

Haemato-immunological responses and effectiveness of feed-based bivalent vaccine against *Streptococcus iniae* and *Aeromonas hydrophila* infections in hybrid red tilapia (*Oreochromis* spp.)

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Abstract

Background Streptococcosis and Motile Aeromonad Septicemia (MAS) are important diseases of tilapia, *Oreochromis* spp. and causes huge economic losses in aquaculture globally. The feed-based vaccination may be an alternative to minimize major infectious diseases in tilapia. Thus, this study aims to evaluate the haemato-immunological responses and effectiveness of a newly developed feed-based killed bivalent vaccine against *Streptococcus iniae* and *Aeromonas hydrophila* in hybrid red tilapia. A total of 495 hybrid red tilapia of 61.23 ± 4.95 g were distributed into 5 groups (each with triplicate). The fish were immunized orally through bivalent (combined *S. iniae* and *A. hydrophila*) spray vaccine (BS group), bivalent formulate vaccine (BF group), monovalent *S. iniae* vaccine (MS group), monovalent *A. hydrophila* vaccine (MA group) and unvaccinated as a control group. The vaccine was orally administered on days 0, 14 and 42 applied feed-based bacterin at 5% body weight. The blood and spleen samples were collected from all groups on 7, 21 and 49 days post-vaccination, and also 96 hours post-infection to assess their haemato-immune responses. Results Compared with the unvaccinated group, leukocyte, lymphocytes, monocytes, granulocytes counts in vaccinated groups were significantly ($P < 0.05$) increased on 21, 49 days post-vaccination and also 96 hours post-infection, while erythrocytes, haemoglobin and haematocrit in vaccinated groups were significantly ($P < 0.05$) enhanced only 96 hours post-infection. Additionally, the lysozyme and phagocytic activity and, serum antibody (IgM) were significantly higher ($P < 0.05$) against *S. iniae* and *A. hydrophila* in vaccinated groups compared to the unvaccinated group in the pre- and post-infection. Results from the challenge through co-infection with *S. iniae* and *A. hydrophila* showed the relative percent survival (RPS) in BF group was $76.37 \pm 5.14\%$, which had the capacity to induce significant protection ($P < 0.05$) compared to others groups. Conclusions This study demonstrates the bivalent formulate (BF) group could elicit significant non-specific and specific immunological responses with higher protection in hybrid red tilapia. In addition, this newly developed feed-based bivalent vaccination can be a promising technique for effective and large scale fish immunization in the aquaculture industry.

Background

According to FAO [1], aquaculture is a fast-growing industry playing an important role in the food producing sector, and has provided good quality and affordable protein source throughout the world [2]. Due to rapid growth, suitability for aquaculture, high acceptability in the market and stable market prices, tilapia (*Oreochromis* spp.) is the most important farmed fish globally next to carps, and is cultured more than 100 countries [3, 4]. The global production of tilapia was calculated around 6.532 million tons in 2018 [5, 6] and is presumed to reach 7.3 million tons by 2030 [7].

Amongst the advantages of culturing tilapia is its general hardiness, farming in high stocking densities, adaptability to different production systems, easier reproduction, owing to its higher growth rate, with advances in easily genetic improvement of brood fish for target breeding having further developed these characteristics [8, 9]. Nevertheless, tilapia sector in globally has been facing currently different health risk for affecting with emerging and re-emerging diseases due to fast growth the tilapia industry, involved with trade globalization, introduced of different new species and, intensive and super-intensive farming under stress conditions [10].

Amongst different important bacterial diseases of tilapia, Streptococcosis and Motile Aeromonad Septicaemia (MAS) are the major obstruction to the sustainable development of tilapia farming mainly in Asia [11-13]. Streptococcosis due to *Streptococcus iniae* and *S. agalactiae*, is considered one of the key tilapia disease with high morbidity and mortality throughout the world including Malaysia [14-16]. It was calculated globally that the yearly economic loss due to Streptococcosis outbreaks was as high as \$150 million [9]. On the other hand, another bacterial disease of MAS mainly by *Aeromonas hydrophila* known to have a great negative impact on the growth with survival rate and significant economic losses in the tilapia industry worldwide [13, 17]. Additionally, mass mortality in tilapia due to bacterial co-infection have also been recorded, such as co-infection of *Aeromonas* sp. and *Streptococcus* sp. [11, 18], *S. agalactiae* and *F. noatunensis* [19], *A. veronii* and *F. columnare* [20] and *F. noatunensis* subsp. *orientalis* and *Shewanella putrefaciens* [21].

To address the losses due to Streptococcosis and MAS, antibiotics are the only option for farmers and the use of antibiotics results in development of antibiotic resistance strains, bio-accumulation, changes the physio-chemical properties of water, imbalance of bacterial microbiota in fish body or in the habitat [22-24]. To solve these difficulties, application of fish vaccines is an alternative to minimize the different infectious bacterial diseases [25, 26].

Vaccines are formulated using either antigens, developed from pathogenic bacterial, or whole bacterial killed cells, which improve the specific immunity of the host [27]. Generally, antigens enhance to activate the innate and the adaptive immune systems, both with cellular and humoral responses. The effectiveness of a good vaccine is related to the appropriate immunization routes for stimulating the fish immune system, with advantages and disadvantages. Among the different immunization routes, intraperitoneal (i.p.) injection has mostly provided good results, although it is stressful to the animals, impractical for farmers level, labour intensive and hence expensive [28, 29]. However, another route of immunization is recently practised; the feed-based oral immunization since it is less tedious, more applicable for mass vaccination at farmers level. This immunization route has also proven efficacy in poultry and mammals, which is associated with enhancing of both mucosal and systemic immune systems [30, 31]. Nevertheless, there is still complexity to understand the mechanisms of oral vaccination that are involved in the uptake of antigens [32, 33]. Although, results from oral immunization in fish are contradictory but few researches showed satisfactory results [19, 34, 35]. Kahieshesfandiari [16] reported a positive findings with using feed-based biofilm vaccine in tilapia after challenge against *S. agalactiae*. According to Nur-Nazifah [33], red tilapia immunized orally which presented 70% RPS after *S. agalactiae* challenge.

The application of *A. hydrophila* vaccine is not commercially available for its bio-chemical heterogeneity and the presence of different isolates or sero-groups and variation in virulence gene expression [36, 37]. Nevertheless, very few findings have proved that different vaccine formulations of *A. hydrophila* may provide protection. Besides on, Aly [38] developed an inactivated *A. hydrophila* vaccine for tilapia and after challenge the RPS was satisfactory. Pasaribu [13] prepared an effective divalent vaccine for tilapia brood stock with formalin inactivated *S. agalactiae* and *A. hydrophila*, and the RPS after challenge by *A. hydrophila* was 73.81%.

