antigen (B) and combined antigens of *S. agalactiae* serotype Ia and III (C).
*Indicates significant difference between treatments in each week ($P < 0.05$).

Antibody titer using serotype Ia as an antigen (Figure 4A) was higher than with serotype III and combined antigen. After the first vaccination (weeks 1–4), antibody titer of all vaccinated groups showed significant differences from the control group ($P < 0.05$), but differences were not significant among vaccinated groups ($P > 0.05$). Antibody titer level reached a peak at week two with highest levels found in vaccinated fish with 20% adjuvant, followed by 10% adjuvant and without adjuvant, then continuously decreased from week 2–16. Only antibody titer of 20% adjuvant was significantly higher than the control group at weeks 4–5 after the first vaccination ($P < 0.05$). In case of the second (booster) vaccination, it was found that the titer of FKC booster reached a peak two weeks after the booster (at week 6), then decreased from week 7–16 with significant differences from other groups ($P < 0.05$). It is interesting to note that serotype Ia antibody appeared to persist until the end of the 16-week trial, while serotype III and combined serotype antibody lasted only for 8 weeks.

The results of antibody titer in this study were quite similar to many reports emphasizing that adjuvant could prolong the antibody expression better than FKC without booster. Antibody titer of FKC with booster was higher than FKC + Aj after the second vaccination. This finding was understandable since a booster vaccine is a re-exposure of the same immunizing antigen which is intended to increase immunity by activated memory cells (Lydyard *et al*., 2011). On the other hand, adjuvant is an oil emulsion which has the ability to extend the distribution of the antigen in the vaccine (Noia *et al*., 2014), to increase immunogenicity by slow release, and to enhance the antigen uptake and presentation (Awate *et al*., 2013; Tafalla *et al*., 2013). In this study, we used different antigens for analyzing antibody titer by agglutination method and found that within the same sample, antigen of serotype Ia yielded the highest level of titer, followed by combined antigen and serotype III antigen, respectively. It could be that serotype Ia is an effective immunogen that induced good antibody response in addition to cross-reaction with serotype III antibody due to correlation between pathogenicity and cross-virulence genes of the two serotypes (Kayansamruaj *et al*., 2014; Kannika *et al*., 2017). However, Kannika *et al*. (2017) mentioned that the virulence test using juvenile tilapia found serotype III to be more virulent than serotype Ia. These studies suggest that not all the antigens associated with virulence and pathogenicity of bacteria are effective stimulators of the immune response. It is interesting to note that the *S. agalactiae* vaccine in this study did not stimulate a high antibody response; however, this antibody level was enough to protect the fish from the experimental challenge. A similar finding was confirmed for other Gram-positive bacteria such as *Lactococcus garvieae*, which showed lower antibody level than the Gram-negative *Aeromonas hydrophila* of bivalent vaccine in rainbow trout but showed non-significant survival rate and RPS (Bastardo *et al*., 2012). Moreover, *L. garvieae* with adjuvant showed lower antibody levels than *L. garvieae* without adjuvant but quite stable levels at later stages of the experiment.

Disease protection

Survival rates after challenge with *S. agalactiae* (Table 3, Figure 5A) indicated that all vaccinated fish showed higher protection than the control group ($P < 0.05$). At the first month, only vaccinated fish with adjuvant showed significant difference from the control ($P < 0.05$). After the booster, the highest protection (survival and RPS) was shown in FKC with booster, but there was no significant difference from the vaccine groups with adjuvant for two months (months 2–3) ($P > 0.05$) (Figure 5A, Table 4). Adjuvant has been found to be an effective tool to enhance vaccine efficacy against pathogenic bacteria infection in tilapia such as *Vibrio vulnificus* (Shoemaker *et al*., 2011), *Francisella noatunensis* (Roldan, 2014), and *Edwardsiella tarda* (Cao *et al*., 2014), and it also could enhance the efficacy of subunit vaccines against *S. agalactiae* (He *et al*., 2014). However, only FKC with booster showed the highest protection in the last month of the trial ($P < 0.05$) (Figure 5).

Although most of the antibody titers did not show significant differences from the control beyond week eight (two months), the efficacy of vaccination was shown by prolonging protection throughout the experimental trial (four months) in terms of survival rate and RPS. Thus, even though no antibody was detected, the memory cells could be stimulated from the challenge process. Furthermore, vaccination can induce the response of both adaptive and innate immune systems (Bøgwald and Dalmo, 2012). The innate immune response might be stimulated by vaccines or inflammation from adjuvant because self-nonself discrimination of the immune system could identify adjuvant as foreign. Some studies reported that fish which were injected with only adjuvant showed higher survival rate than unvaccinated control fish after being challenged with a specific pathogen (Roldan, 2014; Jaafar *et al*., 2015). Furthermore, adjuvant could enhance the phagocytic activity and immune gene expression (i.e. IL-1β and TNF-α) (Huang *et al*., 2014; Wang *et al*., 2014). However, innate immune parameters were not investigated in our study. It may be informative for future research to explain the effect of different concentrations of adjuvant on innate immunity and disease resistance.

