



Streptococcosis in *Oreochromis* sp.: is feed-based biofilm vaccine of *Streptococcus agalactiae* effective?

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Abstract

This study was carried out to determine the potential of a biofilm vaccine of *Streptococcus agalactiae* in protecting tilapia from streptococcal infection. The tilapia were vaccinated orally using the biofilm vaccine with $10^9 10^{10}$ CFU/g (FBV) incorporated in feed and killed whole-cell vaccine (FCV) of *S. agalactiae* also prepared in feed, and the unvaccinated control group (Cx) was fed with standard feed formulation. Throughout the study, serum, mucus and gut lavage fluid were collected to evaluate the antibody responses among the groups. The results showed that tilapia vaccinated with FBV exhibited significantly ($p < 0.05$) stronger immune response and high protection with the relative percentage survival (RPS) of more than 70% (85%) compared with the FCV group, which was only 56%. None of the tilapia in the Cx group survived (0%) post-challenge. Thus, the study revealed that the feed-based biofilm vaccine (FBV) significantly confers the immune protection against the high-dose challenge of *S. agalactiae* by secretion of the protective immunity in the mucosal and humoral in tilapia.

Keywords Biofilm · *Streptococcus agalactiae* · *Oreochromis* sp. · Oral vaccination

Introduction

Streptococcosis, which has been associated with high mortality rates that affected many types of fish species throughout the world including Malaysia, is a devastating disease in the aquaculture industry (Siti-Zahrah et al. 2004). The disease is a challenge to the industry and

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can lead to major losses to farmers and nations. Two major species commonly associated with streptococcosis are *Streptococcus iniae* and *Streptococcus agalactiae* common in tilapia more than in other species. To address the losses due to streptococcosis, antibiotics are the only option for farmers. However, the use of antibiotics results in environmental pollution, development of antibiotic resistance strains and antibiotic residues in fish (Klesius et al. 2000; Soltani et al. 2005; Agnew and Barnes 2007; Liaghat et al. 2011). Therefore, vaccination is an alternative to minimising the infectious diseases (Gudding et al. 1999; Firdaus-Nawi et al. 2014). The use of vaccines on a large farm has to be assessed for its safety, implication of cost, ideal methods of delivery and period of protection which are crucial factors to be taken into consideration.

The most common method of vaccine delivery is injection, followed by immersion and oral vaccination (Moore et al. 1998). Out of the three methods of vaccination administration, the efficacy of oral vaccination has been questionable; in fact, previous studies have reported that the efficacy of the oral vaccine was poor (Iida et al. 1982). The researchers indicated that the ineffectiveness of oral vaccines includes the inability to identify a suitable vaccine candidate and the destruction of the vaccine material in the gastric compartment in the fish, which led to a poor response of immunity and short duration of protection in fish. Nevertheless, with the knowledge advancement of biotechnology and immunology, oral vaccine administration method is been reemphasised for several reasons (Quentel and Vigneulle 1997; Firdaus-Nawi et al. 2014; Nur-Nazifah et al. 2014). The reasons for the preference of using oral vaccine are the following: minimise the stress level of fish and operators, minimum handling of fish and it is simple, easy and suitable for mass vaccination. In addition, the oral vaccination is applicable to all fish sizes (size of the feed pellet can be adjusted according to the size of fish) and is more cost-effective than other methods. To overcome the challenges of using oral vaccines that were mentioned earlier, the present study investigated the use of adjuvant and/or used potential antigenic/immunogenic vaccine materials to induce better and long-term immune response in fish (Firdaus-Nawi et al. 2014; Nur-Nazifah et al. 2014). Streptococcosis tends to cause annihilation of the stomach/foregut enzymes before affecting the immune responsive lymphoid organs (Piganelli et al. 1994; Dalmo et al. 1995). Therefore, to strengthen the gut lining from the damage by streptococcosis, the gut-associated lymphoid tissues (GALTs) are important defence mechanism which act as local immune response in preventing the establishment of the pathogen in the host (Noraini et al. 2013; Firdaus-Nawi et al. 2014; Nur-Nazifah et al. 2014).

Various immunogenic items such as an outer membrane protein, lipopolysaccharide and cytotoxin could be selected as vaccine candidates as they are associated with disease pathogenesis. Another potential vaccine candidate which has not been given enough attention is biofilm. Biofilm is a polymeric glycocalyx matrix assembled by bacterial populations (Beveridge et al. 1997; Davey and O'Toole 2000). Biofilm has a protective character that can be harvested to develop an effective oral vaccine that can resist gastric destruction, facilitating the improvement of antigen delivery (Johnson and Amend 1983; Hart et al. 1988). For example, biofilm has been used in a vaccine against *Aeromonas hydrophila*, which demonstrated a significant immune response and protection in carp (Azad et al. 1999, 2000). Following that, the present study investigated the potential of biofilm vaccine prototype for streptococcosis by incorporating it into the feed. The results were assessed through antibody responses either in the serum, mucus and gut lavage fluids, including the existing of gut-associated lymphoid tissues.

Material and methods

Bacterial strain

The pathogenic strain of *S. agalactiae* SAK2 was obtained from the previous study (Siti-Zahrah et al. 2004; Nur-Nazifah et al. 2014). Bacteria were subcultured on the blood agar (Oxoid, Basingstoke, Hampshire, UK), and the pure colony was grown in 1.5% (w/v) tryptone soya broth (TSB; HiMedia, Mumbai, India) at 37 °C overnight in an incubator shaker. The bacteria were harvested by centrifugation at 10,000 rpm for 10 min. Subsequently, the pellet was suspended in 1.5% TSB with 18% glycerol as stock suspensions and stored in – 20 °C for further use. To confirm the identity of the isolates, biochemical tests (API Rapid ID Strep32) and 16S rRNA gene sequencing were conducted according to the manufacturer protocol before commencing the experiment.

