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RESEARCH ARTICLE

The Efficacy of Nile Tilapia Vaccination by Formalin killed *Streptococcus agalactiae* Encapsulated in Alginate Microcapsules

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Abstract Nile tilapia is one of the major farmed fish that is often infected with Streptococcus. Oral vaccination is the most preferable technique, but the antigen is usually degraded by gastric juice. Encapsulation by alginate could be effective in protecting the antigen. The aim of this study was to assess the effectiveness of vaccinating Nile tilapia using formalin-killed *Streptococcus agalactiae* encapsulated in alginate. The average diameter of fabricated microcapsules was 500±20 µm, with 86% encapsulation efficacy. Fish were then divided into four groups (30 fish per group): group A: fish vaccinated by alginate microcapsules covered by formalin-killed cells (FKC), group B: fish vaccinated by non-covered FKC, group C: fish fed with empty alginate microcapsules and group D: fish feed with commercial pellets as control group. All groups were treated for 14 days and then fed with commercial pellets. Blood samples were collected on days 7, 14, 21, and 28 days post-vaccination. Bactericidal activity, lysozyme activity and serum antibody titer were significantly elevated in group A ($p<0.001$) compared to other treated groups. Then, gene expression analysis was performed at 29 days post-vaccination to evaluate the expression of main immune contributors such IgM, IgT, TCR β, CD4, CD8α, IL 1β, IL 8, IFN 1, and TNFα. The analysis showed significant upregulation of all tested genes in group A compared to other treated groups ($p<0.001$). Lastly, a challenge test was done on day 30 post-vaccination by injecting 4.6×106 CFU mL−1 of virulent *S. agalactiae*. The relative percent of survival (RPS) were 92± 2%, 48± 5% and 4± 3% for groups A, B and C respectively. The obtained result indicated that alginate encapsulation provided antigen protection due to its higher immunogenicity compared to non-covered vaccine. Hence, the fabricated vaccine could be incorporated with food and orally administered to Nile tilapia to prevent *S. agalactiae* infection.

Keywords: *Streptococcus agalactiae*, alginate, microcapsule, Nile tilapia, vaccine, aquaculture.

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Introduction

In developing countries, fishing is a major contributor to social development, revenue generation, and nutrition security [13, 34]. The fish industry recently received a lot of attention and is quickly expanding thanks to the expansion of aquaculture [68]. Fish produced through aquaculture account for over 50% of the total global fish production [29]. The native Egyptian species known as the Nile tilapia, *Oreochromis niloticus*, is the most important socioeconomic species for tilapia aquaculture [52]. Tilapia is the second-most-cultivated fish globally behind carp, it is sold internationally in even more than 135 countries and is in great supply among consumers, furthermore, the global demand for tilapia meat increased by around 2% yearly, meaning the production of this fish should increase by 150 000 tons every year [29].

Despite the tremendous growth of aquaculture industry, the sector faces pathogenic highly problematic infections, with an estimated $6 billion (USD) in global aquaculture disease-related losses per year [59].

Streptococcus spp. infections, particularly *Streptococcus agalactiae* and *Streptococcus iniae*, are currently the major infectious bacterial species and cost the aquaculture sector a great deal of money, however, in tilapia *S. agalactiae* is more prevalent as streptococcosis agent [51, 79]. Streptococcosis plagued tilapia cultures, leading to severe symptoms including spinal curvature, dark skin pigment, dermal hemorrhages, hyperemic gills, corneal opacity, an anorexia phase, erratic swimming, and diffuse epithelial tissue proliferation symptoms followed by high mortality rate and mass economic losses for tilapia culture industry [35, 65].

Disease prevention and control are significant for enhancing the production rate and development of commercial aquaculture [67]. Safeguarding aquatic animal production was concentrated on using antibiotics to protect aquaculture health and earn higher production, but the excessive use of antibiotics may contribute to a global problem by raising the multi-resistant bacteria, hazards to food health and environmental issues because of releasing antibiotics into surrounding water during treatment [16, 22, 57]. One of the really crucial—and likely the most urgent—strategies for the management and prevention of pathogenic fish sickness is the advancement of vaccination [1]. Also, using vaccines in aquaculture is recognized as environmental friendly disease control strategy by minimizing the use of antibiotics, which may outcome ecologically dangerous side effects, besides minimizing their residues in human food chain [24, 58]. Over disease protection due to augmentation, cellular and humoral responses, it is well known that various immunostimulants used in aquaculture induce additional advantages, such as growth enhancement and increased survival rates under stress [78].

Vaccination is a widely accepted and effective method to control *S. agalactiae* infection and prevent mass fish mortalities [24, 52]. The first vaccine against *S. agalactiae* in humans was described in the 1930s [49]. While the interest in vaccines to control *S. agalactiae* epidemic in aquaculture expanded by vastness of Nile tilapia aquaculture productions by the 1990s [58]. Currently, researcher seeking vaccines that are friendly for users and environment, economical, effective, suitable for small and large fish, protect against wider range of infectious pathogens, are easy to administer, and provide long-lasting protective immunity [22, 47]. The vaccine's effectiveness varied depending on the pathogen, route of delivery, vaccine production techniques, status of immunological memory, type and life stage of fish, water temperature during the immunization process and labor`s experience in vaccination [1, 84]. Most manufactured vaccines are in four shapes: inactivated bacterial cells, live attenuated bacteria, recombinant immunogenic proteins, heterologous vectors and DNA vaccines.

In fact, vaccines can be administered to fish by three main routes: injection, immersion and orally [57, 77]. Despite the higher efficiency of the injection and immersion methods, their usage is constrained, especially when fish need to be immunized on a wide scale because of the excessive fish handling and high labor expenses [57]. In contrast, oral delivery makes it possible to vaccinate any fish size on large scales, reduces time consumption, and avoids handling stress, additionally, it is more economical way due to requiring minimal equipment without experienced labor [57, 77]. Notwithstanding previous advantages, the oral method has demonstrated the lowest immune protection [80]. The low efficacy resulted from vaccine degradation in the digestive tract before inducing an immune response, where vaccine was broken down by acid-sharp condition in the stomach [54].

