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Growth Performance, Palatability and Water Stability of Oral Feed-Based Vaccines Against *Streptococcus Agalactiae* in Red Tilapia *(Oreochromis* sp.)

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Abstract

In aquaculture the oral route of vaccination is more feasible than immersion and injection routes. In oral vaccination, the vaccine is incorporated in feeds. In this case, palatability and water stability of the feed incorporated with vaccines and the influence of the feed incorporated with vaccines on growth performance of fish is important. In this study, for water stability test, 2 g of biofilm feed-based, free-cell feed-based and commercial pellets as control was put in 100 ml of water and were incubated at 30°C. Water stability of the feed was measured for various times from 1 to 7 hours. Also for evaluating the palatability of the different kinds of pellets, fishes in different groups were fed with 50 pellets of different kind of feeds in each aquarium. No any significant difference was observed in different hours of water stability of vaccine incorporated and commercial pallets. For determining the growth performance of the different type of the feed on red tilapia 50 ± 2 g fish were divided in different aquarium and fed with different kind of feeds for four weeks. Results showed no any significant difference between palatability of the vaccine incorporated and commercial pellets. In addition growth performance parameters showed no any significant differences between fish fed by commercial and feed incorporated with vaccines.

Keywords: Palatability, Water Stability, Growth Performance, Red Tilapia

Introduction

Tilapia is the most cultured fish worldwide and is second to carp as a farmed fish food (Fitzsimmons, 2015). The production of tilapia in 2000 was more than 1.5 million MT compared with that in 1980 (28,260 million MT). This information shows that tilapia is one of the most important species for aquaculture in the 21st century. The introduction of tilapia in international markets in the first half of 2015 was 200,000 MT. Moreover, more than 75,000 MT of whole tilapia were exported to the international market from Asian countries (FAO, 2015). The global production of tilapia is estimated to exceed 5 million MT with a growth rate of 6% compared with that in 2014 (FAO, 2015).

Streptococcu agalactiae is a Gram-positive bacterium under group B *Streptococcus* (GBS). It is a major agent of meningoencephalitis and infection in fishes (Evans et al., 2006). GBS is one of the important pathogenic species which cause infection in a wide range of animals, such as reptiles, mammals, amphibians and fish (Elliot et al., 1990). *S. agalactiae* infection leads to enormous mortality in several wild and cultured fishes, including both freshwater and marine species (Pasnik et al., 2005b). Tilapia is highly prone to *S. agalactiae* infection. Streptococcosis caused by *S. agalactiae* can lead to huge mortality in tilapia farms annually. Thus, this disease has become a major problem in the aquaculture industry causing up to 100% mortality (Ferguson et al., 1994; Agnew and Barnes, 2007. A

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wide range of fish species exposed to streptococcosis with high mortality rates include freshwater fish, golden shiner (Robinson and Meyer, 1966), tilapia (Miyazaki et al, 1984) and rainbow trout (Humphrey et al., 1987; Bragg et al., 1989). In addition, blue fish, striped mullet, striped bass (Baya et al, .1990), eels (Plumb et al., 1974), yellowtail (Kimura and Kusuda, 1979) and menhaden (Cook and Lofton, 1975) are affected by streptococcosis. *S. agalactiae* occurs in tilapia farms, especially in large tilapias (Amal et al., 2008), and has caused significant economic losses in the tilapia industry (Pasnik et al., 2005a). The economic losses due to streptococcosis amounted to USD 150 million in 2000 and reached USD 250 million in 2008 (Othman et al., 2015; Klesius et al., 2008 & 2000). Recently, this disease has become a critical problem in tilapia farms. Among different routes of vaccination, application of oral route is more demand by fish farmers as it is less cost-effective and less stressful. However, the maintaining of water quality and palatability of feed incorporated with the vaccine are important issues and need to be assessed. To our knowledge there is no such data in the literature review and thus, this study addressed the palatability, water stability, growth performance and protective efficacy of feed-based biofilm and free-cell vaccines against *S. agalactiae* in red tilapia.