Preparation of *S. agalactiae* biofilm and whole-cell vaccines

Streptococcus agalactiae biofilm was prepared according to the method of Azad et al. (1999) with some modifications. A conical flask containing TSB (0.225%) and chitin flakes (0.3% w/v) (CIFT, Cochin) was autoclaved at 121 °C for 15 min. *Streptococcus agalactiae* was inoculated in the medium and placed in an incubator shaker at 180 rpm at 37 °C and shaken for 6 h per day for 4 days. The resulting bacterial pellet intact with chitin was harvested by centrifugation at 10,000 rpm for 20 min at 4 °C. The harvested biofilm cells were suspended in sterile phosphate-buffered saline (PBS, pH 7.2) and inactivated via heating at 100 °C for 50 min. The biofilm was washed thrice using sterile PBS. For the preparation of whole-cell vaccine, the described strain was inoculated in TSB before incubation in an incubator shaker at 180 rpm at 37 °C for 24 h. The pellet was harvested by centrifugation at 10,000 rpm for 10 min, followed by thrice washing using PBS. Following centrifugation, inactivation was performed by heating at 90 °C for 10 min. In order to achieve final concentration of 10^{10} CFU/mL of *S. agalactiae*, the bacterial density was determined by measuring optical density at 550 nm using a spectrophotometer (BioPhotometer, Eppendorf, Germany) by assuming that an optical density of 1.000 corresponds to 1.2×10^9 CFU/mL of the McFarland standard (BioMerieux, France). The bacterial suspension was later diluted to the target concentration in PBS. To confirm that the chitin was encapsulated with the bacteria, the biofilm cells were viewed using scanning electron microscopy (SEM).

Preparation of biofilms for SEM

Samples of biofilm incorporated with chitin were fixed in 2.5% buffered glutaraldehyde (pH 7.4) for 12 to 24 h at 4 °C. Then, the samples were washed three times with 0.1 M sodium cacodylate buffer (pH 7.3) for 10 min each. The samples were dehydrated in ascending series of acetone (35%, 50%, 75% and 95%) for 10 min at each concentration. Finally, 100% acetone was used to dehydrate the samples three times for 10 min, respectively. The samples were dried by critical point dryer (CPD 030 Bal-TEC, Switzerland) and mounted onto the aluminium stub before sputter-coated with gold-palladium (SEM coating unit ES100, Polaron equipment, England). The samples were viewed under JSM JOEL 6400 (Japan) scanning electron microscope at 15 kV and between 15 and 17 mm of working distance, and photographs were taken.

Preparation of feed-based biofilm and whole-cell vaccines

The biofilm and whole-cell vaccines were re-suspended in PBS solution to a concentration of 10^{10} CFU/g. Then, each of the suspended solutions was mixed thoroughly before spraying onto the pellet mixture using a homogeniser (ESCO-Labor AG, Germany) to incorporate the vaccines into the pellet. Finally, the prepared feed was pelleting using a pelletiser machine and dried at 70 °C for 24 h in the oven before the experiment.

Experimental design

Two hundred seventy Red hybrid tilapia (*Oreochromis* sp.) weighing approximately 50 ± 10 g each were acclimatised in 2000-tonne tanks at the Aquaculture Experimental Station, UPM, Puchong, Selangor. After 2 weeks of acclimatising in fibreglass tanks, the fish were divided into three groups in triplicate into the 2000-L glass aquariums with 50 fish each. Group feed-based biofilm (FBV) was fed with feed-based biofilm vaccine of *S. agalactiae*, and group whole-cell vaccine (FCV) was fed with feed-based whole-cell vaccine of *S. agalactiae* on week 0 and a booster feed on week 2. Meanwhile, group Cx, the unvaccinated control group, was fed with standard feed. Prior to commencing the study, 3 fish from each group were sacrificed for bacterial and parasitic screening to ensure that fish were pathogen-free.

Preparation of live bacteria inoculums for challenge

Streptococcus agalactiae was subcultured in blood agar and incubated at 37 °C for 24 h, before subculturing further in brain heart infusion broth (BHIB) at the same temperature for the next 18 h with gentle shaking. To activate the virulence of the bacteria, about 3 mL of broth was passaged into the tilapia by intraperitoneal inoculation. *Streptococcus agalactiae* was then isolated from the eye, brain and kidney of the dead tilapia within 24 h. The identity of the isolates was confirmed by biochemical tests and reinoculated into 100 mL of BHIB until the logarithmic phase. Subsequently, a tenfold serial dilution and colony counting technique were performed to determine the bacterial concentration. Briefly, 1 mL of *S. agalactiae* broth was added into 9 mL of peptone water with a series of highest dilution (10^1) to the lowest (10^9). Again, blood agar was used to spread 0.1 mL of each dilution before incubation at 37 °C for 24 h. Colonies between 25 and 250 were counted before the concentration was expressed as colony-forming unit per millilitre (CFU/mL) (Alcama 1997). The last concentration of live *S. agalactiae* was recorded, and the challenge dose was 1.1×10^9 CFU/mL of live *S. agalactiae* and used immediately.