Currently, many research has centered on biotechnological solution by covering vaccines for improving oral immune-modulators delivery system [14]. In this context, many polymers like chitosan, alginate, polylactic-co-glycolic acid (PLGA), polyethylene glycol (PEG) and other polysaccharides have been utilized to encapsulate vaccines in nano/microcapsules in purpose shield antigens productively from the stomach's sharp environment [1, 75]. In particular, alginate as natural polymer remains an attractive material for biomedical applications because of its numerous favorable properties, this biopolymer is linear and anionic polysaccharide derived from brown seaweed and can be produced by bacterial sources [12]. Alginate is composed of alternating blocks of α-1,4-l-guluronic acid (G) and *β-1,4-d-manurunic* acid (M) units and there are different grades of purity available depending on the application [12, 17]. Its availability and its unique ability to form stable pH-sensitive hydrogels in aqueous media and mild conditions by addition of multivalent cations makes this biopolymer very useful for drug delivery [4, 19, 50]. Also, alginate is commercially available in form of salt (e.g., sodium alginate) and preferable due to its solubility in water at room temperature, while other anionic marine-source polysaccharides like agar and carrageenan require heating and cooling cycles for the formation of gel [10, 76]. Fabricated alginate microcapsules have several qualities that enable this polymer to perform pharmaceutical functions efficiently, including stability in acidic pH and disassociation in basic conditions, which make it ideal suit to cover drug active principles from gastric harsh conditions and release it at basic environment in the intestine as the target organ [3, 6, 76]. In addition to biodegradability, low toxicity, and chemical versatility, mucoadhesiveness makes this biomaterial one of the most applicable biopolymers used in the capsulation of oral drug delivery [4, 26].

In the current study, *S. agalactiae* formalin-killed cells were encapsulated in alginate microcapsules using our research's approach and administered orally to Nile tilapia. Then measurements of serum immunological constituents, the production of immune-related genes, and challenge the treated fish with virulent bacteria, and subsequent quantification of fish mortality were used to assess the effectiveness of vaccination. Ultimately, our research strives to advance the field of fish immunology and provide practical solutions for disease management in aquaculture and shed light on the effectiveness of this vaccination strategy.

Materials and Method

Bacteria Preparation

The strain *S. agalactiae* KT869025 was obtained from fish disease laboratory of University Malaysia Terengganu, the bacteria was previously isolated from a streptococcosis outbreak authorized by DNA sequencing and stocked in 25% glycerol at −80°C. The bacteria were thawed using heating block and grown for 48 hours at 30 °C in Trypticase Soy Broth TSB; (Difco, USA). Furthermore, the bacteria were cultured on blood agar plates and incubated at 30°C for 24 h to confirm hemolytic activity. In order to cultivate bacteria, hemolytic colonies of *S. agalactiae were* inoculated from the blood agar plate into tryptic soy broth TSB (Merck, Germany) and incubated in shaker incubator (IKA KS 4000, China) at 30 °C with constant shaking at 150 rpm for 24 h. After incubation, the bacterial concentration was determined using the standard plate count method.

Vaccine Preparation

The *S. agalactiae* pellets were harvested from cultured *S. agalactiae* broth by centrifugation with 5000 rpm at 4 °C for 15 min (Eppendorf 5810, Germany), then the pellets washed twice with phosphate-buffered saline (PBS) followed by centrifugation (5000 rpm 15 min at 4 °C) to remove remaining broth media particles.

The vaccine of formalin-killed cell FKC prepared according to [30], Pathogens cells inactivated by immerse the pellet with 0.5 % buffered formalin overnight at room temperature, afterwards the suspension were washed twice with PBS and centrifuged by 5000 rpm for 15 min at 4 °C in order to remove the formalin residues. The formalin-treated bacteria were re-suspended in sterile PBS to the final concentration of 6.5×10^8 CFU mL^{-1}, then 0.1 ml of suspension was streaked in tryptic soy agar plate (Merck, Germany) and incubated for 24 h at 30 °C, where no growth noticed and the inactivation of *S. agalactiae* confirmed.

Microcapsule Fabrication

Alginate microsphere that encapsulate *S. agalactiae* FKC vaccine was prepared following [45] with some modifications. The FKC bacterial suspension (BS) of (10^8 CFU/mL) mixed with sodium alginate solution (1.9 %) with constant stirring to form Alg- BS homogenous mixture, then, the microsphere fabricated by drop-wising Alg- BS mixture via automatic liquid feeder into sterile $CaCl_2$ (500 mM), as a cross-linker, with 200 rpm constant orbital stirring to prevent microcapsules agglomeration. Figure 1 describes briefly the procedures that were followed to encapsulate *S. agalactiae* FKC inside alginate microcapsules. Then, the produced microcapsules were then washed with sterile PBS separated on a Whatman cellulose filter, dried at 25 °C and stored at 4 °C until use.

Encapsulation Efficacy

The FKC number in alginate microcapsules after loading determined referring to the procedure of [33] with slight changes. Briefly, 1 ml of alginate microspheres solubilized in 9 ml of digestion solution (50 mM sodium citrate, 0.45% NaCl). The suspension was kept at 35°C with shaking 100 rpm for 15 minutes in air shaker bath until microspheres diapered, then samples were centrifuged at 10,000×*g* for 20 min and the bacterial cells concentration in supernatant determined using usual spectrophotometric protocol, the experiment carried out in three independent replicate and the encapsulation efficacy calculated by the following equation:

Encapsulation efficacy = FKC concentration in microspheres / FKC concentration in starting bacterial suspension ×100%

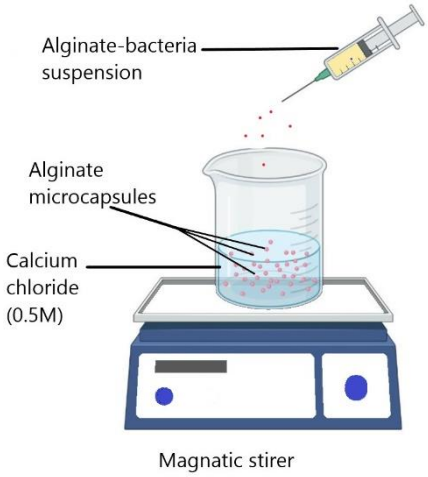


Figure 1. Technical illustration describe alginate microcapsules fabrication

Release Efficacy of Encapsulated Vaccine in Fluids Simulating Gastrointestinal Conditions