Material and Methods

Polystyrene plate biofilm formation assay of Streptococcus agalactiae

Biofilm formation was determined by the ability of cells to produce extracellular polymeric substances and adhere to the 96-well polystyrene plate using the modified method of Boddey et al. (2006). Briefly, a 100 µl of LB broth (Pronadisa, Spain) was added into each well of sterile 96-well polystyrene plate (SPL, Korea) followed by the addition of 1 µl of *S. agalactiae* culture which was grown at 37°C overnight with shaking at 150 rpm. The plate was incubated without shaking at 37°C for 18 hours. Thereafter, 1 µl from each well was transferred into the clean and fresh wells of 96-well plate containing 100 µl of fresh LB before the plates being incubated without shaking at 37°C for 24 hours. The supernatant was discarded carefully and the wells were stained with 150 µl of 1% crystal violet (BD, Ireland) at room temperature for 30 minutes. The wells were washed twice with 175 µl sterile deionized water prior to addition 175 µl of dimethyl sulfoxide (DMSO) (Vivantis, USA) to solubilize the crystal violet. The absorbancy of the well was read at wavelength 570 nm using spectrophotometry (Thermo Scientific, VARIOSKAN LUX, USA). Wells containing the medium was used as control. The same protocol was followed to quantify the biofilm after prolonged incubation at 37°C for 48 hours and the results were compared to the biofilms formed for 24 hours post-incubation.

Vaccine Development

Free-Cell Vaccine Preparation

For the preparation of free-cell vaccine, 1.5% (w/v) TSB medium was placed in a conical flask and sterilised by autoclaving at 121°C for 15 min. The *S. agalactiae* strain was inoculated into TSB and placed under shaking condition (180 rpm) at 30 °C for 24 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min followed by three washing with sterile phosphate buffer saline (PBS) at pH 7.4 prior to inactivation at 90 °C for 10 min in a water bath. The concentration of whole inactivated-cells was adjusted at 10^{10} CFU/mL.

Biofilm Vaccine Preparation

S. agalactiae biofilm was prepared as described by Azad et al. (1997) with some modifications. A conical flask containing 0.225% (w/v) Trypticase soy broth (TSB) and chitin flakes (0.3% w/v) (CIFT, Cochin, India) was prepared. Conical flask along with 0.225% (w/v) TSB and 0.3% (w/v) chitin was autoclaved at 121 °C for 15 min for sterilization. After cooling, *S. agalactiae* was inoculated in the medium, placed at 30 °C with a shaking condition (180 rpm) for 6 h per day. On the 4th day, the supernatant was discarded and biofilm cells along with chitin flakes were collected. Then, the chitin flakes were washed three times with sterile PBS at pH 7.4 to remove the loose bands and free-cell. PBS was then added and the flasks were vortexed gently for 3–5 min. Cell counting was performed by dilution method and culture on media to determine the concentration of biofilm cells on the chitin (10^{10} CFU/g). Biofilm cells were inactivated by putting in a water bath and heating at 100°C for 50 min. The safety of vaccine was also confirmed by culturing of the inactivated cells on Trypticase soy agar (TSA) and blood agar incubated at 30 °C for 24 h.

Incorporation of Biofilm and Free-Cell Vaccines in Feed

The biofilm and free-cell vaccines were homogeneously mixed with red hybrid tilapia feed. The prepared feeds were pelletized using a pelletiser machine (Bosch, Germany) and then sun dried. A control feed with the same ingredients without vaccine was used.

Water stability of the feed

The feed incorporated with biofilm vaccine, whole-cell vaccine and control feed without any vaccine was prepared for water stability test as described by Obaldo et al. (2002) with some modifications. Briefly, 2 g of each kind of feed was added into 100 ml of water in the flasks in three replicates and the flasks were incubated at 30°C. Water stability of the feed was measured for various times from 1 to 7 hours. After incubation time all solids were collected by whatman filter and dried in oven and weighted. The water stability of feed was calculated as the ratio of the remained solid in the water and the dry matter of the original feed at the start of the experiment and was expressed as percentage.

Fish and growth condition

Healthy red tilapias were collected from a commercial farm in UPM aquaculture site, Puchong, Malaysia. They were acclimated for 2 weeks in 200-L capacity fiberglass tanks. The samples were aerated with freshwater at 27± 2 °C and fed twice a day with 5% of their body weights. The water parameters were: pH, 7.2–8; temperature, 27± 2 °C; hardness, 75–100 mg/L; dissolved oxygen, 7–8 mg/L; and ammonia concentration, <0.1 mg/L with renewal of 10% of the water daily to remove waste feed and faecal materials.