Bacterial challenge

On week 4, 30 fish from each group were challenged with 0.15 mL of the prepared inoculum of live virulent *S. agalactiae* with the concentration of 10^9 CFU/mL through intraperitoneal (ip) route. The ip route was adopted to evaluate the systemic protection accorded by the standard vaccination. The challenged fish were observed for the next 14 days' post-challenge (pc), and all clinical findings and mortalities were recorded daily.

Sampling of mucus, serum and gut lavage

Serum, mucus and gut lavage were collected at weekly interval as described by Nur-Nazifah et al. (2014). Fish were anaesthetised with Tricaine MS-222 before blood was drawn from the caudal peduncle vein. Mucus was swabbed using a clean, sterile cotton swab on one side of the fish from head to tail. Then, the cotton swabs were immersed into the Bijou bottles, containing 1 mL of sterile PBS/sodium azide. Blood was allowed to clot for at least 4 h; after which, serum was extracted by centrifugation for 5 min. Meanwhile, the hindgut was cut approximately at 10-cm length. Then, the gut was infused with 1 mL of sterile PBS/sodium azide prior to transfer into sterile microcentrifuge tubes with a gentle massage. Finally, after the collection of gut lavages, the gut tissues were cut and fixed in 10% formalin-buffered solution for histological examination.

Enzyme-linked immunosorbent assay

Samples collected above were subjected to enzyme-linked immunosorbent assay (ELISA) (Firdaus-Nawi et al. 2014) to assess IgM antibody responses against *S. agalactiae*. In brief, microtitre plates were coated with *S. agalactiae* antigen and kept overnight at 4 °C. For each step, the plates were incubated at 37 °C for 1 h and washed three times with PBST. Nonspecific binding was blocked with blocking buffer (PBST/BSA). One hundred microlitre of diluted serum, mucus and gut lavage fluid (1:1000) was titrated into the wells. Subsequently, 100 µL of goat anti-tilapia hyperimmune serum diluted at 1:5000 was added to each well. Following that, same dilution (1:5000) of 100 µL of rabbit anti-goat IgM-horseradish peroxidase (Nordic, The Netherlands) was used. One hundred microlitre of TMB One Solution (Promega, USA) was afterwards added for 30 min. The reaction was stopped by 50 µL of 2.5 M sulfuric acid. Absorbance was read with iMark microplate absorbance reader (Bio-Rad, USA) at 450 nm.

Bacterial isolation, Gram stain and PCR

The dead fish were necropsied, and several organs such as kidney, brain, liver and eye were collected aseptically for bacterial isolation on post-challenge. The grown bacteria were stained with the Gram stains and subjected to 16S rRNA forward (5'-GCT ATA CAT GCA GTA GAA CGC TGA-3') and reverse primer (5'-ACG ACT TCA CCC CAA TCA TCT AT-3'). DNA was extracted by lysing one colony of bacteria in 1 µL of DNazol before mixing with the PCR mixture as a template. Each master mix of 50 µL contains 1× PCR buffer, 200 mmol/L of each dNTP, 2.0 mmol/L MgCl₂, 1 U Taq DNA polymerase and 50 pmol of each primer. The PCR for 16S rRNA region of *S. agalactiae* was denatured at 94 °C for 4 min followed by 34 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min. The final extension was at 72 °C for 10 min. The 1% gel was stained with GelRed Nucleic acid gel stain (Biotium, USA) and viewed under an ultraviolet light transilluminator (Bio-Rad, Germany) before photographed by Gene Snap program, UK.

Collection of gut tissue

The guts were fixed in 10% neutral-buffered formalin solution for at least 24 h. The samples were then dehydrated in a series of alcohol before impregnated with paraffin at the melting point of 56–58 °C. Serials of 4- μ m thick paraffin sections were cut from the paraffin blocks using a Jung Multicut microtome (Leica, Germany) and placed carefully on the glass slides. The cut sections were dried overnight at 40 °C. All samples were then subjected to routine haematoxylin and eosin (H&E) staining. The presence of GALTs on the slides was identified using FIVE Image Analyzer (Olympus, Japan).

Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 18.9 (MedCalc Software, Ostend, Belgium). The data were analysed between the antibody responses among the treatment groups. The clustered multiple comparison graph shows the standard error mean (SEM) for each group of treatment. The results revealed significance in Student-Newman-Keuls tests pairwise comparison under one-way ANOVA. Significant differences were determined at $P < 0.05$.

Results

Scanning electron microscopy

Before the oral administration of biofilm vaccine was evaluated, *S. agalactiae* was coated with chitin to produce a biofilm. Figure 1 shows the encapsulation of bacteria in chitin, a typical feature of biofilm, after 4 days of incubation.