In order to test microspheres stability in fish gastrointestinal conditions, 4 solutions with different pH were made simulating fish stomach and intestine fluids as suggested by [69] with slight modification, Briefly 0.3 g of microcapsules incubated at room temperature in 4 test tube contain 1.7 ml of sterile solutions with the following pH 2.0, 4.5 (deionized water adjusted with HCl), 7.4 (PBS) and 9.0 (Tris–buffer), after incubation for 12 h with gentle orbital shaking, test tubes content filtrated in order to observe microspheres under optical microscope after incubation in simulated gastric juice, while the filtrate centrifuged at 500 rpm for 10 min to remove microcapsules residues and then the supernatant transferred to determine the amount of released bacteria utilizing Nano-drop spectrophotometer.

Scanning Electron Microscopy

To determine the shape and size variation of dehydrated microspheres, scanning electron microscopy (SEM) was used. The microspheres were re-distributed in distilled water and put onto aluminum stubs after being freeze-dried. The mixture was then dried by air movement in a natural environment. Microspheres were lastly coated in gold particles using auto fine coater (JEOL 1600, Japan), and observed under a scanning electron microscope (JEOL 7001, Japan) under 20 kV until being seen.

Experimental Diets Preparation

Pellets incorporated with alginate – FKC microcapsules were prepared by grinding commercial pellets into powder and mixed uniformly with alginate microspheres that encapsulate FKC and subsequently mixed with PBS (50%, w/w) with 0.1% guar gum as binder, then the homogeneous paste applied to food pelletizer. The same procedure repeated in order to prepare food pellet incorporated with alginate empty microsphere, while pellets incorporated with FKC (non-capsulated vaccine) were synthesized by mixing the powdered pellets with FKC suspension diluted in sterile PBS with 0.1% guar gum afterward the paste applied into food pelletizer. Taking into consideration each fish will be fed 3% of body weight per day, the concentration of FKC that incorporated in pellets modified to be 1.0×10^6 cells/g of food.

Experimental Design

Nile tilapia *O. niloticus* with weight 25±2 g were acclimatized for 14 days prior the beginning of the research in aerated fiberglass tanks with running water and fed a commercial diet at 3% of body weight daily with 12-h light and dark periods, the fish were kept in temperatures ranged 27–29 C, dissolved oxygen (DO) of 5.33–6.55 mg. L^{-1} and a pH at (7.5-7.7), water quality parameters checked daily by air-calibrated YSI model 57 oxygen meter (YSI, Yellow Springs, OH, USA). Before the start of the experiment five fish were randomly selected and dissected, samples from skin, kidney and brain were collected for microbiological examination to ensure that they were free from *S. agalactiae* infection.

Fish were distributed among four treatment groups in 200 L containers by 45 fish per group. Before vaccination, fish fasted for 24 h, afterward, fish feed the following diets group A feed by pellets incorporated with alginate particles encapsulate FKC, group B feed by pellets incorporated with FKC (non-capsulated cells), group C feed pellets incorporated with empty alginate microcapsules and group

D fish feed by commercial pellets diet (negative control), pellets was dispersed in the aquaria slowly and uniformly to allow all fish uptake relatively equal dosage of the feed, feeding diet performed one time per day at 3% of body weight for 14 consecutive days. After vaccination all groups feed commercial diet throughout remaining examination period, experiment performed in three replicate Figure 2.

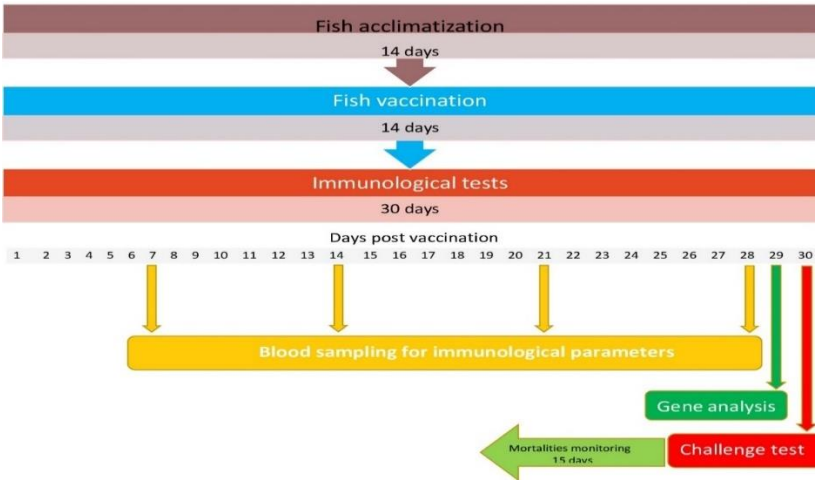


Figure 2. Experimental design and workflow including vaccination, blood sampling, gene analysis and challenge test

Blood Sample Collection

On days 7, 14, 21, and 28 post vaccination, 3 fish from each group randomly selected and evacuated from the caudal peduncle using an insulin needle to analyze the serum immune components. Following centrifugation (13,700 g for 10 min), sera were isolated, and stored at -80 °C until immunological testing. Afterward sera samples tested to detect antibody titer, lysozyme activity and bactericidal activity.