As the diversity of infectious bacterial agents is very high intensity in the tilapia culture or production systems, bivalent or multivalent vaccines can be conferred the advantage of protection to tilapia against a wide variety of Gram-negative and Gram-positive bacterial strains [39]. Nevertheless, to date from our knowledge, there is no available study on haemato-immunological responses and protection in hybrid red tilapia immunized with feed-based formalin killed bivalent vaccine against Streptococcosis and MAS. Hence, this study aimed to assess the influence of a feed-based formalin killed bivalent *S. iniae* and *A. hydrophila* vaccine on the haematological and immunological parameters and, protective efficacy in immunized tilapia against challenge with *S. iniae* and *A. hydrophila* each bacterium, independently or co-infection.

Results

Haematological parameters

Results of haematological studies are presented in Tables 1-6. After 7 days post-vaccination the levels of erythrocytes, thrombocytes, lymphocytes, haemoglobin, MCH, MCHC and haematocrit were not statistically different ($P > 0.05$) among the groups, but only the number of leucocytes in immunized groups were significantly higher ($P < 0.05$) than the unvaccinated group. On day 21 post-immunization, the leucocytes count, monocytes and granulocytes were significantly higher in vaccinated groups than in the unvaccinated, and those from the BF group ($45.39 \pm 1.34 \times 10^3/\mu\text{l}$) presented significantly the highest ($P < 0.05$) leucocytes and granulocytes values ($7.19 \pm 0.23 \times 10^3/\mu\text{l}$). The number of leucocytes, lymphocytes and granulocytes were significantly increased ($P < 0.05$) in all the vaccinated groups at 49 days post-vaccination compared with the unvaccinated group. Nevertheless, leucocytes count was the highest in MS group ($47.45 \pm 3.22 \times 10^3/\mu\text{l}$), while the level of granulocytes was the highest in BF ($8.09 \pm 0.71 \times 10^3/\mu\text{l}$) group ($P < 0.05$) at 49 days post-vaccination (Tables 1-2).

After challenged with *S. iniae*, the thrombocytes, MCH and MCHC of vaccinated groups compared with the unvaccinated group showed a numerical increase but there was no significant difference ($P > 0.05$). However, the number of erythrocytes, leucocytes, monocytes, granulocytes, haemoglobin and haematocrit in the groups of vaccinated showed a meaningful difference compared with the unvaccinated group ($P < 0.05$) (Tables 3-4) after the challenged. Moreover, the level of erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes, hemoglobin and hematocrit in unvaccinated fish after challenged with *A. hydrophila* were also significantly ($P < 0.05$) lower than that of the vaccinated groups, and the other haematological parameters after the challenge were not significantly different ($P > 0.05$) (Tables 5-6).

Immunological parameters

The value of lysozyme activity and phagocytic activity showed a statistically different effect ($P < 0.05$) between the groups of vaccinated and unvaccinated after 7 days post-immunization, but the antibody level (IgM) did not show statistically difference ($P > 0.05$) among the groups. On day 21 post-vaccination, the value of lysozyme activity, phagocytic activity and antibody level (IgM) were significantly higher in vaccinated groups than in the unvaccinated group, and those from the BF (0.62 ± 0.02) group presented the highest antibody level against *S. iniae* ($P < 0.05$) among the different groups. At 49 days post-vaccination, both lysozyme activity and phagocytic activity were significantly higher in vaccinated groups compared to the unvaccinated group, whereas BF group also showed the statistically highest ($P < 0.05$) lysozyme (325.90 ± 6.02 units/ml) activity comparisons with other groups. On the other hand, the antibody (IgM) level of vaccinated groups were also significantly higher against *S. iniae* and *A. hydrophila* ($P < 0.05$) in comparison to the unvaccinated group, whereas the BF (0.78 ± 0.02), MS (0.71 ± 0.01) and MA (0.63 ± 0.03) groups were obtained significantly the highest level among the groups at 49 days post-vaccination (Table 2).

After challenged with *S. iniae*, the unvaccinated fish group presented significantly lower ($P < 0.05$) lysozyme activity, phagocytic activity and antibody level compared to the vaccinated group. Additionally, the lysozyme activity was significantly higher in both BF (327.83 ± 6.38 units/ml) and MS (323.57 ± 3.04 units/ml) groups, while the antibody level was higher only in BF (0.86 ± 0.07) group ($P < 0.05$) when compared with others groups (Table 4). Furthermore, it was observed that after challenged with *A. hydrophila*, the value of lysozyme activity, phagocytic activity and antibody level (IgM) showed a significant ($P < 0.05$) difference compared to the unvaccinated group. Consequently, the antibody level was also significantly higher ($P < 0.05$) in both BF (0.75 ± 0.04) and MA (0.65 ± 0.04) groups, followed by BS (0.53 ± 0.08) and unvaccinated group (0.30 ± 0.01) (Table 6).

Mortality and Relative Percentage of Survival (RPS)

Percent cumulative mortalities (mean±SE) and RPS (mean±SE) up to 96 h after infection with *S. iniae* and *A. hydrophila* in vaccinated and unvaccinated groups are shown in Fig. 1 (a, b) and Fig. 2 (a, b). After challenge (i.p. injection) with *S. iniae*, the cumulative mortality percentage was statistically ($P < 0.05$) higher in the unvaccinated group than in the vaccinated groups, and those from the BF (10±4.71%) and MS (13.33±0.00%) groups presented the lowest cumulative mortality against *S. iniae* ($P < 0.05$) among the different groups. Similar results were obtained in the challenge with *A. hydrophila*, where the cumulative mortality percentage was also significantly lower ($P < 0.05$) in the groups of BF (13.33±0.00%) and MA (16.67±4.72%) compared with the unvaccinated group. Consequently, the cumulative mortality of the unvaccinated group (70.00±4.71%) showed significantly higher ($P < 0.05$) than other bivalent groups after co-infection with both *S. iniae* and *A. hydrophila*. Nevertheless, after challenged by *S. iniae*, the RPS was significantly higher ($P < 0.05$) in BF (85.35±5.00%) group compared with the BS group (54.04±3.57%). Furthermore, after challenged with *A. hydrophila*, the RPS was also obtained significantly higher protection against *A. hydrophila* in BF (78.89±1.57%) compared with the group of BS (57.77±3.14%). Simultaneously, under the challenge of co-infection with both *S. iniae* and *A. hydrophila*, BF group produced significantly higher RPS (76.37±5.14%), indicating that bivalent vaccine candidate of BF group could confer much better protection against *S. iniae* and *A. hydrophila* compared to the other immunized groups.