Palatability of the feed

After two weeks of acclimatisation in fiberglass tanks, 90 red tilapias weighing 50 ± 2 g were divided into the cleaned glass aquariums, each with 10 fishes in three replicates per treatment. Groups included biofilm feed, whole-cell feed, and control feed. Palatability was conducted by using Dong et al. (2016) method with some modifications. One day before growth performance trial, for comparison of the palatability of the different kind of feeds, fishes in different groups were fed with 50 pellets of different kind of feeds in each aquarium. They were monitored for 1 h and after 1 h the uneaten pellets were collected and counted. Ingestion ratio was utilized for evaluating the palatability by counting the number of ingested pellets divided by the number of fed pellets.

Growth performance of red tilapia

The growth performance was followed using the below equations. The water was filtered using top filters and was changed twice a week along with water syphoning to remove the faeces. Fish were fed at 5% body weight/ day. First group was fed using feed incorporated with biofilm, second group using whole-cell vaccine and control group was normal feed. The experiment was run for four weeks and clove oil was used as anesthetizer during the biometry works.

Weight gain (WG) (g) = FBW (g) – IBW (g)

Daily weight gain (DWG) (g/day) = $\frac{FBW\ (g) - IBW\ (g)}{t}$

Relative growth rate (RGR) (%/day) = $[\frac{FBW\ (g) - IBW\ (g)}{IBW \times t}] \times 100$

Percent weight gain (PWG) (%) = $[\frac{FBW\ (g) - IBW\ (g)}{IBW}] \times 100$

Specific growth rate (SGR) (%/day) = $[\frac{Ln\ FBW\ (g) - Ln\ IBW\ (g)}{t}] \times 100$

Where FBW and IBW are final and initial body weight respectively, t is the days of culture, and Ln is natural log

Bacterial Challenge and vaccine protection

S. agalactiae was grwon into brain heart infusion broth (Merk, Germany) incubated in 37 ºC for 24h. Fish in different groups were challenged with 1 mL of live virulent *S. agalactiae* at 10^9 CFU/mL via IP injection. The challenged fish were observed for 7 days post-challenge.

Statistical Analysis

One–way analysis of variance (ANOVA) was applied to address the differences among treated groups and control group at confidence interval of 95% ($p < 0.05$). Multiple comparisons as tukey test was conducted to explore the level of difference among the groups while the P value indicated the value lower than 0.05. All Statistical tests were done using SPSS version 19 computer software.

Results

In vitro biofilm formation by Streptococcus agalactiae

After staining the wells of 96 well polystyrene plate with crystal violet (Fig1) and reading the OD of the wells at 570 nm, the results revealed that the higher OD measured for the samples after 48 hours. The mean OD of the control including only media was 0.130 ± 0.021. The mean ODs of the samples which produced biofilm at 24 and 48 hours were 0.437 ± 0.052 and 0.686 ± 0.015 respectively (Fig2). The results showed that the OD values of the biofilm formation after 24 and 48 h was significantly ($P< 0.05$) higher than control. In addition, the OD value of the biofilm formation after 48 h was significantly ($P< 0.05$) higher than that for 24 h.

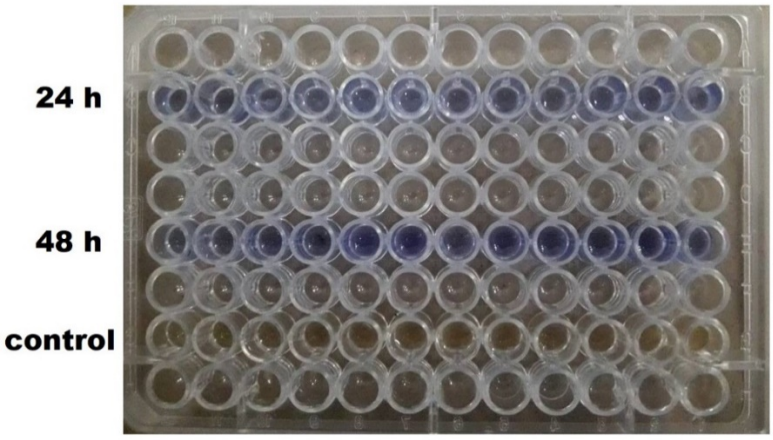

Fig 1**:** Biofilm formation assay of *Streptococcus agalactiae* in 96 well polystyrene plates with crystal violet staining. The first line is the biofilm formation after 24 h, the second line is the biofilm formation after 48 h, and the last line is the control including only media.