Enzyme-Linked Immunosorbent Assay (ELISA) Antibody Titers

An enzyme-linked immunosorbent test was used to objectively quantify the plasma antibody titers against *S. agalactiae*. Using [74] protocol ELISA dishes (Nunc, Denmark) were first coated for 18 hours at 4 °C with 50 µl of sonicated *S. agalactiae* (100 g/ml) antigen that had been diluted 1:50 in covering buffers (carbonate bicarbonate at pH = 9.6). plates were then blocked with 2.5 percent non-fat milk in phosphate-buffered saline plus Tween-20 (PBS-T) for 1 hour at 25 °C after being rinsed with phosphate-buffered saline (PBS) containing 0.05 percent of Tween-20 (PBS-T). Serum products (100 µl) were diluted (1:25) in PBS-T-S (phosphate-buffered saline + 0.05 percent Tween-20 (PBS-T) containing 0.1 percent skim milk) and put on ELISA plates after the preceding stage had been washed three times with PBS-T. The plates were held at 25 °C for 90 minutes while being shaken, and they were then washed once more as before. After washing, 100 µl of monoclonal antibody anti-Nile tilapia immunoglobulin was added to the wells of the ELISA plates at a concentration of 1:7500 in PBS-T-S. The shaking of the dishes took place for hour, and then they were PBS-T washed three times. Following that, 60 minutes were spent hatching the wells with 50 µl of goat anti-mouse IgG HRP conjugated (Sigma-Aldrich) diluted (1:2500) in PBS-T-S. After washing with PBS-T, 50 l of the chromogen solution that contains TMB (3,3′, 5,5; -tetramethylbenzidine-H2O2) was added to the wells, where it was left to react for 10 minutes at 25 °C. Finally, 50 µl of 2 N H_2SO_4 was used to stop the activity and ELISA reader was used to detect the serum antibody titers at 450 nm.

Bactericidal Activity

Using the technique described by Budino *et al*., the bacterial activity was assessed with some changes. [15]. Essentially, 25 µl of serum sample were diluted in 25 µl of pure PBS before being mixed with 50 ml of bacterial solution (10^6 CFU/ml). This suspension was allowed to brood for 6 hours at room temperature. 50 µl of MTT (dimethylthiazol-diphenyl tetrazolium bromide) (Sigma, M5655) was added to the suspension then incubated at room temperature for 15 minutes. Finally, the live bacteria (Formazan positive cells) were measured spectrophotometrically at 600 nm using an ELISA reader.

Lysozyme Activity

Depending on the lysis of the Gram-positive bacterium *Micrococcus lysodeikticus*, which is lysozyme-sensitive, serum lysozyme action (U/ml) was measured by turbidimetric analysis [28]. Regarding this, serum products were followed by the addition containing 0.15 mg/ml of M. lysodeikticus in 0.1 M acetate buffer (pH = 5), and the optical density of the wells was assessed by an ELISA reader (Accu Reader, Taiwan) after 3 min at (RT) at 540 nm. One grade of Lysozyme action was defined as a decrease in absorption of 0.001 1/min.

Immune Related Gene Transcription Analysis

Immune related gene transcription analysis evaluated at 29 day post immunization from tilapia spleen. Three fish form each group were sacrificed by anesthetized, and then euthanized via an overexposure of Benzocaine, then the spleen was removed aseptically. Total RNA was extracted from spleen tissue using easy-spin™ Total RNA extraction kit (Qiagen, Germany), following the manufacturer's instructions. Briefly, 600 µl Trizol reagent was homogenized with 100 mg of spleen tissue in a mortar, after the final wash with ethanol the RNA samples were treated with diethylpyrocarbonate treated water (DEPC). The amount and purity of the extracted RNA measured via Nanodrop Uv–Vis spectrophotometer (ColeParmer, Jenway, USA). The cDNA generation was carried out using 10 µl of RNA through Reverse Transcription Kit (Qiagen, Germany) according to manufacturer's instruction, the synthesized cDNA was stored at −80°C for further use.

To normalize the expression of the immune related genes specific primers were designed for q-PCR to amplify the candidate genes with reference to the known sequences of Nile tilapia. All primers sequences of the innate and adaptive immune related genes IgM, IgT, TCR β, CD4, CD8α, IL-1β, IL-8, IFN 1, TNFα as well as the housekeeping gene (reference gene) β-actin are detailed in Table 1.

Table 1. Primers used for real-time PCR analysis of gene expression TNF-α = tumor necrosis factor alpha, INF-γ = interferon gamma, IL-1β = interleukin 1 beta, IL-8= interleukin 8, IgM, IgT, CD4, CD8 and β-actin = internal reference gene (house-keeping gene)

Gene	Primer	Accession number
IL-1β	F:5-GTGCCTCTAACAGTTTCCTGC-3 R:5-GGTTTGTGCCTTTGATGCCC-3′	XM_003460625.2
IL-8	F:5- GCACTGCCGCTGCATTAAG-3' R:5- GCAGTGGGAGTTGGGAAGAA-3′	NM001279704
INF-γ	F:5-AAGAATCGCAGCTCTGCACCAT-3' R:5-GTGTCGTATTGCTGTGGCTTCC-3′	XM_005448319.1
TNF-α	F:5-GGAAGCAGCTCCACTCTGATGA-3′ R:5-CACAGCGTGTCTCCTTCGTTCA-3′	JF957373.1
IgM -H	F:5'- CCACTTCAACTGCACCCACT-3′ R:5'- TGGTCCACGAGAAAGTCACC- 3'	KC677037.1
IgT	F-5′-TGACCAGAAATGGCGAAGTCTG-3′ R-5′-GTTATAGTCACATTCTTTAGAATTACC-3′	XM_025904470.1
CD4	F-5′-GCTCCAGTGTGACGTGAAA-3′ R-5′-TACAGGTTTGAGTTGAGCTG-3′	XM_025911776.1,
CD8	F-5′-GCTGGTAGCTCTGGCCTTT-3′ R-5′-TGTGATGGTGTGGGCATCTC-3′	XM_005450353.3
β-actin	F:5'-CCACACAGTGCCCATCTACGA-3′ R:5'-CCACGCTCTGTCAGGATCTTCA- 3'	EU887951.1

The analyses of the relative expressions of the immune-related genes was performed by CFX Connect™ Real-Time PCR (Biorad, USA). All the qRT-PCR reactions were conducted using SensiFast ™ SYBR Green master mix (Bioline, UK), according to manufacture protocol and each sample was run in triplicate. Thermo cycling conditions were performed at 95 °C for 5 min as initial denaturation followed with 40 amplification cycle consist of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. After the cycling, the relative RNA expression level of each immune-related gene was normalized to that of β-actin and presented as a fold change by dividing the values of the vaccinated tissues by those of the control as described previously [82].