Discussion

Haematological parameters have been usually used for observing the health status and immunological responses of fish and aquatic animals [40]. Among the different haematological parameters, the leukocyte count is very important for functioning in the non-specific and specific immune system of the fish [41]. In this study, the leukocyte count in all vaccinated group was higher than the unimmunized ones before and after challenge with *S. iniae* and *A. hydrophila*. Bailone [42] observed that vaccinated tilapia had higher leukocyte count compared to the unvaccinated fish both before and after challenge with *A. hydrophila*. Furthermore, the leukocyte count in immunized rainbow trout (*Oncorhynchus mykiss*) were higher against *A. hydrophila*, respectively [43, 44]. According to Ashfaq [45], the increase in leukocyte count positively affects antibody production, leading to body resistance response against the extraneous substance. Likewise, Silva [29] established, a higher count of leukocyte was strongly correlated with the increasing of phagocytic activity, lysozyme function and antibody titers. In the present experiment, leukocyte count was higher as a result of vaccine can indirectly increase natural immune response which is marked by an increase in phagocytic cells. On the contrast, it indicates in this study that the immunized fish were capable to show better defence response against infection with *S. iniae* and *A. hydrophila*.

In other assays, the total number of erythrocyte was higher in vaccinated red tilapia compared to unvaccinated fish after challenge. Zhang [46] stated that the erythrocytes count of immunized tilapia with formalin killed vaccine via i.p. injection was higher compared with unvaccinated fish after challenge with *A. hydrophila*. On the other hand, previous findings demonstrated that the decreasing erythrocytes count after challenge in unvaccinated fish may be signs of bacterial infection [47, 48]. Subsequently, previous reports also show that the number of erythrocytes decreased in unimmunized salmon (*Salmo salar*) and rainbow trout (*O. mykiss*) fish after infected with *V. anguillarum*, *Aeromonas* sp. and *Streptococcus* sp., respectively [49, 50]. However, in this study, erythrocyte count in the unvaccinated group significantly decreased, this might be due to *S. iniae* and *A. hydrophila* had hemolysin that can cause erythrocyte lysis [51].

Haemoglobin is a pigment in erythrocyte and has a function to bind oxygen to be further circulated to all over the body [52]. Thus, haemoglobin and haematocrit percentage are good signs for the oxygen transportation capacity of fish [53, 54]. Shoemaker [38] and Sukenda [55] observed that the haemoglobin level and haematocrit percentage were significantly increased in vaccinated broodstock tilapia after post-infection compared with the unvaccinated fish. Once again, haemoglobin level of tilapia was decreased after infection with *S. iniae* [54, 56] and *F. columnare* [57]. Thus, the reductions of haemoglobin level and haematocrit percentage indicate that fish infected with the bacterium are developing anemic states [57]. In the present case, the haemoglobin and haematocrit percentage of vaccinated fish showed significantly higher compared to the unvaccinated fish after infection with *S. iniae* and *A. hydrophila*. Nevertheless, this indicates that there was no anemic states develop in feed-based immunized fish after challenged with both bacteria.

Monocytes play a key role in the innate immune response by producing cytokines which can stimulate the activity of lymphocytes [58, 59]. In the current study, the number of monocytes and granulocytes were significantly increased in the vaccinated tilapia on 21, 49 days post-vaccination and after infection with *S. iniae* and *A. hydrophila* as well. Chin and Woo [60] observed that vaccinated salmon (*S. salar*) with live *Cryptobia salmositica* showed a significant increase in the number of monocytes and granulocytes on days 28 and 42 post-immunization, respectively. Moreover, Garcia [61] reported that the number of neutrophils and monocytes in *Piaractus mesopotamicus* were increased after infection with *A. hydrophila*. In contrast, Ranzani-Paiva [62] claimed, the number of neutrophil and monocyte were not changed after challenged with *Mycobacterium marinum*. Nevertheless, increased number of monocytes might be related with a stimulate immune response, phagocytic activity [61] as well as in presenting antigen to lymphocytes [58, 63].

Lymphocytes counts did not differ significantly on 7 days post-vaccination, but an increase in the lymphocyte counts was observed only in vaccinated tilapia on 21, 49 days post-vaccination and also after infection with both bacteria. The lymphocytes counts was significantly higher in the vaccinated *Ictalurus punctatus*, fish [59], which was similar to the current findings. Sirimanapong [64] showed that the number of lymphocyte was also significantly higher in the vaccinated fish by 1, 14 and 21 days post-immunization. Jirillo [65] reported that neutrophils and monocytes increased in a very short time after days 4 and 7 post-vaccination, while lymphocytes increased after 21 and 28 days post-vaccination. These trends indicate that the neutrophils and monocytes played a significant role in the early immune response followed by the lymphocytes.

Lysozyme is a bacteriolytic enzyme produced in the lysosome of the phagocytic cell [66] and lysozyme activity is an essential part of the non-specific immune response of fish [55]. To date, vaccinated tilapia showed a significant increase of lysozyme activity compared to the unvaccinated fish in both pre- and post-infection. Subsequently, previous reports show that the lysozymes activity was significantly higher in vaccinated than unvaccinated tilapia on 3 weeks post-vaccination [67]. In contrast, Silva [68] and Pereira [26] reported, the lysozyme activity did not show a significantly different effect after post-vaccination compared with unvaccinated. Likewise, Sukenda [69] observed that vaccinated broodstock tilapia delivered a significant lysozyme activity in broodstock, eggs, and fry compared to the unvaccinated and showed significant survival in immunized fry. However, this indicates that the increasing reaction of the immune response system in vaccinated tilapia might be co-related with increasing of lysozyme activity.

Fish phagocytes, acting as accessory cells with adaptive immune function, also play a significant role in the innate immune system and are attached in combatting pathogen infection at all steps [60]. The phagocytic cells are functioned in stimulating an inflammatory response, phagocytosis and bactericidal action and nitric oxide (NO) for killed pathogens [64]. In the current study, the phagocytosis activity in vaccinated fish was higher both before and after infection with *S. iniae* and *A. hydrophila* compared with the unvaccinated fish, which was similar to record by Pereira [26]. Additionally, similar results have been also shown in vaccinated yellowtail (*Seriola lalandi*) and striped catfish (*Pangasianodon hypophthalmus*) those were immunised with *Pasteurella piscicida* and *A. hydrophila*, respectively [64]. However, these findings indicate that the peripheral blood of vaccinated fish have phagocytic activity and therefore might also play a significant role in the early innate immune response.

Antibody level is the major parameter to evaluate the specific immune response [70]. The findings of this experiment demonstrate that the antibody level (IgM) of the monovalent and bivalent vaccinated tilapia was increased significantly than the unvaccinated fish when tested for both bacterial antigens. In line with this, Nur-Nazifah [33] observed that tilapia vaccinated with the feed-based vaccine improved a strong and significantly higher antibody response in blood serum samples compared to the unvaccinated fish. Sugiani [18] and Sumiati [11] also showed that tilapia vaccinated with the bivalent *A. hydrophila* and *S. agalactiae* vaccine and the vaccine delivered a significantly higher level of antibodies against both the antigens. Additionally, as previously reported by Sumiati [11] and Pasaribu [13] observed that significantly higher levels of antibody were found in vaccinated tilapia compared with control fish. However, this finding revealed that feed-based monovalent or bivalent vaccine can develop protective specific immune responses in hybrid red tilapia.