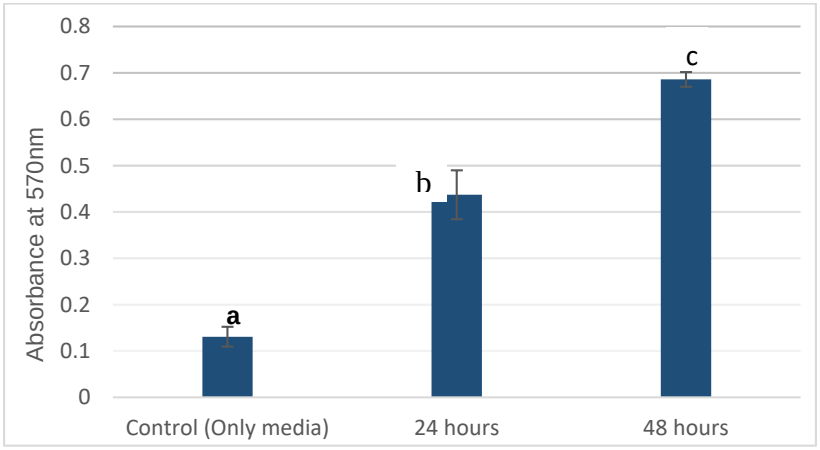

Fig 2: Optical density of the production of biofilm by *Streptococcus agalactiae* at 24h and 48h. The different lowercase superscripts indicate significant differences between the 24, 48h biofilm production and the control.

Validation of the Developed Biofilm Vaccine

The cultured vaccines on TSA and blood agar was observed for bacterial growth. No growth was observed on TSA and blood agar. This result proved that the bacteria were killed with heating and further confirmed vaccine development.

Growth performance of red hybrid tilapia fed by different types of feed

The fish of each group was weighted weekly and their weight was recorded (Table 1). After analysis, revealed that no significant differences was ($P> 0.05$) observed in fish weight fed with different types of feeds (fig 3). Also the weight gain (WG), daily weight gain (DWG), relative growth rate (RGR), and specific growth rate (SGR) (Table 2 and fig 4) showed no significant differences ($P> 0.05$) among different groups. The results of the growth performance between different groups revealed that vaccines do not influence or decrease the growth performance of the vaccinated fish compared with control group. Thus, this fact was shown that biofilm and whole-cell oral vaccines have no negative influence on nutrient intake in vaccinated fish.

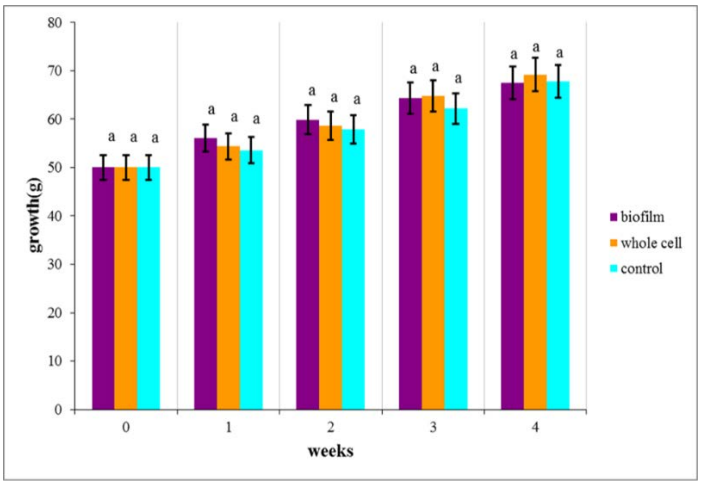

Fig 3: Growth (weight) of red tilapia fed different diets including feed incorporate with biofilm Vaccine, feed incorporated with whole-cell vaccine, and feed without vaccine as control group. Significant differences among treatments in each week are indicated by different lowercase superscripts. The same lowercase superscript indicates no any significant differences between the treatments in each week.

Table 1- Growth (weight) of red tilapia fed different diets including feed incorporated with biofilm Vaccine, feed incorporated with whole-cell vaccine, and feed without vaccine as control group.