Challenge Test

Challenge test were performed at 30 day post vaccination where remaining fish in each group (n= 30) were anesthetized using Tricaine methanesulfonate (MS-222) (Sigma, Aldrich) and interperitonealy

injected with 0.1 mL normal saline suspension containing 4.6×10^6 CFU mL^{-1} of virulent *S. agalactiae*, fish were monitored for 14 days, disease signs, behavioral abnormalities and mortality, were recorded twice daily and dead fish were removed to avoid deterioration. The relative percentage of survival (RPS) was calculated through equation of [7], RPS = (1- % mortality in vaccinated/% mortality in control) × 100. The experiment was performed in triplicate.

Animal's Ethics

According to a proposal reviewed and approved by the University Malaysia Terengganu, ethic committee under animal ethic approval number: UMT/ JKEHMK/ 2022/67 in accordance with Malaysian code of practice for the care and use of animals for scientific purposes. All guidelines for the care and use of living fish in this research, like appropriate housing conditions, avoiding unnecessary harm, minimizing stress during handling, and minimizing pain during experimental procedures, which include anesthesia when necessary, were followed.

Data Analysis

The data were analyzed using SPSS software V.23. Two-way analysis of variance (ANOVA) were employed. To compare the means of parameters among the groups Post-hoc tests were performed using Dunnett's t-tests where control group was the reference group, while multiple comparison analysis of serum parameters levels at different sampling times post-vaccination was conducted using the Tukey HSD test.

Results and Discussion

Microspheres Ultrastructure

Microsphere shape and size determined utilizing scanning electron microscope. The produced microspheres appeared in similar shape and size, with an average diameter of 500 ±20 µm. The external surface ultrastructure was in uniform appearance with a similar degree of roughness and porosity, as shown in Figure 3. Usually, the size of microcapsules that are applied in drug delivery range between 1 and 1000 µm. Even though there is no maximum limit of microparticle size for oral administration, smaller particles provide faster and easier passage through the upper digestive tract into the intestines [4]. Since the current study not only aimed to protect vaccine antigenicity from digestive system conditions but also preserve vaccine during fabrication, therefore, in view of the potential negative impact on vaccine antigenicity during the fabrication of very tiny microcapsules, this research employed technique to produce medium-sized microcapsules.

Coating Efficacy and Microsphere Stability in Gastric Fluids

To evaluate the stability of microcapsule in gastrointestinal conditions in vitro experiment held simulating the same conditions in tilapia digestive system, since food particles travels through the tilapia digestive tract on average for about 12 h, next to this period ingested particles travelling through pH conditions ranging from 2 to 9, where the stomach pH equal 4.5 before feeding and decreased directly upon food arrival to 2 due the excretion of pepsinogen and hydrochloric acid by oxyntopeptic cells and return to 4.5 until next feeding while the pH in tilapia intestine ranged normally in basic condition between pH 7 and 9 [69]. In accordance with the previous principles, the test of microspheres stability was designed, the experiments involved four different solutions simulating the pH of tilapia gastrointestinal fluids. Table 2 describes the result of FKC releasing from alginate microcapsules in gastrointestinal fluids. There were no notable morphological changes in most microcapsules that were incubated in acidic conditions (pH = 2 and 4.5), with vaccine releasing was less than 8%, however, in intestine simulating fluids, alginate microparticles showed much higher releasing rate of 41 ±3% and 62 ±2% for 7.4 pH and 9.0 pH respectively. The low release of FKC in the simulated stomach conditions mirrors alginate microcapsules resistance to degradation in acidic pH. Conversely, in intestine simulating fluids (basic fluids), produced microparticles showed much higher releasing rate reaching 41 ±3% and 62 ±2% for 7.4 pH and 9.0 pH respectively. The higher release of FKC in basic pH resulted from the disassociation of alginate microparticles. Thus correlated with alginate behavior in the basic condition where alginate microspheres swell and burst, releasing the entrapped agents [46, 76]. Our results are in line with previous research [33, 69, 70]. The variation in releasing efficacy of alginate microcarrier between different studies can be related with microsphere size, alginate concentration and cross-linker type that used in microsphere synthesis [63]. Saving the antigenicity from gastric juice by microencapsulation has been also reported in similar conditions. [79] Confirmed the protection of the three-dimensional structure of the recombinant vaccine (rsrr protein of *S. iniae*) that released from alginate microparticles after incubation in fluids simulating catfish digestive system.