The low percent of cumulative mortality in vaccinated fish are usually caused due to the development of the specific immune response that followed by an increase of non-specific immune system [55]. In our present work, significantly lower percent cumulative mortality was in vaccinated fish compared with unvaccinated. In some previous experiments were used *Bacillus subtilis* spores expressing sip and *S. agalactiae* ghost and confirmed their effectiveness in preventing mortalities in immunized tilapia [71, 72]. A similar low mortality rate was also documented in rainbow trout immunized with bivalent formalin-inactivated whole cells *A. hydrophila* [37]. Additionally, an oral vaccine of *A. hydrophila* was applied in catfish (*Clarias batrachus*) showing also positive findings [73].

The results of RPS demonstrate the effectiveness of a vaccine. Throughout the study, bivalent formulate (BF group) vaccinated tilapia showed a significant higher RPS against *S. iniae*, *A. agalactiae* and co-infection (with *S. iniae* and *A. hydrophila*) than other groups. Some previous works observed that the increase of antibody in fish related to the survival rate or RPS of fish [74]. Similar results have been observed by Laith [76] whom estimated the efficacy of a formalin-inactivated and live attenuated vaccine of *S. agalactiae* in tilapia using booster dose with a 21 days interval. Additionally, several works confirm that the application of feed-based vaccination in fish increases the RPS or survival significantly, as in tilapia immunized against *S. agalactiae* [33, 35].

The RPS of the BS group in this study was significantly lower compared with the BF group after challenged with co-infection (*S. iniae* and *A. hydrophila*). For bivalent spray (BS) vaccine, commercial pellet feed was directly top-dressed with bivalent FKCs suspension, whereas the formulate bivalent or monovalent vaccines was developed by incorporating or mixing the FKCs on feed powder and finally loaded into the feed pellet machine to make formulated vaccinated feed. Eventually, spraying or top dressing of antigens suspension on pellet feed is quite easy to apply but have the disadvantage of irregular distribution, leaching the antigens during feeding time and also the threat that the antigens are directly exposed to hostile stomach environment upon feeding, leading to degradation [77]. By contrast, the BF group in our findings showed higher protection might be regular distribution antigens inside the vaccine pellet feed and also protecting the antigens against the hostile stomach environment for impregnating antigens inside the feed.

The success of a bivalent or multivalent vaccine is often controlled by the amount of individual antigens, cross reactivity and competition between or among different antigens [44]. In the current study, the protection achieved and the antibody (IgM) response to *S. iniae* and *A. hydrophila* showed the lack of antigenic cross reactivity and competition between these pathogenic bacteria. Bastardo [37] showed that rainbow trout (*O. mykiss*) presented higher immune responses and survival after the immunization of bivalent vaccines, even better than the monovalent vaccines. As a result, throughout the findings further suggest that the bivalent formulate (BF group) vaccine is capable of inducing protective immunity against *S. iniae*, *A. hydrophila* or co-infection. Nevertheless, in combination with the haematological parameters, immunological responses and protection results in this study, we considered that this was due to the activation of innate and specific immunity after feed-based bivalent immunization.

In conclusion, the findings of this study demonstrate that the feed-based formalin killed bivalent vaccine can significantly induce non-specific and specific immune responses and, provided strong protection against Streptococcosis and MAS in hybrid red tilapia. These results also suggest that feed-based oral bivalent vaccination can be a promising technique for effective and large scale fish immunization in the aquaculture industry.

Methods

Fish and experimental conditions

The study was carried out at Fish Hatchery Unit in Laboratory of Marine Biotechnology (MARSLAB), Institute of Bio-Science (IBS), Universiti Putra Malaysia, Malaysia. A total of 525 apparently healthy hybrid red tilapia (*Oreochromis* spp.) with an average weight of 61.23 ± 4.95 g, were collected from a local fish farm (Kam Sing Fish farm, Selangor, Malaysia). The collected red tilapia were randomly distributed into 15 tanks with 400 litres capacity. The fish were acclimatized for 14 days before vaccination and fed with an available commercial diet (Star Feed, Star Feed Mills SDN. BHD, Malaysia) with 32% protein containing feed at 3% body weight per day. The fish faces and waste materials were siphoned out 3 h after feeding. Prior to commence the experiment, thirty fish were randomly dissecting for screening pathogenic bacteria and checking the antibody level to confirm that they were free from *Streptococcus* sp. and *Aeromonas* sp. One day prior to vaccination or challenge fish were taken off feed. The water quality of the rearing tanks like temperature, pH, dissolved oxygen, ammonia and nitrites were observed throughout the study period. Anesthetics were applied on the experimental fish using 120 mg/L of tricaine methanesulfonate, MS-222 (Aldrich, USA) prior to collect blood samples and bacterial challenge protocols.

Formalin-killed bacteria preparation

The pathogenic strains of *S. iniae* and *A. hydrophila* were isolated from diseased red tilapia and obtained from the previous study [78]. The formalin killed bacterin were prepared as stated in the previous studies [79]. Briefly, the bacterial strains of *S. iniae* and *A. hydrophila* were cultured individually on 5% blood agar (Oxoid, UK) and further grown in separate flasks of 500 ml containing Brain Heart Infusion Broth (BHIB, Oxoid, UK) at 30°C in a shaker incubator at 150 rpm for overnight. The following incubation, the bacterial concentrations were calculated by applying the establish plate count. The individually cultured bacteria cells were then inactivated by treating with neutral-buffered formalin to the concentration of 0.5% formalin in PBS (phosphate buffered saline) and kept at 4°C for 24 h. After that, the bacterial cells were washed four times with the sterile PBS by centrifugation at 6000 x g for 15 min to remove the medium and formalin residue from the culture. Afterwards, the inactivated bacteria were again suspended in sterile PBS to keep the final concentration of 6.7×10^9 CFU/ml. The bacterial suspension was again streaked onto BHIA and incubated at 37°C for overnight to confirm that all *S. iniae* or *A. hydrophila* cells were inactivated. For formulations of bivalent vaccine, formalin killed whole cells (FKCs) of two vaccine strains were combined at a ratio of 1:1 (v/v) and kept at 4°C. Subsequently, for improving the vaccine antigenicity, palm oil (Vesawit, Malaysia) as an adjuvant was mixed to a final concentration of 10% [80] before it was thoroughly sprayed on commercial pellet or formulate feed to obtain a final concentration of 6.7×10^9 cells/g of feed [35, 79].