Clinical findings and relative percentage survival

The fish showed normal behaviour after post-vaccination in weeks 0 and 2, indicating that the prepared vaccines were safe for the host. Nevertheless, the clinical signs and mortality were monitored in all groups after 12-h post-challenge. Fish particularly in group Cx showed

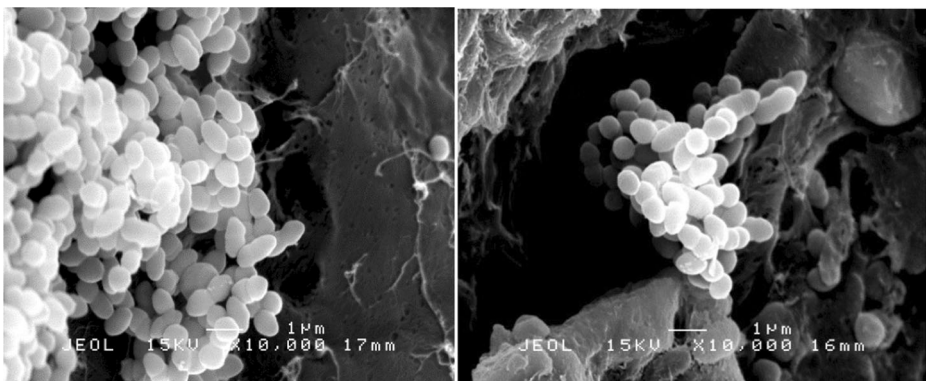


Fig. 1 Scanning electron microscopy (SEM) micrograph showing the cluster-like *Streptococcus agalactiae* biofilm-coated with chitin (mag. $\times 200$).

exophthalmia, erratic swimming, lethargy and inflamed body, which are all common signs of streptococcosis. The relative percentage survival (RPS) was counted for all groups; group FBV showed the highest percentage (87%), followed by group FCV (57%), and all fish in group Cx died within 24 to 48 h (0%) (Fig. 2). Overall, group FBV showed a significantly higher protection ($P < 0.05$) following challenged with 1.1×10^9 CFU/mL of live *S. agalactiae* than groups FCV and Cx.

Antibody response

Prior to vaccination, antibody levels of serum, mucus and gut lavage fluid in all fish were low ($OD = \sim 0.1++$)

Serum antibody response

Post-vaccination, groups FBV and FCV showed a significant increment ($P < 0.05$) of antibody levels at week 1 (Fig. 3). However, group Cx showed no significant changes ($P > 0.05$) in antibody levels throughout the study period. The serum IgM levels constantly increased until week 4 in both FBV and FCV groups; however, after the challenge on week 4, a decreasing pattern was observed. On week 5, the serum IgM level starts to rise back. In general, the antibody level of group FBV was significantly higher ($P < 0.05$) than that of groups FCV and Cx.

Mucus antibody response

The body mucus IgM levels of groups FBV and FCV revealed a significant increase ($P < 0.05$) as early as week 1 post-vaccination (Fig. 4). However, group Cx showed no

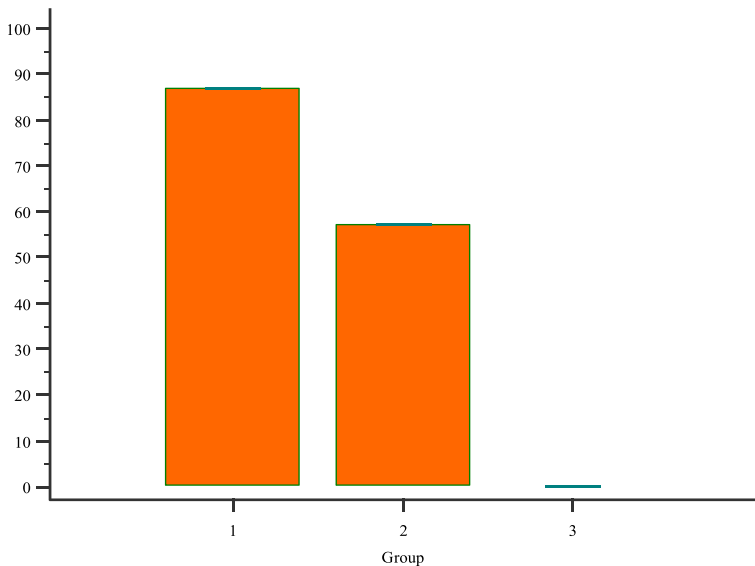


Fig. 2 The relative percentage survival (RPS) rate in Red hybrid tilapia post-challenge with 10^9 CFU/mL live virulent strain of *Streptococcus agalactiae* by ip injection. Group 1 (feed-based biofilm vaccine; FBV), group 2 (feed-based whole-cell vaccine; FCV) and group 3 (unvaccinated control; Cx)