Mean of fish / Weight/week Group	Week 0	Week 1	Week 2	Week 3	Week 4
Feed incorporate with biofilm vaccine	50 ± 2.89	56.1 ± 2.23	59.9 ± 3.10	64.3 ± 3.30	67.5 ± 3.53
Feed incorporate with whole-cell vaccine	50 ± 2.32	54.4 ± 3.13	58.7 ± 1.76	64.8 ± 2.29	69.2 ± 4.07
Feed without any vaccine (control)	50 ± 2.68	53.6 ± 2.87	57.9 ± 2.55	62.2 ± 2.97	67.8 ± 3.52

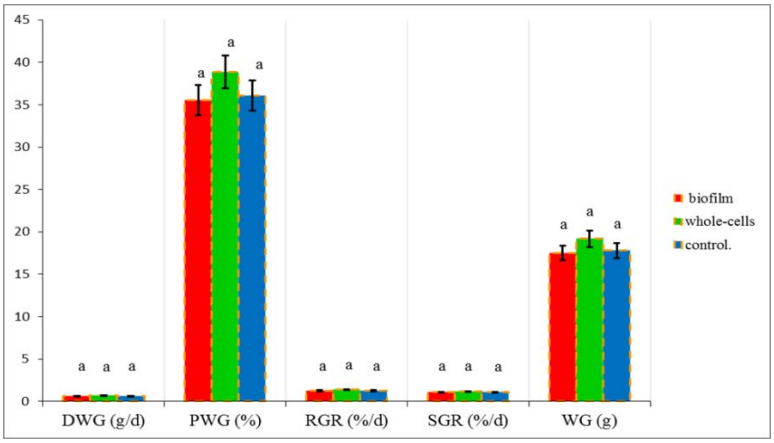

Fig 4: Growth parameters of red tilapia fed different diets including feed incorporated with biofilm vaccine, feed incorporated with whole-cell vaccine, and feed without vaccine as control group. Significant differences among treatments in each week are indicated by different lowercase superscripts. The same lowercase superscript indicates no any significant differences between the treatments for each growth parameter.

Table 2- Growth parameters of red tilapia fed different diets including feed incorporated with biofilm vaccine, feed incorporated with whole-cell vaccine, and feed without vaccine as control group.

Growth parameter / Group	WG (g)	DWG (g/day)	RGR (%/day)	PWG (%)	SGR (%/day)
feed incorporate with biofilm vaccine	17.5 ± 5.03	0.625 ± 0.17	1.26 ± 0.42	35.52 ± 11.83	1.07 ± 0.31
Feed incorporate with whole-cell vaccine	19.2 ± 5.55	0.685 ± 0.19	1.38 ± 0.44	38.84 ± 12.52	1.15 ± 0.32
Feed without any vaccine (control)	17.8 ± 4.98	0.635 ± 0.17	1.28 ± 0.41	36.07 ± 11.70	1.08 ± 0.30

Palatability of the different types of feed including incorporated with biofilm, whole-cell vaccines, and without vaccine as control

Palatability of the different types of feed was calculated 1 hour after feeding the fish in different groups. The results of the palatability test showed no any significant differences (P> 0.05) in biofilm vaccine incorporated in feed, whole-cell vaccine incorporated in feed, and feed without any vaccine as control group where the palatability percentages of the different groups were 98.66 ± 1.33, 98 ± 1.15, and 98.66 ± 0.66 respectively . The results revealed this fact which all types of feed have same palatability and the vaccines are not able to change the palatability of the feed (fig 5).

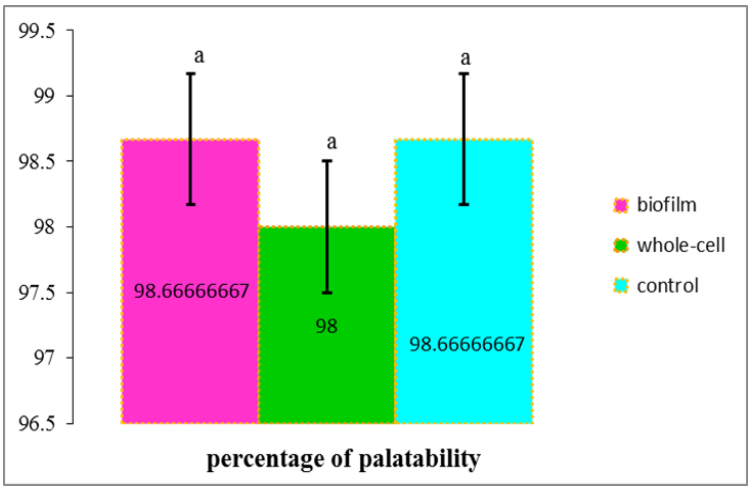

Fig 5: Palatability percentage of the different types of feeds including feed incorporated with biofilm vaccine, feed incorporated with whole-cell vaccine, and feed without any vaccine as control. Significant differences between the different treatments are indicated by different lowercase superscripts. The same lowercase superscript indicates no any significant differences between the treatments.