Feed-based vaccine preparation

Bivalent spray vaccine

The feed-based vaccine was formulated according to the method described earlier Firdaus-Nawi [79] with some modifications. Briefly, the formalin-killed bacterin (FKB) of *S. iniae* (6.7×10^9 CFU/ml) and *A. hydrophila* (6.7×10^9 CFU/ml) with 10% palm oil were mixed and re-suspended properly in PBS for preparing bivalent vaccine. Next, the bivalent FKB solution was directly sprayed onto the commercial floating pellet feed (Star feed, containing 32% protein) to obtain the individual FKB a final concentration of 6.7×10^9 cells/g of feed. A homogenizer or mixer (Golden Bull B10-A Universal Mixer, Malaysia) was used to distribute and impregnate the bivalent FKB vaccine properly into the pellet feed. Finally, the vaccine added pellet was dried up at 30°C for overnight in the oven prior to the experiment.

Preparation of formulated vaccine

A commercially available pellet feed (Star feed, containing 32% protein) was blended with a blender machine to form a very fine mesh powder. To incorporate the vaccine, the formalin-killed bacterin (FKB) of monovalent or bivalent vaccine with 10% palm oil was re-suspended in PBS to a final concentration of 6.7×10^9 CFU/ml. Afterwards, the FKB solution of monovalent or bivalent vaccine was sprayed properly onto the fish feed powder to obtain the individual FKB a final concentration of 6.7×10^9 cells/g of feed. A homogenizer was used to distribute and impregnate the monovalent or bivalent FKB vaccine properly onto the fish feed powder. In unvaccinated group, only 10% palm oil was added in fish feed as a control group. Finally, the vaccine added feed paste was loaded into the auto mini pellet machine (Golden Avill, China) to make the pellet size of 4 x 4 mm and kept at 30°C for overnight in the oven prior to the feed-based immunization.

Experimental Design

A total of 495 hybrid red tilapia (*Oreochromis* sp.) were randomly distributed in five experimental groups with each of three replicates. Group-1 (unvaccinated) was fed non-vaccine containing commercial pellet feed (incorporated only 10% palm oil); group-2 (Bivalent Spray, BS) was

vaccinated by bivalent mixture of *S. iniae* and *A. hydrophila* vaccine directly sprayed on commercial pellet feed; group-3 (Bivalent Formulate, BF) was vaccinated by bivalent vaccine incorporated in feed; group-4 (Monovalent *S. iniae*, MS) was vaccinated by only monovalent *S. iniae* vaccine incorporated in feed and group-5 (Monovalent *A. hydrophila*, MA) was vaccinated by only monovalent *A. hydrophila* vaccine incorporated in feed. At the start of the vaccination, the feed-based vaccine was orally applied in all vaccination groups only on day 0 at 5% body weight four times daily up to 5 consecutive days. Except for unvaccinated control group, all other groups were double boosted with the same immunization on 14 and 42 days after first dose vaccination (Fig. 2).

Challenge test

The challenge tests were performed on 70 days post-vaccination with single bacterial infections of *S. iniae* (3.4×10^8 CFU/ml), *A. hydrophila* (6.8×10^9 CFU/ml) and the co-infection of both pathogenic bacteria with a composition ratio of 1:1. The fish were challenged via intraperitoneal (i.p.) route at a dose of 0.5 ml. Besides the four vaccinated groups, four sub-control groups were set up for challenge test, including control (negative)-1 (unvaccinated, without challenge), control-2 (unvaccinated, but challenged with *S. iniae*) and control-3 (unvaccinated, but challenged with *A. hydrophila*) and control-4 (unvaccinated, but challenged with co-infection, *S. iniae* and *A. hydrophila*). For each bivalent vaccinated group, fish were separated into three sub-groups: The sub-groups were challenged separately with *S. iniae*, *A. hydrophila* and another with co-infection. In every vaccinated group or sub-group was two replicates and each replicate had consisted of 15 fish. All of the unvaccinated or non-challenged each fish was also injected with 0.5 ml PBS. Cumulative mortality of tilapia (%) was recorded up to 96 hours and the RPS were estimated according to Li [81]; $RPS = [1 - (\% \text{ mortality in vaccinated fish} / \% \text{ mortality in unvaccinated fish}) \times 100]$. All remaining fishes at the end of the challenge trials and experiment were euthanized in overdose concentration of 400 mg/L of tricaine methanesulfonate, MS-222 (Aldrich, USA) for at least 10 min and soaked in 25% sodium hypochlorite for 30 minutes prior disposed as clinical waste.

Sample collection

Sampling of fish blood and spleen organ were done from five groups on 7, 21 and 49 days post-vaccination (dpv) and also 96 hours post-infection. The blood sample was collected through the caudal veins from 9 fish of each group and the collected blood were kept in two different type tubes, in one EDTA-containing tubes while another without EDTA-containing tubes. The EDTA-containing blood samples were immediately sent to the laboratory for haematological parameters analysis but without EDTA-containing samples were used to assess immune responses. The spleen samples were collected to determine the phagocytic activity.

Haematological assays

The anti-coagulate blood samples were used to determine the erythrocyte, thrombocytes, leukocyte, haemoglobin, lymphocytes, haematocrit, MCH (mean corpuscular haemoglobin), MCHC (mean corpuscular haemoglobin concentration), monocytes and granulocytes count using an automatic Mythic 18 Vet Haematology analyser machine (Woodley Veterinary Diagnostics, England and Wales).

Immunological assays

Serum lysozyme activity

The lysozyme assay was done according to the methods of Anderson and Swicki [82] and Abdollahi [75]. The pH of the PBS was adjusted to 6.2 at 25°C using 1M HCL and 1M KOH. This (0.01 M PBS) was used to prepare 0.4 mg/ml of *Micrococcus lysodeikticus*. Firstly, 100 µl blood serum and then prepared 100 µl *M. lysodeikticus* was put into the microplate. Afterwards, the resulting absorbance was read at 450 nm (optical density, OD) using microplate reader (Multiskan™ GO Microplate Spectrophotometer, USA) at the time interval of 30 sec and after 30 min. The lysozyme activity was calculated based on a decrease in OD of 0.001/minute. The following formula was used to estimate the units of enzyme activity per one ml of the serum. (see Formula 1 in the Supplementary Files)

Phagocytosis activity

Phagocytosis by spleen performed using the method of Anderson and Swicki [82]. Briefly, in this method; yeast cells were the particulate cells, where equal volumes of spleen cell suspension and yeast cells (0.1 ml) were mixed well with a pipette and incubated for 20 min at 25°C. Five microliters of the incubated solution was placed on a glass slide (pre-coated with 10 % Poly L-Lysine (PLL) solution and dried) and made a smear, air dried and fixed with 95 % methanol for 1 min, transferred into May-Grunwald (MG) solution for 5 min. Finally, the cells were stained with 7% Giemsa stain for 20 min, air-dried. The cells were set under oil immersion (100 x magnification) and one hundred cells were counted from different portions of the slide and finally, the percentage of phagocytic cells were determined.