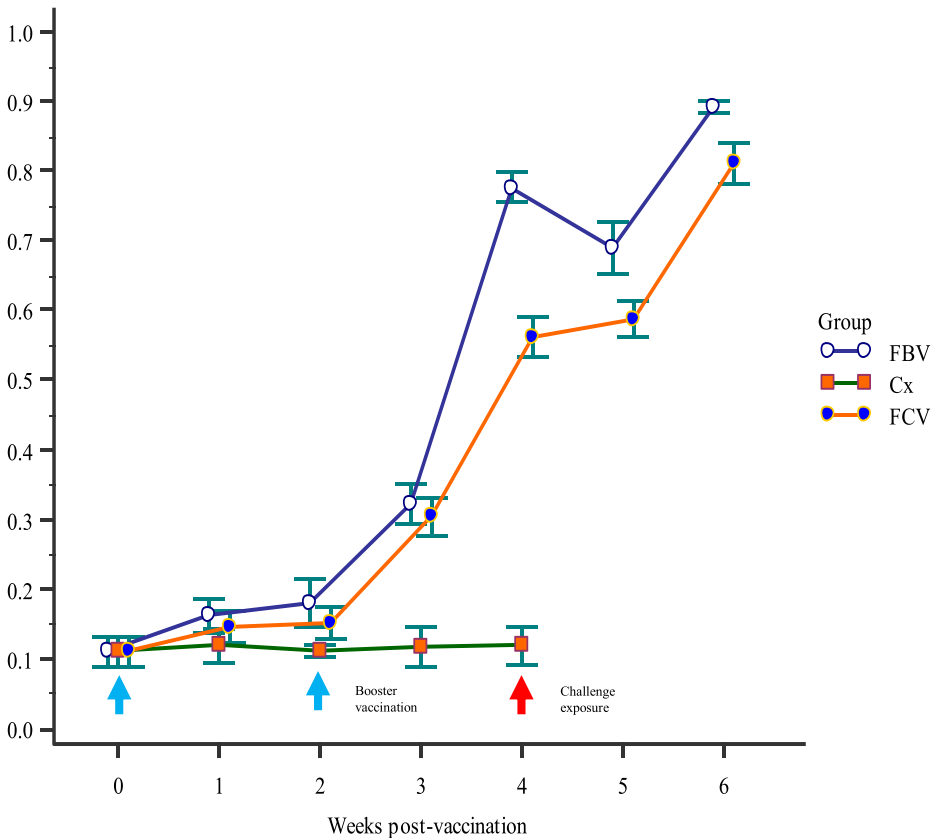


Fig. 3 Antibody response of serum IgM against *Streptococcus agalactiae* following vaccination using feed-based biofilm vaccine (FBV), feed-based whole-cell vaccine (FCV) and unvaccinated control group (Cx)

significant changes ($P > 0.05$) in mucus antibody level throughout the study. Similar to serum IgM antibody levels, the mucus antibody levels constantly increased until week 4 but decreased after the challenge until week 5. On week 5 post-challenge, the mucus antibody levels start to increase as expected. Again, in general, the antibody level of group FBV was significantly higher ($P < 0.05$) than that of groups FCV and Cx.

Gut lavage antibody response

Following vaccination, the IgM levels in the gut lavage fluid of group FBV started to increase significantly ($P < 0.05$) as early as week 1 compared with groups FCV and Cx (Fig. 5). Similar to serum and mucus IgM, group Cx showed no significant changes ($P > 0.05$) during the period of the study. Similarly, the gut lavage fluid IgM levels constantly increased until week 4 but decreased after the challenge until week 5. On week 5, the antibody response starts to pick-up by the increasing pattern. The antibody level of group FBV was significantly higher ($P < 0.05$) compared with groups FCV and Cx.

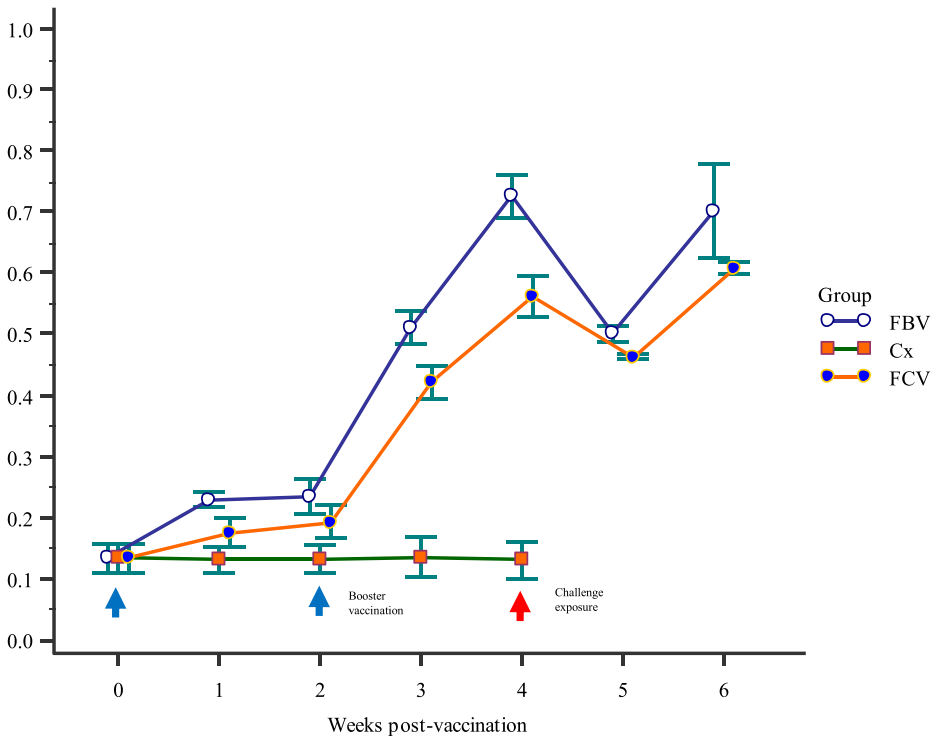


Fig. 4 Antibody response of mucus IgM against *Streptococcus agalactiae* following vaccination using feed-based biofilm vaccine (FBV), feed-based whole-cell vaccine (FCV) and unvaccinated control group (Cx)

Bacterial isolation and PCR

Streptococcus agalactiae was successfully isolated from the brain, eye, kidney and liver from the dead fish of groups FCV and Cx. The stained isolates were Gram-positive and cocci in shape. Molecular identification was carried out using 16S rRNA PCR to confirm the isolates, where positive bands are shown in the agarose gel at 1429 bp (Fig. 6).