Water stability of the different types of the feed incorporated with biofilm and whole-cell vaccine, and feed without any vaccine as control

Water stability between different groups of feed in various hours from 1 hour to 7 hours showed no any significant differences (P> 0.05). So all types of the feed applied in the experiment of the present study have similar water stability which this results can prove that vaccines do not have any effect on water stability of the feed that they are incorporated with. The amounts and percentage of water stability of the feed is showed in Table 3 and Fig 6.

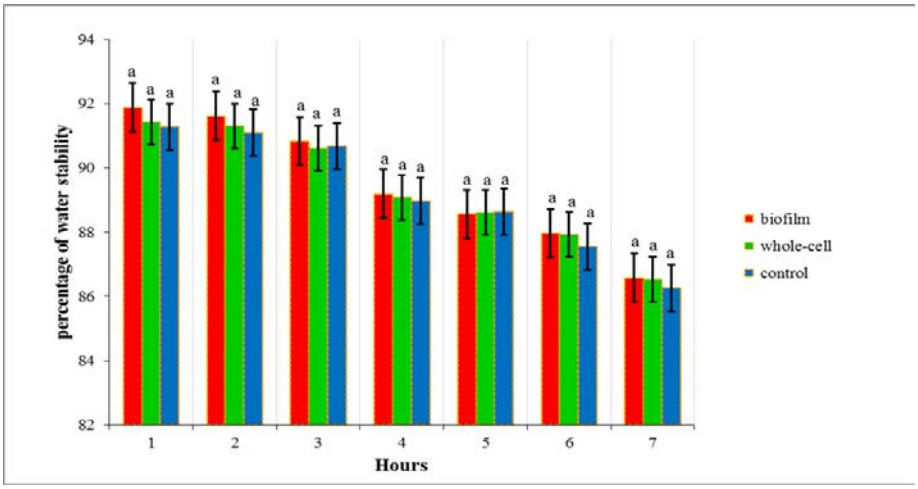

Fig 6: Percentage of water stability of the different types of feed including feed incorporated with biofilm vaccine, feed incorporated with whole- cell vaccine, and feed without any vaccine as control group in different hours from 1hour to 7 hours. Significant differences between the different treatments are indicated by different lowercase superscripts. The same lowercase superscript indicates no any significant differences between the treatments.

Table 3- Percentage of water stability of the different types of feeds involving biofilm vaccine incorporated in feed, whole-cell vaccine incorporated in feed, and no any vaccine incorporated in feed as control in various hours from 1 to 7 hours.

Hours / Group	1 h Percentage (%)	2 h Percentage (%)	3 h Percentage (%)	4 h Percentage (%)	5 h Percentage (%)	6 h Percentage (%)	7 h Percentage (%)
Feed incorporate with biofilm vaccine	91.88 ± 0.81	91.61± 0.53	90.83 ± 0.76	89.20 ± 0.31	88.56 ± 0.17	87.96 ± 0.07	86.58 ± 0.16
Feed incorporate with whole-cell vaccine	91.43 ± 0.33	91.30 ± 0.22	90.61 ± 0.12	89.08 ± 0.42	88.61 ± 0.17	87.93 ± 0.12	86.53 ± 0.12
Feed without any vaccine (control)	91.28 ± 0.30	91.10 ± 0.25	90.68 ± 0.27	88.96 ± 0.25	88.63 ± 0.10	87.55 ± 0.15	86.26 ± 0.17

Survival rate and vaccine protection efficacy

At 7 days post-challenge, the survival rates of the biofilm, free cell and control groups were 93, 63 and 27%, respectively. The fish in control group started dying from first day post-challenge. The results revealed that survival rate in the biofilm vaccinated and free-cell groups were significantly higher ($P < 0.05$) than in control group (table 4). This fact illustrated which developed vaccines had protection effect against *S. agalactiae* in red tilapia. Also the survival rate of the fish in biofilm vaccine was significantly ($P < 0.05$) higher than free-cell vaccine.

Table 4- Survival rate of red tilapia challenged by IP injection of live virulent strain of *S. agalactiae* at 10^9 CFU/ml.