Enzyme-linked immunosorbent assay (ELISA)

The samples of serum were subjected to indirect ELISA to determine the antibody titre against *S. iniae* and *A. hydrophila* using the method described by Firdaus-Nawi [79] and Li [81] with minor modification. Coating antigen was prepared by culturing *S. iniae* and *A. hydrophila* into BHIB and incubated for overnight in shaker incubator for 150 rpm at 30°C. The concentration of the cultured bacteria was calculated with the flowing of

the standard plate count method prior to harvest through centrifugation and washed with PBS. After that, the bacterial pellets were suspended in carbonate-bicarbonate buffer (pH 9.6). The prepared bacteria was inactivated through boiling in a water bath at 90°C for 20 min and incubated at room temperature before to use as coating antigen (2.5×10^5 CFU/ml). Then, 100 µl coating antigen was coated into the microtitre plates and kept at 4°C for 24 h before washed two times with PBST (PBS + 0.05 % Tween 20). This was followed by adding 200 µl of 1% BSA to block unspecific binding sites and kept at 37°C for one hour. Thereafter, 100 µl of diluted serum (1:1000) was added into the reaction and incubated. Afterwards, goat anti-tilapia hyperimmune serum (*Aquatic Diagnostics Ltd*, Scotland) was diluted at the ratio of 1:10000, added 100 µl into the reaction and incubated at 37°C for one hour again. Then, 100 µl of conjugated rabbit anti-goat IgM horseradish peroxidase (Nordic, Netherland), diluted 1:10000 was added and incubated. The following microtitre plates was added 100 µl of TMB (Promega, USA) after washed for thrice with PBST and finally added 100 µl of TMB (Promega, USA) before 0.2 mol/l sulphuric acid. The absorbance was calculated by setting microplate reader (Multiskan™ GO Microplate Spectrophotometer, Finland) at 450 nm wavelength.

Statistical analysis

Data were analysed using SPSS-16 software (SPSS Inc., Chicago IL). Differences in haemato-immunological parameters and RPS between unvaccinated and vaccinated groups were examined using one-way ANOVA with Duncan post hoc tests. Statistical significance was considered at p values < 0.05.

Abbreviations

MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; ELISA: Enzyme-linked immunosorbent assay; IgM: Immunoglobulin M; i.p.: Intraperitoneal; FKB: Formalin killed bacteria; dpv: Days post vaccination; hpi: Hours post infection; BS: Bivalent spray vaccine; BF: Bivalent formulate vaccine; MS: Monovalent *S. iniae* vaccine; MA: Monovalent *A. hydrophila* vaccine.

Declarations

Ethics approval and consent to participate

All procedures in this study involving animals were approved by the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee, approval number #UPM/IACUC/AUP-R076/2019.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analysed during the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MSM collected and analysed data, and drafted the manuscript. MSMY, ZMZ, HAH and IMY participated in designing the experiment, supervised the project and revised manuscript. MSM, AM and MSMHN conducted all the experiments. All authors provided critical feedback, read and approved the final manuscript.

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Tables

Table 1 Haematological parameters (mean±SD) of hybrid red tilapia on days 7, 21 and 49 post-vaccination, before challenge trial

Groups	Days after	Erythrocytes (10 ⁶ /μl)	Thrombocytes (10 ³ /μl)	Leucocytes (10 ³ /μl)	Lymphocytes (10 ³ /μl)	Monocytes (10 ³ /μl)	Granulocytes (10 ³ /μl)	Haemoglobin (g/dl)
Unvaccinated	7 (dpv)	4.22±0.55 ^a	22.43±3.33 ^a	26.71±4.51 ^c	25.49±1.95 ^a	0.30±0.07 ^a	4.76±0.46 ^a	5.45±1.18 ^a
BS		4.98±1.51 ^a	23.37±3.59 ^a	31.90±1.92 ^b	26.35±2.59 ^a	0.33±0.05 ^a	4.52±0.73 ^a	5.38±1.47 ^a
BF		5.51±1.96 ^a	24.49±3.91 ^a	37.23±0.46 ^a	28.46±5.71 ^a	0.36±0.05 ^a	5.45±1.66 ^a	5.80±1.49 ^a
MS		5.22±2.14 ^a	25.56±6.35 ^a	36.46±1.20 ^a	27.43±5.65 ^a	0.35±0.07 ^a	5.30±1.13 ^a	5.82±1.30 ^a
MA		4.46±0.86 ^a	24.13±3.92 ^a	35.20±0.63 ^{ab}	27.26±3.89 ^a	0.33±0.06 ^a	4.90±0.20 ^a	5.69±1.49 ^a
Unvaccinated	21 (dpv)	3.92±1.52 ^a	19.90±3.10 ^a	29.21±4.83 ^c	17.82±1.52 ^c	0.37±0.06 ^b	4.29±0.47 ^c	4.90±1.84 ^a
BS		5.93±2.50 ^a	21.77±2.96 ^a	37.03±2.08 ^b	24.39±1.10 ^c	0.39±0.10 ^{ab}	5.71±1.15 ^b	4.98±1.04 ^a
BF		7.55±2.06 ^a	25.34±5.06 ^a	45.39±1.34 ^a	27.63±1.40 ^{ab}	0.46±0.03 ^{ab}	7.19±0.23 ^a	6.58±1.55 ^a
MS		6.36±2.66 ^a	27.30±4.99 ^a	38.75±2.00 ^b	28.65±2.84 ^a	0.49±0.03 ^a	5.69±0.55 ^b	6.71±2.31 ^a
MA		8.01±2.02 ^a	23.31±4.89 ^a	39.78±2.49 ^b	25.93±1.44 ^{ab}	0.43±0.02 ^{ab}	5.73±0.40 ^b	6.35±1.05 ^a
Unvaccinated	49 (dpv)	4.22±0.94 ^a	17.80±5.13 ^a	27.92±1.59 ^c	18.66±2.84 ^c	0.39±0.02 ^b	3.96±0.60 ^c	5.11±0.66 ^a
BS		4.64±1.58 ^a	23.62±5.37 ^a	39.35±0.86 ^b	23.57±1.95 ^b	0.49±0.02 ^{ab}	5.74±0.31 ^b	5.73±2.12 ^a
BF		6.52±2.10 ^a	26.55±5.26 ^a	43.18±2.14 ^b	27.65±1.88 ^{ab}	0.52±0.09 ^{ab}	8.09±0.71 ^a	6.79±2.13 ^a
MS		7.14±1.51 ^a	24.83±6.25 ^a	47.45±3.22 ^a	28.80±1.73 ^a	0.55±0.06 ^a	6.16±0.69 ^b	7.01±2.66 ^a
MA		6.35±2.32 ^a	21.11±3.11 ^a	42.19±2.99 ^b	27.10±2.15 ^{ab}	0.53±0.03 ^{ab}	6.11±0.63 ^b	6.57±2.26 ^a