Table 3. Percent survival of vaccinated Nile tilapia after challenge with *S. agalactiae.*

Treatment	Survival rate (%)			
	1st month	2nd month	3rd month	4th month
Control	31.67 ± 7.64^{a}	28.33 ± 11.55^{a}	11.67 ± 7.64^{a}	12.50 ± 3.54^{a}
FKC (w/o booster)	46.67 ± 7.64^{ab}	55.00 ± 5.00^{b}	31.67 ± 5.77^{b}	35.00 ± 5.77^{b}
FKC (with booster)	41.67 ± 2.89^{ab}	73.33 ± 5.77^{c}	56.67 ± 7.64^{c}	77.50 ± 3.54^{c}
FKC + 10% adjuvant	48.33 ± 10.41^{b}	61.67 ± 10.41^{bc}	48.33 ± 2.89^{c}	40.00 ± 7.07^{b}
FKC + 20% adjuvant	50.00 ± 10.00^{b}	63.33 ± 5.77^{bc}	46.67 ± 10.41^{c}	47.50 ± 10.61^{b}

Data represent mean value ± SD, significant differences are indicated by different letters within the same month ($P < 0.05$). Second vaccination of FKC booster group was administered at week 4 of the trial.

Table 4. Relative Percentage Survival (RPS) of vaccinated Nile tilapia after challenge with *S. agalactiae*.

Treatment	RPS (%)			
	1st month	2nd month	3rd month	4th month
Control	-	-	-	-
FKC (w/o booster)	21.95 ± 11.18^{a}	37.21 ± 6.98^{a}	22.64 ± 6.54^{a}	25.71 ± 8.08^{a}
FKC (with booster)	14.63 ± 4.22^{a}	62.79 ± 8.06^{b}	50.94 ± 8.65^{b}	74.29 ± 4.04^{b}
FKC + 10% adjuvant	24.39 ± 15.23^{a}	46.51 ± 14.52^{ab}	41.51 ± 3.27^{b}	31.43 ± 8.08^{a}
FKC + 20% adjuvant	26.83 ± 14.63^{a}	48.84 ± 8.06^{ab}	39.62 ± 11.78^{b}	40.00 ± 12.12^{a}

Data represent mean value ± SD, significant differences are indicated by different letters within the same month ($P < 0.05$).

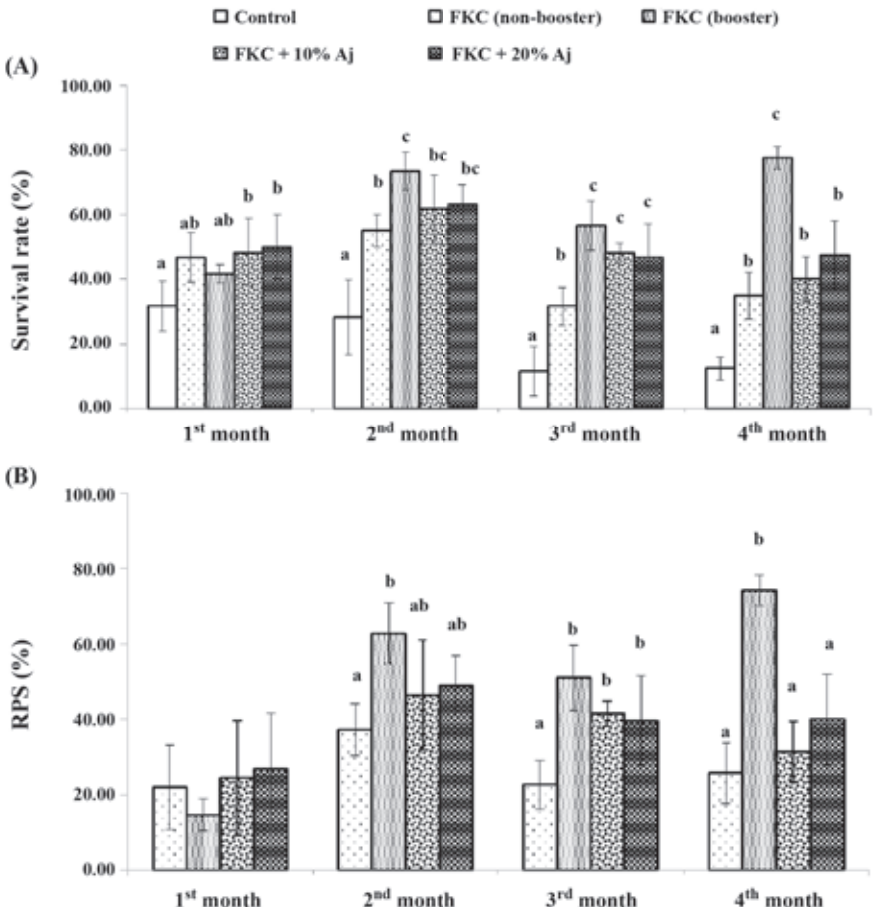

Figure 5. Percent survival rate (A) and RPS (B) of vaccinated Nile tilapia after challenge with *S. agalactiae*. Significant differences are indicated by different letters within the same month ($P < 0.05$).

CONCLUSION

Oil emulsion adjuvant, Montanide ISA 763 A VG, enhanced vaccine efficacy by the slow release of antigen. This research demonstrated the benefit of 10% adjuvant application in the *S. agalactiae* vaccine for prolonged protection in Nile tilapia. In addition, it might reduce trauma in fish from booster vaccination and side effects such as internal adhesions caused by high concentrations of adjuvant. Although the usage of low concentrations of adjuvant could not protect fish throughout the experimental trial (four months), the use of 10 and 20% adjuvant showed protection (survival and RPS) as good as the vaccine with a booster until the third month of the trial ($P > 0.05$). It may be interesting to study adjuvant levels higher than 20% without a booster, which may prolong protection throughout the crop period.

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