Group	Number of Fish	Number of Survived fish	Survival (%)	Mortality (%)
Biofilm vaccine	30	28	93	7
Free cell vaccine	30	19	63	37
Control	30	8	27	73

Discussion

Antibiotics are currently unavailable for curing this disease (Agriculture Research Service, 2010). Thus, development of an applicable and sufficient vaccine is crucial. Vaccination dispenses pathogens to enhance the immune system against infection in aquatic animals and protect them. This method is more complex in aquatic animals compared with other terrestrial species because of the aquatic environment (Vandenberg, 2004). Thus, several routes of vaccination, such as IM, IP, immersion and oral vaccination, were applied to promote vaccination. The most suitable and preferable method for mass vaccination of fish for all sizes is oral vaccination along with antigen and incorporation into the feed (Firdaus-Nawi et al., 2013; Davey and O'toole, 2000; Hart et al., 1988). However, for developing a feed-based oral vaccine, some issues should be researched on. One is damage of antigen due to pepsin and other enzymes in the stomach, another is palatability and water stability of the feed incorporated with vac cine and growth performance of the feed-based vaccine on the target fish. To resolve the first concern, coating and bio-encapsulation of the antigen were found to be useful (Azad et al., 1999; Sommerset et al, 2005). For the next concerns, after developing vaccine the palatability, water stability, and growth performance of the feed-based oral vaccine should be tested. Natural bacterial populations benefit from food concentration and are protected against toxic agents, and predators prefer to make assemblages in a polymeric glycocalyx matrix called biofilm (Davey and Toole, 2000; Beveridge et al., 1997). This protective property of bacteria was established to develop a helpful oral vaccine that can resist gastric destruction, thereby facilitating improved antigen delivery. In this study the biofilm formation of *S. agalactiae* was confirmed by producing biofilm *in vitro* in 96 well polystyrene plates and crystal violet staining. The results showed *S. agalactiae* is capable to produce biofilm. In addition, some other studies have worked on forming biofilm of *S. agalactiae in vitro*. Borges et al. (201)2 and Kaur et al. (2009) formed biofilm of *S. agalactiae* in TSB at 24, 48, 72, and 96 h in different range of PH. They revealed that the better formation of biofilm of *S. agalactiae* was in neutral PH after 48 h without sugar (glucose and sucrose) supplement in culture broth. However, some researches illustrated that better growth of biofilm of *S. agalactiae* occurred in acidic PH (D'Urzo et al., 2014; Ho et al., 2013) and supplemented with sugar (Rosini and Margarit., 2015; Konto-Ghiorghi et al., 2009). Some others found the biofilm formation of *S. agalactiae* in different kinds of media such as LB, RPMI (nutrient limited media) and THB (rich nutrient media) (Park et al., 2012; Rinaudo et al., 2010; Konto-Ghiorghi et al., 2009). It is exhibited that *S. agalactiae* is a biofilm producer (Rosini and Margarit., 2015; Ho et al., 2013; Borges et al., 2012; Park et al., 2012; Rinaudo et al., 2010). The bacteria within biofilm can endure PH changes, antibiotics, nutrient deprivation, toxic and enzymes. Moreover, it has been reported that one of the important factors which can induce and enhance biofilm formation of *S. agalactiae* is starvation and nutrient-limited condition (D'Urzo et al., 2014; Ho et al., 2013; Borges et al., 2012; Yang et al., 2012; Kaur et al., 2009). Furthermore, in this study due to either biofilm's glycocalyx, which resists gastric destruction (Azad et al., 1999; Vinay et al., 2016), or antimicrobial agents (Isiaku et al., 2017; Mah and O'Toole, 2001), a biofilm oral vaccine was

developed against streptococcosis in red tilapia. Some previous studies such as Siriyappagouder et al. (2014) and Vinay et al. (2013) developed an efficient biofilm oral vaccine against *A. hydrophila* in *Channa striatus* and *Labeo rohita*, respectively.

Likewise in present study the different types of feeds of different groups were compared to indicate whether palatability, water stability of the feed and the growth rate of the fish can influenced by vaccines incorporated in feeds or not. in current study palatability and water stability of the different types of feeds , biofilm vaccinated feed, whole-cell feed, and unvaccinated feed as control were measured. The results of palatability showed no any significant differences (P>0.05) between the different types of feeds and all types of feeds revealed the palatability more than 98%. Moreover, the water stability of the different types of feeds was recorded from 1h to 7 h and as already mentioned no significant differences (P>0.05) were observed in different groups.