The different superscript letter within the same column shows a significantly different effect ($P < 0.05$)

Table 2 Haematological and immunological parameters (mean±SD) of hybrid red tilapia on days 7, 21 and 49 post-vaccination, before challenge

Groups	Days after	Haematological parameters			Immunological parameters		
		MCH (pg)	MCHC (g/dl)	Haematocrit (%)	Lysozyme activity (units/ml)	Phagocytic activity (%)	Antibody level (IgM) (450 nm)
Unvaccinated	7 (dpv)	14.24±2.24 ^a	29.43±2.94 ^a	17.32±4.21 ^a	93.73±2.08 ^d	23.41±2.03 ^c	0.23±0.02 ^a
BS		13.75±5.32 ^a	31.38±3.82 ^a	17.94±5.26 ^a	158.35±9.62 ^c	27.74±1.52 ^b	0.27±0.06 ^a
BF		13.90±4.22 ^a	28.96±5.89 ^a	19.71±4.97 ^a	212.77±8.98 ^a	32.00±1.40 ^a	0.27±0.04 ^a
MS		14.12±3.96 ^a	30.63±6.67 ^a	18.52±2.74 ^a	202.17±4.08 ^{ab}	32.10±1.51 ^a	0.28±0.03 ^a
MA		13.24±4.87 ^a	29.91±6.65 ^a	18.72±5.25 ^a	192.96±5.50 ^b	30.84±1.35 ^a	0.26±0.05 ^a
Unvaccinated	21 (dpv)	16.71±2.07 ^a	38.58±3.15 ^a	17.79±2.24 ^a	93.60±2.80 ^d	24.21±3.54 ^b	0.25±0.03 ^c
BS		15.95±1.52 ^a	39.28±1.74 ^a	18.58±4.01 ^a	204.16±9.10 ^{ab}	34.55±2.53 ^a	0.40±0.08 ^b
BF		14.98±3.23 ^a	40.91±3.28 ^a	20.17±5.85 ^a	264.78±6.99 ^a	38.27±4.12 ^a	0.62±0.02 ^a
MS		16.63±2.48 ^a	43.99±4.86 ^a	19.44±3.80 ^a	253.68±6.78 ^{ab}	39.67±6.51 ^a	0.49±0.09 ^b
MA		13.68±1.45 ^a	40.92±3.80 ^a	17.85±6.67 ^a	242.55±6.26 ^c	36.28±4.32 ^a	0.50±0.03 ^b
Unvaccinated	49 (dpv)	17.10±3.01 ^a	36.27±2.17 ^a	16.49±4.41 ^a	94.75±4.43 ^d	23.66±3.92 ^b	0.21±0.04 ^c
BS		16.97±4.10 ^a	38.39±2.47 ^a	18.86±2.75 ^a	207.84±9.43 ^c	33.17±5.10 ^{ab}	0.58±0.03 ^b
BF		15.93±4.16 ^a	40.75±4.29 ^a	19.69±4.39 ^a	325.90±6.02 ^a	40.88±5.02 ^a	0.78±0.02 ^a
MS		17.01±4.43 ^a	40.13±6.58 ^a	17.86±2.04 ^a	313.34±5.19 ^{ab}	39.63±7.81 ^a	0.71±0.01 ^a
MA		15.92±4.05 ^a	43.45±5.83 ^a	17.92±3.27 ^a	303.05±8.57 ^b	38.92±7.22 ^a	0.63±0.03 ^{ab}

The different superscript letter within the same column shows a significantly different effect ($P < 0.05$)

Table 3 Haematological parameters (mean±SD) after 96 h challenged with *S. iniae* of hybrid red tilapia

Groups	Hours after	Erythrocytes (10 ⁶ /μl)	Thrombocytes (10 ³ /μl)	Leucocytes (10 ³ /μl)	Lymphocytes (10 ³ /μl)	Monocytes (10 ³ /μl)	Granulocytes (10 ³ /μl)	Haemoglobin (g/dl)
Unvaccinated	96 h (hpi)	2.98±0.29 ^b	15.87±4.17 ^a	18.70±4.50 ^c	21.55±3.57 ^b	0.24±0.04 ^b	3.34±0.51 ^b	3.97±1.64 ^b
BS		5.19±0.55 ^a	20.01±6.21 ^a	24.02±1.74 ^{bc}	27.67±1.00 ^a	0.30±0.03 ^a	4.97±0.62 ^{ab}	5.87±0.55 ^a
BF		6.9±0.99 ^a	22.76±3.63 ^a	33.19 ± 2.48 ^a	29.50±1.10 ^a	0.34±0.15 ^a	6.24±1.48 ^a	6.25±0.56 ^a
MS		7.02±1.81 ^a	24.63±3.84 ^a	29.03±4.57 ^a	30.17±2.10 ^a	0.33±0.30 ^a	6.06±0.53 ^a	7.02±0.75 ^a

The different superscript letter within the same column shows a significantly different effect ($P < 0.05$)

Table 4 Haematological and immunological parameters (mean±SD) after 96 h challenged with *S. iniae* of hybrid red tilapia

Groups	Hours after	Haematological parameters			Immunological parameters		
		MCH (pg)	MCHC (g/dl)	Haematocrit (%)	Lysozyme activity (unit/ml)	Phagocytic activity (%)	Antibody level (IgM) (450 nm)
Unvaccinated	96 (hpi)	15.58±2.82 ^a	21.99±1.38 ^a	13.45±2.71 ^b	96.50±4.16 ^c	27.45±6.14 ^c	0.30±0.02 ^c
BS		13.08±2.26 ^a	28.79±3.32 ^a	18.45±2.78 ^a	253.37±6.11 ^b	36.50±3.49 ^b	0.59±0.06 ^b
BF		12.90±5.03 ^a	29.46±7.82 ^a	20.26±1.57 ^a	327.83±6.38 ^a	47.60±4.50 ^a	0.86±0.07 ^a
MS		14.79±2.20 ^a	30.29±7.83 ^a	21.06±2.65 ^a	323.57±3.04 ^a	39.00±9.05 ^{ab}	0.72±0.03 ^{ab}

The different superscript letter within the same column shows a significantly different effect ($P < 0.05$)

Table 5 Haematological parameters (mean±SD) after 96 h challenged with *A. hydrophila* in hybrid red tilapia