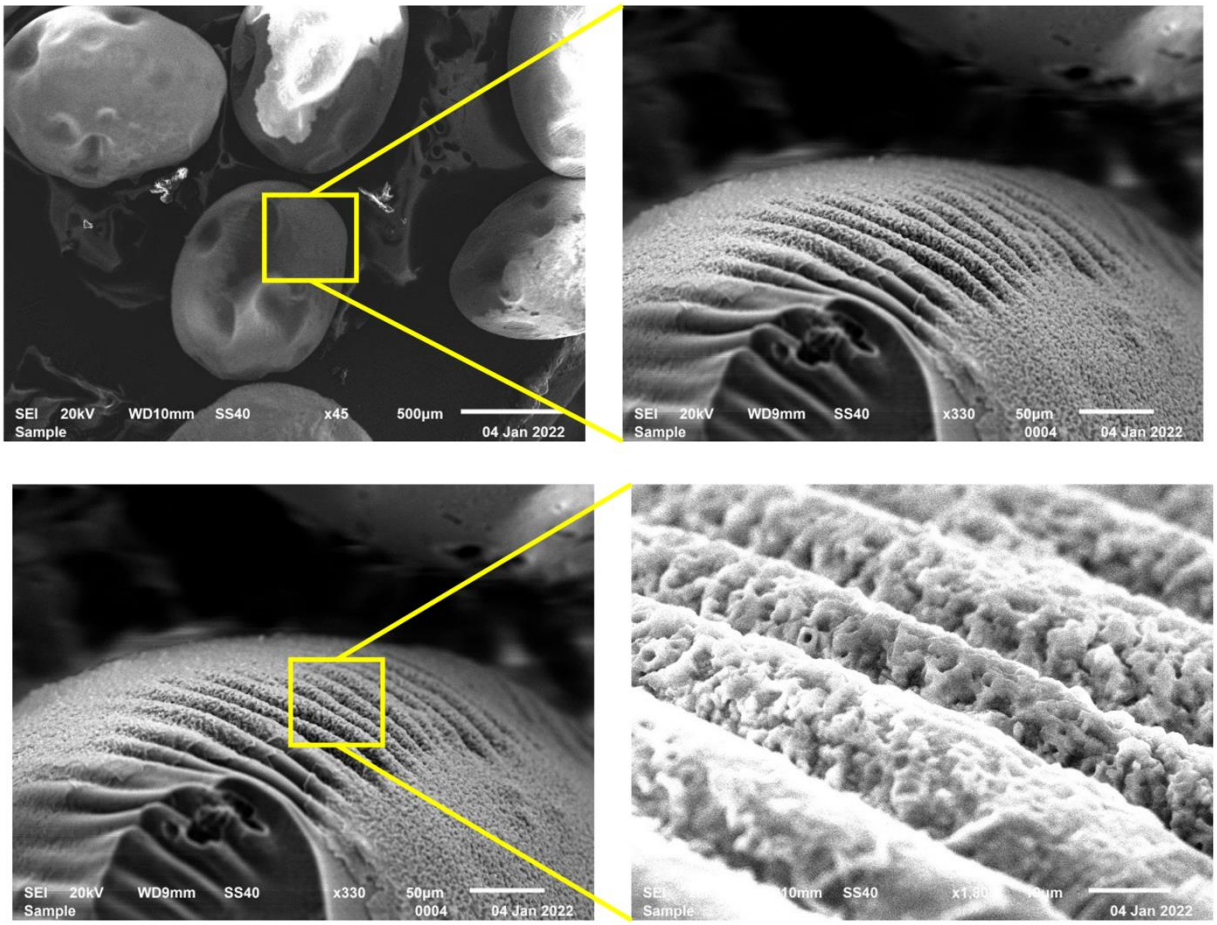


Figure 3. Alginate microcapsules under scanning electron microscope (SEM), with different magnification describe the microcapsules ultrastructure

Table 2. Releasing efficacy of FKC from alginate microspheres in different simulated gastrointestinal condition

Simulated condition	Solution pH	Releasing efficacy
Stomach upon food arrival	2	6±1%
Stomach before feeding	4.5	7 ±2%
Minimum intestine pH	7.4	41 ±3%
Maximum intestine pH	9.0	62±2%

The concentration of FKC in each ml of alginate microsphere which reflects the efficiency of the encapsulation process was 86 ±3%. In agreement with our findings [25] reported 87% efficiency for chitosan-alginate microcarriers that entrap FKC of *Y. ruckeri*, which is considered acceptable efficacy for microencapsulation of inactivated fish pathogens compare with similar works that earned much lower encapsulation efficacy which may be correlated with the type and concentration of the utilized polymer. According to [2], alginate showed higher efficacy compared to chitosan in propose to encapsulate the same fish vaccine. Also, [79] found that increasing the concentration of alginate and $CaCl_2$ in a specific ratio can increase the encapsulation efficacy of pathogen antigen in fabricated microcapsules as fish vaccine.

Immunological Assays

The mucosal secretions of fish contain several natural immunity molecules, including complement, lysozyme, proteases, and antimicrobial peptides [9, 38]. In the current study, to evaluate the immune-stimulatory effect of the alginate-covered vaccine sera were collected at 7, 14, 21 and 28 dpv. The collected samples were subjected to lysozyme and bactericidal activity tests to detect innate immune responses, while ELISA antibody titer were measured to evaluate adaptive immune response in treated fish sera.

Antibody Titer

Immunoprotection against bacterial infection is importantly dependent on the production of antigen-specific antibodies. To study the adaptive immune response for vaccination, antibody titer was evaluated using the ELISA protocol, which reflect levels of specific antibody production in fish sera. Several studies have demonstrated increased antibody titer in fish immunized with encapsulated vaccines compared to non-coated vaccine. For instance, samples from rainbow trout serum, intestines, and skin mucus showed a significant increase in specific antibody titer after vaccination with encapsulated FKC and LPS of *Y. ruckeri* [25]. As well as significant increase in serum antibodies has been confirmed in fish that have been orally vaccinated with encapsulated antigens compared to non-coated antigens, such as rainbow trout [42, 2], channel catfish [79] and sea bass [55]. However, in agreement with the previous results, the serum antibody titer against *S. agalactiae* was elevated in vaccinated groups through the experimental period, with the highest serum antibody titer values recorded for group A, Figure 4 shows the elevations of antibody production in the treated groups. The SPSS analysis for the ELISA results showed that there was a high significant difference between all groups ($p < 0.001$). The results showed that both vaccinated groups (A and B) had significantly higher mean differences compared to the control group ($p < 0.001$). On the other hand, there was no significant difference between group C (empty alginate microcapsules) and the control group (p-value = 0.971). Importantly, fish fed with alginate-encapsulated FKC (group A) had significantly higher antibody levels ($p < 0.001$) compared to group B (non-covered FKC). The test also showed that there were significant differences in the mean antibody titer levels between the different sampling points ($p<0.001$). Additionally, the serum antibody titer peaked in vaccinated groups at 14 dpv, where group A was up to 4 folds higher than group C and D (non-vaccinated groups) Figure 4. . These increased antibody titers are clearly outcome of adaptive immunity inducing more efficiently by oral vaccination with encapsulated antigens, thus, adaptive immunity enhancements were driven by the influence of microencapsulation on the bacterial antigens stability in gastrointestinal environments, as well as more effective antigen delivery and sustain releasing and processing in the intestinal mucus, which trigger the production of specific antibodies. Furthermore, key immunological gene upregulation in immunized fish revealed B and T cell activation and consequently antibody production.