Gut-associated lymphoid tissue

The gut-associated lymphoid tissue (GALT) diameter and number of lymphocytes were measured by observing the slides under light microscopy (Leica, Germany). Vaccination using FBV showed evidence of the aggregations of lymphoid cells within the lamina propria in group FBV with high intensity (Fig. 7) in the mucosa as early as week 2 post-vaccination. FCV also showed the development of GALT but with less intensity (Fig. 8). There was no lymphoid cell aggregation found in the fish gut of the unvaccinated control group (Cx) (Fig. 9). Higher GALT diameter and number of lymphocytes were observed in group FBV followed by group FCV but none in group Cx at post-booster vaccination point.

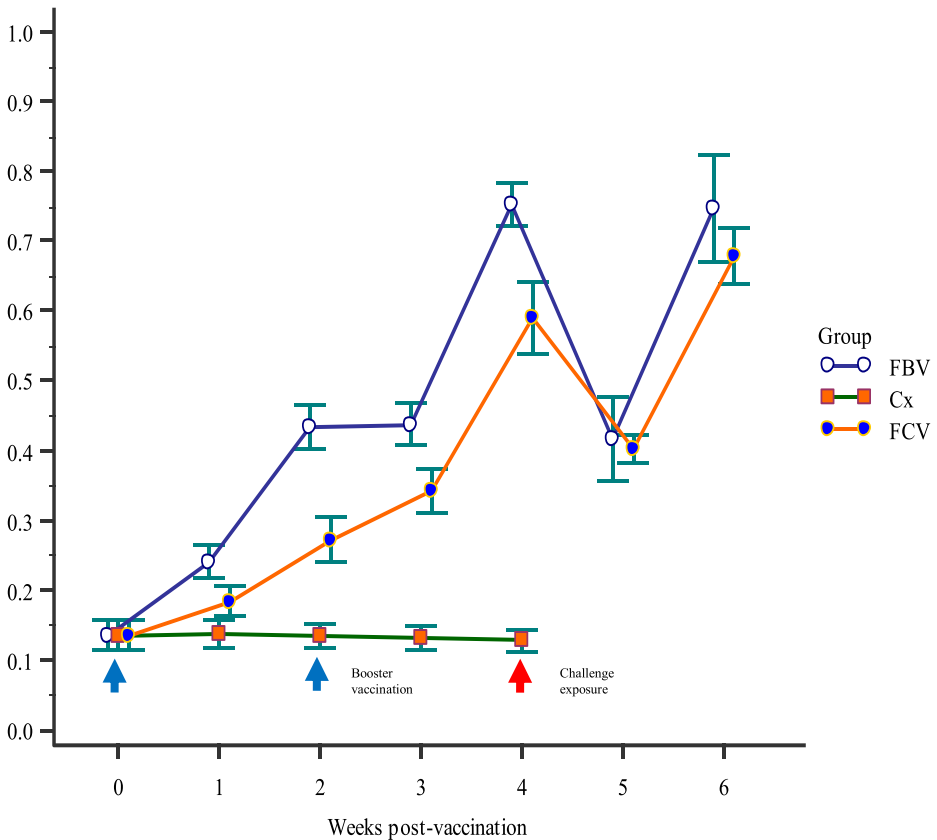


Fig. 5 Antibody response of gut lavage fluids IgM against *Streptococcus agalactiae* following vaccination using feed-based biofilm vaccine (FBV), feed-based whole-cell vaccine (FCV) and unvaccinated control group (Cx)

Discussion

Vaccination is a method of dispensing antigens to enhance the immune system against infection in aquatic animals; subsequently, protection is primed in their immune system. This method is more complex in aquatic animals compared with other species because of the aquatic environment (Vandenberg 2004). Oral vaccination applies to all sizes of fish (Davey and O'Toole 2000; Firdaus-Nawi et al. 2014) and is simple compared with injectable vaccine. Also, it is cost-effective, easy to administer and less stressful. In fact, oral vaccination has been reported to induce mucosal immunity particularly in the gut, which was associated with the gut-associated lymphoid tissues (GALTs) (Rombout et al. 1985; Firdaus-Nawi et al. 2014). However, it has been reported that the antigens are being degraded in the foregut before exposure to the lymphoid tissues in the hindgut leading to the inconsistent efficacy of the oral vaccination in fish (Johnson and Amend 1983; Rombout et al. 1985). Therefore, a batch of antigens is required to reach the lymphoid tissue in the hindgut to be uptake and processed by the memory cells, and finally, the immune response will be activated for optimum protection against the pathogens (Rombout et al. 1985; Davidson et al. 1993; Jenkins et al. 1994). To avoid gastric destruction, either the antigens or vaccine candidate should be incorporated into