Furthermore, the different groups of fish were fed by different types of feed including feed incorporated with biofilm vaccine, whole-cell vaccine, and feed without vaccine as control. Previous researches have revealed that oral vaccines can locally activate and induce inflammatory cells at the sites which contact with antigen, lead to secretion of antibody from different tissue surfaces like liver, skin, and intestinal mucosa (Tobar et al., 2011). Oral vaccination stimulate a local specific IgM response, thus, the influence of oral vaccination on fish nutrition and performance should be determined. This is owing to a successful oral vaccination must not impact entire gut function to maintain the intake rate the intestine of vaccinated fish (Tobar et al., 2011). So, for determination whether oral vaccine can interfere with nutrient absorbance or not, the growth performance of the vaccinated groups was compared with the control group. The growth performance of the fish was indicated by weighting the fish weekly and growth parameters such as weight gain (WG), daily weight gain (DWG), relative growth rate (RGR), percent weight gain (PWG), and specific growth rate (SGR) was measured. The results indicated which the differences between different groups in weekly mean weight of the fish and the growth parameters were not significant (P> 0.05). These results exhibited that growth of the fish was not influenced or decreased by the different types of vaccines. Similar results were explained by other researches. Tobar et al. (2011) developed an oral vaccine of Salmonid rickettsial septicaemia (SRS) and 30 gr Atlantic salmon (*Salmon salar*). Fish were fed by vaccinated feed and also commercial feed for control group. Their results showed same weight gain in vaccinated and unvaccinated group which revealed no any significant differences in weight gain of the fish in both groups. They illustrated that SRS oral vaccine have no effect in nutrient assimilation in vaccinated fish. In another research 32.2 g rainbow trout (*Oncorhynchus mykiss*) fish was fed by different dosage of *Lactobacillus rhamnosus* against furunculosis. Results exhibited that no any significant differences observed in the growth rate of the fish in various group with different dosage of *Lactobacillus rhamnosus* and fish in control group fed with commercial feed (Nikoskelainen et al., 2011). In addition, in some researches the growth performance of the IP injected fish was compared with those in unvaccinated group. Suwannasang et al. (2017) vaccinated Nile tilapia (*Oreochromis niloticus*) with the average weight of 34.45 ± 0.08 g with IP injection of formalin killed *S. agalactiae*. In the end of experiment they found no any significant differences (P>0.05) in fish weight between vaccinated and non-vaccinated group. In another study Atlantic salmons were injected by commercial vaccine of furunculosis against *A. hydrophila*. As a result no any significant differences was observed between the growth of vaccinated and unvaccinated fish (Chalmers et al., 2016). Also, As a result, post-challenge, the survival rate for biofilm vaccine group (93%), followed by the free-cell vaccine group (63%) and the Control group (27%). The results obtained revealed that the survival rate in vaccinated groups were significantly higher ($P < 0.05$) than those in the control group. In comparison between the survival rates of the vaccinated groups, the biofilm vaccinated group exhibited significantly ($P < 0.05$) higher survival rate than free-cell group due to protection of biofilm against destruction of antigen in fish stomach. The results of survival rate was similar to those conducted by Siriyappagouder et al. (2014), Nayak et al. (2004) and Azad et al. (1999, 2000). A highly significant study by Nayak et al. (2004) on walking catfish (*Clarias batrachus*) vaccinated with a biofilm oral vaccine against *A. hydrophila* revealed a survival rate of 93–100%. Meanwhile, the survival rates of the free-cell vaccinated and control groups were 46–56% and 24–35%, respectively (Plant and Lapatra, 2011). In a study conducted by Siriyappagouder et al. (2014), oral biofilm and free-cell vaccines were applied against *A. hydrophila* in *Clarias striatus.* After challenge, the survival rates in the biofilm, free cell and control groups were found to be 92, 49.3 and 28%, respectively.

In conclusion, successfully developed feed-based oral biofilm and free-cell vaccines against streptococcosis which didn't change in the palatability and water stability of the fish feed and also didn't have any negative effect on the growth performance of the fish. We believe that feed-based oral biofilm vaccine can be an ideal vaccine to encounter streptococcosis more than free-cell vaccine. The survival rate of the biofilm vaccine was significantly ($P < 0.05$) higher than free-cell vaccine resulted in protection feature of the biofilm layer against enzymatic destruction in stomach.

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