Groups	Hours after	Erythrocytes (10 ⁶ /μl)	Thrombocytes (10 ³ /μl)	Leucocytes (10 ³ /μl)	Lymphocytes (10 ³ /μl)	Monocytes (10 ³ /μl)	Granulocytes (10 ³ /μl)	Haemoglobin (g/dl)
Unvaccinated	96 (hpi)	2.36±0.78 ^b	14.15±1.64 ^a	19.76±1.95 ^c	23.36±4.43 ^b	0.26±0.03 ^b	2.96±1.10 ^b	3.60±0.62 ^b
BS		4.54±0.99 ^a	18.90±4.28 ^a	23.93±1.34 ^b	29.55±1.26 ^a	0.31±0.03 ^a	5.47±0.69 ^a	5.86±0.43 ^a
BF		5.91±0.67 ^a	19.38±3.04 ^a	28.10±1.75 ^a	30.52±1.93 ^a	0.33±0.02 ^a	6.10±0.77 ^a	6.54±0.97 ^a
MA		5.36±1.63 ^a	20.13±4.81 ^a	25.93±1.56 ^{ab}	29.08±2.64 ^a	0.34±0.02 ^a	5.99±0.68 ^a	5.95±0.68 ^a

The different superscript letter within the same column shows a significantly different effect ($P < 0.05$)

Table 6 Haematological and immunological parameters (mean±SD) after 96 h challenged with *A. hydrophila* in hybrid red tilapia

Groups	Hours after	Haematological parameters			Immunological parameters		
		MCH (pg)	MCHC (g/dl)	Haematocrit (%)	Lysozyme activity (unit/ml)	Phagocytic activity (%)	Antibody level (IgM) (450 nm)
Unvaccinated	96 (hpi)	15.17±0.65 ^a	20.00±1.75 ^a	12.85±1.39 ^b	97.84±4.24 ^d	26.39±5.50 ^b	0.30±0.01 ^c
BS		14.75±0.81 ^a	23.15±3.79 ^a	18.38±1.01 ^a	235.54±6.84 ^c	37.15±2.96 ^{ab}	0.53±0.08 ^b
BF		15.36±2.05 ^a	19.38±3.04 ^a	19.45±1.12 ^a	323.07±4.59 ^a	48.04±6.61 ^a	0.75±0.04 ^a
MA		14.97±1.52 ^a	22.67±3.03 ^a	16.59±2.72 ^a	301.03±5.41 ^b	47.07±7.45 ^a	0.65±0.04 ^a

The different superscript letter within the same column shows a significantly different effect ($P < 0.05$)

Figures

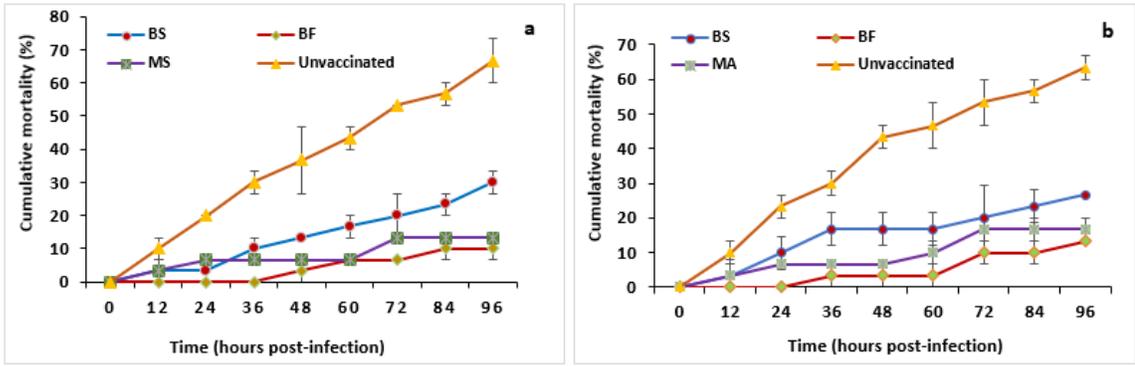


Figure 1

Cumulative mortalities of red tilapia after challenged with (a) *Streptococcus iniae* and (b) *Aeromonas hydrophila*

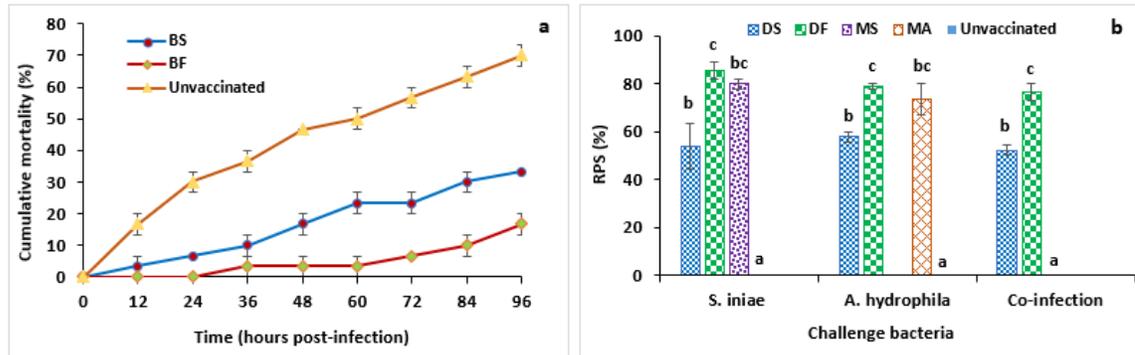


Figure 2

Cumulative mortalities after challenged with (a) co-infection, and (b) RPS of hybrid red tilapia

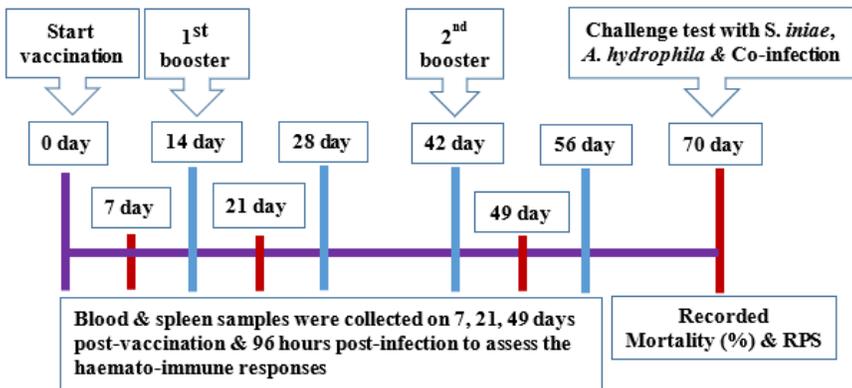


Figure 3

Timeline of vaccination regime and challenge assay

Supplementary Files

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