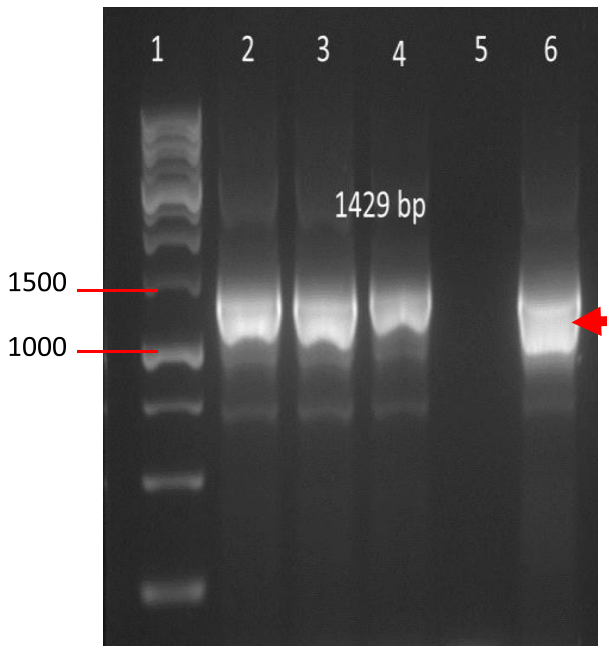


Fig. 6 Agarose gel electrophoresis of PCR amplification of 16S rRNA gene of *Streptococcus agalactiae* assay products. The amplicon was resolved in a 1.5% (w/v) agarose gel and stained with RedSafe Nucleic Acid staining (Intron, South Korea). Lane 1: contains a 1-kb DNA Ladder (Promega, USA); lane 2: a colony of *Streptococcus agalactiae* from the brain; lane 3: a colony of *Streptococcus agalactiae* from the eye; lane 4: a colony of *Streptococcus agalactiae* from the kidney; lane 5: negative control; lane 6: a colony of *Streptococcus agalactiae* from the liver. The arrow at the right indicates 1429 bp amplified product

the feed or encapsulated with artificial materials such as capsule or lipid before they reach the target organs (Wong et al. 1992; Campbell et al. 1993; Joosten et al. 1995; Azad et al. 2000; Irie et al. 2005; Klesius et al. 2006).

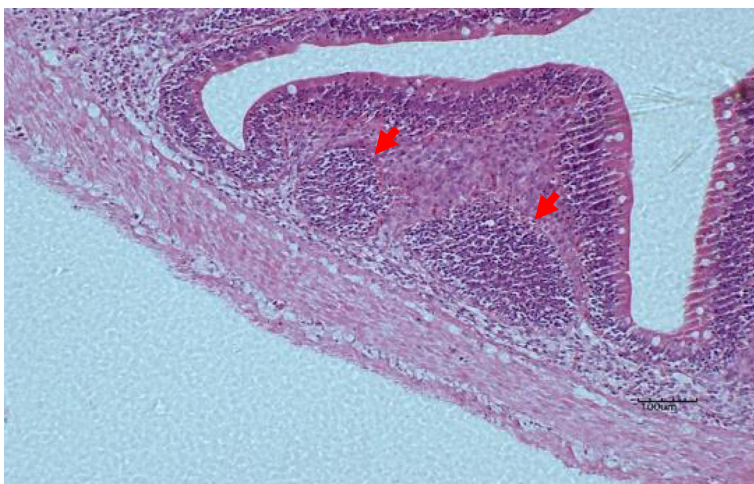


Fig. 7 The gut of Red hybrid tilapia 2 weeks post-booster vaccination. Arrows show two focal aggregations of lymphoid tissue (GALT) in lamina propria of group FBV (HE; bar, 100 μ m)

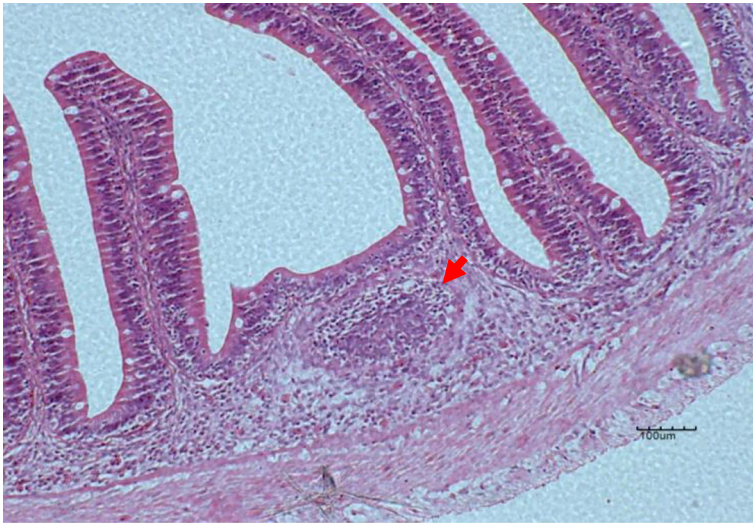


Fig. 8 The gut of Red hybrid tilapia 2 weeks post-booster vaccination. Arrow shows focal aggregation of lymphoid tissue (GALT) in lamina propria of group FCV (HE; bar, 100 μ m)

As mentioned earlier, biofilm is reported to be one of the effective vaccines in fish due to either property of glycocalyx, which is resistant to gastric destruction (Azad et al. 1999) or to antimicrobial agents (Mah and O'Toole 2001; Isiaku et al. 2017). Compared with previous studies, biofilm vaccine of *S. agalactiae* was evaluated and administered orally in Red hybrid tilapia for 6 weeks. As a result, the immune responses of the fish were significantly higher ($P < 0.05$) in groups FBV and FCV compared with group Cx. The high antibody response can be seen either in serum, mucus or gut lavage fluid (Figs. 3, 4 and 5), and the antibody levels increased gradually throughout the study. At the point of challenge, specifically in week 4, both FBV and FCV groups showed a decline pattern of antibody responses either in serum or