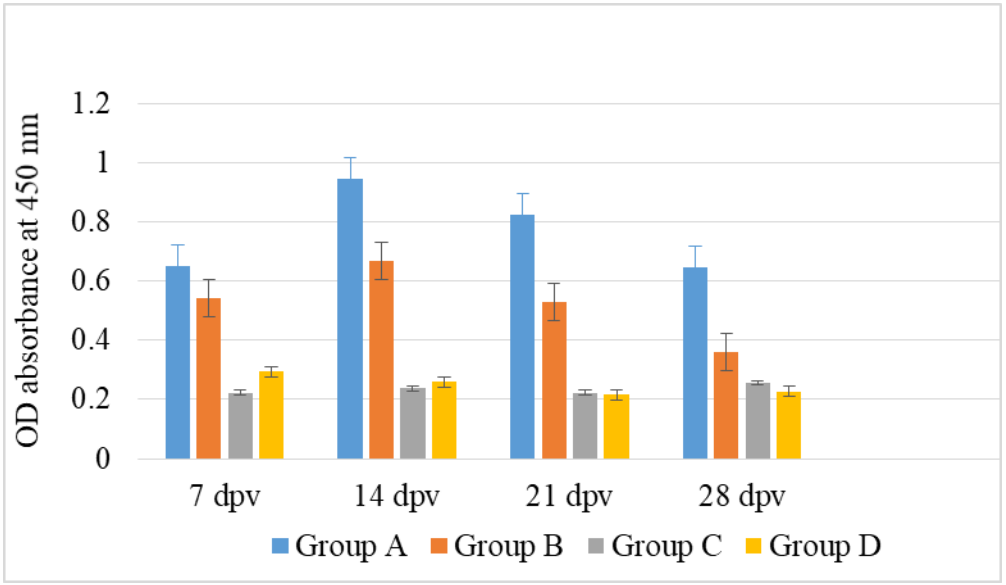


Figure 4. Serum ELISA antibody titer (OD absorbance values at 450 nm) of Set A: fish vaccinated by alginate microcapsules coated FKC injection, Set B: fish vaccinated by non-covered FKC vaccine, Set C: fish feed by empty alginate microcapsules and Set D: fish feed by commercial pellets diet (negative control)

Bactericidal Activity

Bactericidal activity is important part of innate immunity that contributes in elimination of bacterial invasion and is considered an important variable to determine immunization success [73, 77]. In the current research, the bactericidal activity was elevated in vaccinated groups, Figure 5 describes the changes in bactericidal activity in treated groups at four sampling points. ANOVA analysis showed that there was a significant difference in the bactericidal activity between groups ($p<0.01$). The post hoc tests using the Dunnett t-test showed that groups A, B and C had significantly higher in bactericidal activity than the control group ($p < 0.001$). Moreover, the post hoc analysis compared between group A (alginate-covered FKC) and group B (non-covered FKC) showed a significant difference ($p<0.01$), reflecting the effect of encapsulation. Sampling date also affect the bactericidal activity levels, the highest levels of bactericidal activity were recorded at 21 dpv. As well as, the bactericidal activity of vaccinated fish (A and B) showed significant differences between sampling points ($p<0.001$), while unvaccinated groups (C and D) didn't show significant differences throughout the 28 days experimental period ($p>0.05$) as shown in Figure 5**.**

Similarly, in a previous study, the values of bactericidal activity and complement activity increased after 20 days post oral vaccination of rainbow trout with chitosan-alginate microcapsules that enclosed killed *L. garvieae and S. iniae* [42]. Nevertheless, according to [25], bactericidal activity wasn't affected in rainbow trout (*O. mykiss*) vaccinated with chitosan-alginate microspheres encapsulated *Y. ruckeri*. The contrast in bactericidal activity between the last two mentioned studies is probably related to the used pathogens. However, fish with higher bactericidal activity reflect superior delivery and adsorption of bacterial antigens [56].

Lysozyme Activity

The efficacy of the vaccination in enhancing innate immunity was assessed by measuring levels of lysozyme at different time points. Figure 6 shows the lysozyme activity variation in the treated fish sera at four different sampling points. The ANOVA test showed significant differences between groups ($p<0.001$), the analysis also indicate that groups A and B had significantly higher lysozyme levels ($p<0.001$) than the control group, but group C showed no significant difference ($p > 0.05$) compared to the control group. Other comparison between group A (alginate covered FKC) and group B (non-covered FKC) showed significant differences between these groups ($p<0.001$). Besides that, to assess the effect of sampling date, statistical analysis indicated that there were significant differences between all sampling points ($p<0.001$), however, lysozymal activity reached the highest values at the first two sampling points, as shown in Figure 6. In general, lysozyme and bactericidal activity had different peak points, indicating that these two reactions occurred at different rates during the innate immune response.

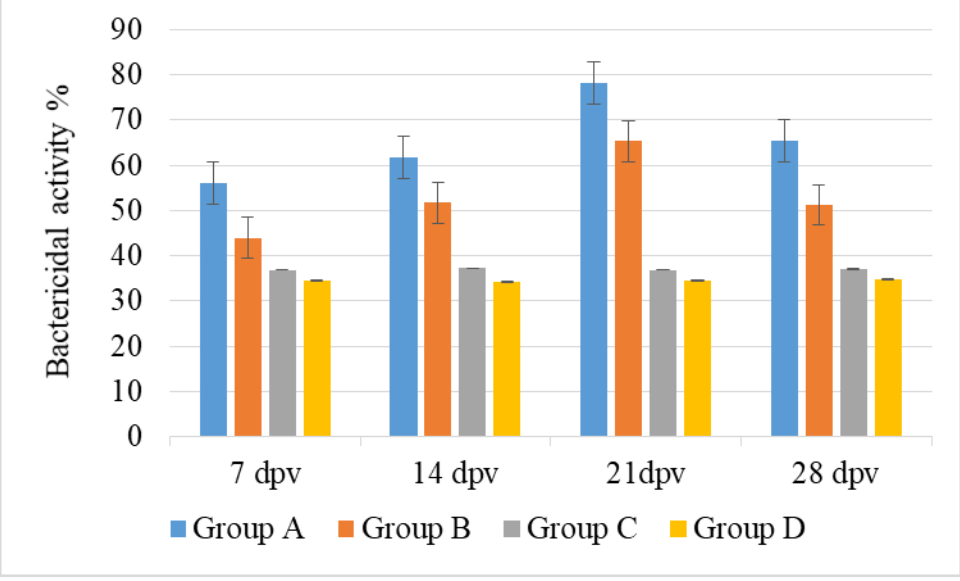


Figure 5. Serum bactericidal activity (%) of Set A: fish vaccinated by alginate microcapsules coated FKC, Set B : fish vaccinated by non-covered FKC vaccine, Set C: fish feed by empty alginate microcapsules and Set D : fish feed by commercial pellets diet (negative control)