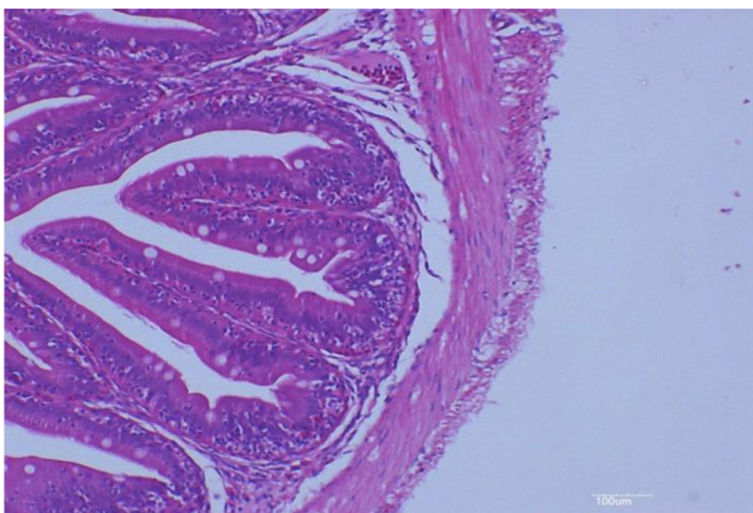


Fig. 9 The gut of Red hybrid tilapia 2 weeks post-booster vaccination. No aggregation of lymphoid tissue (GALT) in lamina propria of group Cx (HE; bar, 100 μ m)

mucus or gut lavage fluid before they rise to optimum levels. Assessment of antibody responses in group Cx was not carried out as the fish had shown signs of sickness and eventually died. Similar studies were observed in Noraini et al. (2013), Firdaus-Nawi et al. (2014) and Nur-Nazifah et al. (2014). Consistently, group FBV still demonstrated a significantly higher ($P < 0.05$) immune response than group FCV until post-challenge (Figs. 3, 4 and 5). This study illustrated similar results that have been reported using biofilm vaccine administration in carps against *A. hydrophila* by assessing the antibody-agglutination titre method (Azad et al. 1999, 2000; Nayak et al. 2004; Plumb and Hanson 2011), as well as in ELISA (Siriappagounder et al. 2014). In fact, the gut-associated lymphoid tissues (GALTs) seem to gradually response and correlate to the gut lavage fluid in this study. As a result, post-challenge revealed the highest relative percentage survival (RPS) rate for the FBV group (87%), followed by the FCV group (57%) and Cx group (0%), respectively (Fig. 2). Thus, the biofilm vaccine is suggested to be more efficacious than other products since the survival rate in FBV group was significantly higher ($P < 0.05$) than others. In other studies, similar results were also reported (Azad et al. 1999, 2000; Nayak et al. 2004; Firdaus-Nawi et al. 2014; Nur-Nazifah et al. 2014; Siriappagounder et al. 2014). In more than 70% of RPS, the FBV is considered satisfactory and successful. Even though the FCV group elicited high antibody responses, it did not confer enough protection as seen in this study. The fish mucosal immune response can be induced by vaccination. Results gained in the current study were also supported by Firdaus-Nawi et al. (2014) where this level of protection might be due to high antibody responses in the gut, which increase the number of GALTs in the vaccinated group. The results of the present study indicate that FBV has the highest antibody level in fish blood serum, closely followed by FCV that was in line with the number of cells in GALTs and its diameter. The gut histology section demonstrated the aggregation of GALTs in the lamina propria of the fish, which is ($P < 0.05$) significantly different between vaccinated and unvaccinated control fish, fed with only commercial feed (Figs. 7, 8 and 9). According to Tafalla et al. (2013), the introduction of a vaccine to the fish stimulates the mucosal responses by increasing the number of lymphocytes with the capability to memorise and identify the specific pathogen by giving sturdy and vigorous responses from pathogen invasion.

If we look at mammals as an example, the best route to induce local immunity such as bronchus-associated lymphoid tissues (BALTs) is through the intranasal vaccination (Kiyono et al. 1992; Effendy et al. 1998), whereby the mucosal immunity will be stimulated as well as the humoral immunity. In the end, animals were conferred protection from the invasive pathogens. A similar concept applies to fish, whereby introduction of antigens to the hindgut via oral feeding can lead to mucosal immunity such gut-associated lymphoid tissues (GALTs). Therefore, either GALT can manufacture optimum antibody against the pathogens, which can be assessed in the mucus, or serum or gut lavage fluid (Noraini et al. 2013; Firdaus-Nawi et al. 2014; Nur-Nazifah et al. 2014).

On the whole, we support the findings by Azad et al. (1997) that biofilm vaccine induces higher antibody levels and gives protection to fish compared with whole-cell or free-cell vaccine. This study showed high levels of antibody responses and protection against *S. agalactiae* in FBV group due to the property of biofilm, which has not been demolished by the digestive enzymes as they are resistant to chemical (e.g. antibiotics, gastric juices). At the tissue level, the lymphoid organs of fish have been exposed to the biofilm vaccine leading to the development of mucosa-associated lymphoid tissues known as gut-associated lymphoid tissue, which gradually increased in size or number of cells in the GALT (Fig. 7) and subsequently confers protection from the pathogens. All these findings suggested that FBV

has the potential to be one of the effective vaccines in fish against *S. agalactiae* infection. Based on this study, we believe that using biofilm as a vaccine candidate can enhance the immunity of fish and demonstrate high antibody levels through the mucosal and humoral immunity to confer maximum protection. However, further studies are also required to challenge the fish with heat stress to determine the vaccine efficacy. This is because streptococcosis outbreak is always associated with the hot and dry seasons in Malaysia. In addition to that, to strengthen the findings, determination of IgT levels in fish is necessary for the future. To optimise the cost and protective effectiveness of this biofilm vaccine, a larger scale study and dosage determination are necessary for further use.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional guidelines for the care and use of animals were followed by the authors.

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