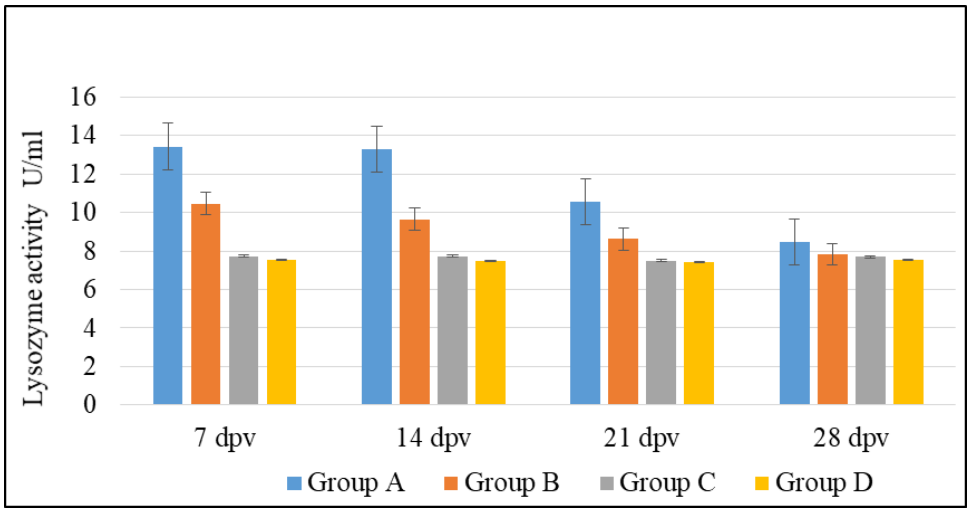


Figure 6. Serum lysozyme activity (U/ml) of Set A: fish vaccinated by alginate microcapsules covered FKC vaccine, Set B: fish vaccinated by non-covered FKC injection, Set C: fish feed by empty alginate microcapsules and Set D: fish feed by commercial pellets diet (negative control)

Lysozyme is a vital protein and an important component of fish nonspecific immunity that degrades bacterial cell walls, activates complement system and increases opsonization of invaded microbes [8, 31]. Generally, vaccines, immune stimulants, and some probiotics have been associated with an increase in lysozyme activity in fish [60]. For instance, lysozyme gene expression increased in different organs of Atlantic salmon after immunization with lipopolysaccharide [62]. Consequently, in several studies, higher levels of serum lysozyme were reported after oral vaccination with encapsulated vaccines, such [25] in rainbow trout (*O. mykiss*) that vaccinated with chitosan-alginate microsphere encapsulated (*Y. ruckeri*), also [11] surface membrane protein of *Aeromonas hydrophila* microcapsulated with PLGA to vaccinate rohu fish, [39] in common carp vaccination with oligonucleotides encapsulated by PLGA and similarly [79] in catfish.

Despite the obtained values beginning to decrease at the subsequent sampling points, lysozymal activity in group A remained higher than the values obtained in the other groups at all sampling points. In fact, a variety of reasons may lead to increase in lysozyme activity during fish immunization. Firstly, the direct contact of lipopolysaccharide of inactivated bacteria stimulates lysozyme transcription within macrophages, additionally, it induces macrophage differentiation and maturation, therefore increasing the total lysozyme production [23, 77]. As well, exposure to lipopolysaccharide triggers cytokine synthesis, which consequently leads to proliferation of lymphocytes, enhanced phagocytic activity, respiratory burst and lysozyme release [18]. Thus, increasing the bacterial lipopolysaccharide that is delivered safely to MALT, lead to an increase in lysozyme activity [40]. On the other hand, several factors can explain the differences in the findings from various studies on the impact of encapsulated vaccines on fish innate immunity indices, such as the biopolymer type (chitin, chitosan, and alginate), microparticle size, fish species, temperature, vaccine specificity of adjuvant and the rate and duration of the regime [25].

Immune-related Gene Expression

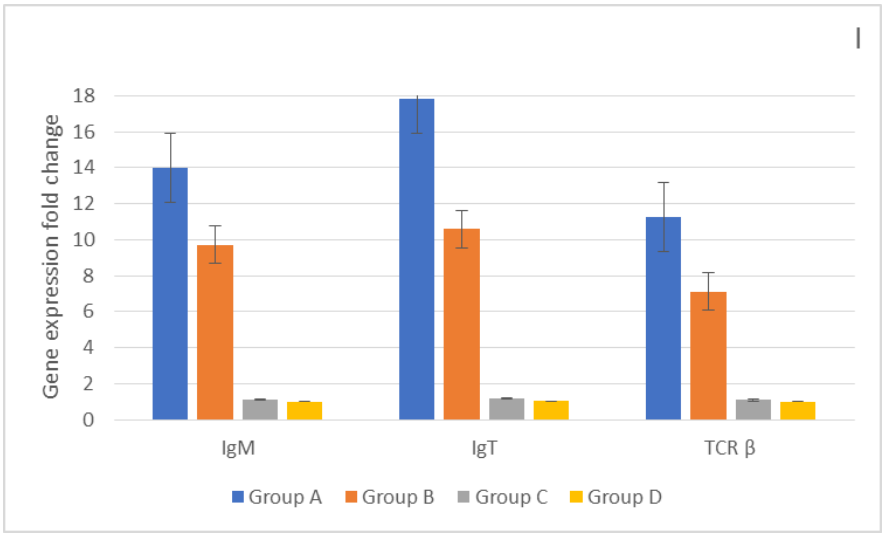
Immune-related gene transcription analyses from tilapia spleen were sampled once after 4 weeks post-vaccination. In fact, the importance of this experiment comes from its ability to detect any increase in the immunological contributors, even though these immunological proteins are difficult to evaluate in fish serum. These immune proteins are usually more abundant in mucosal organs than blood serum [53].

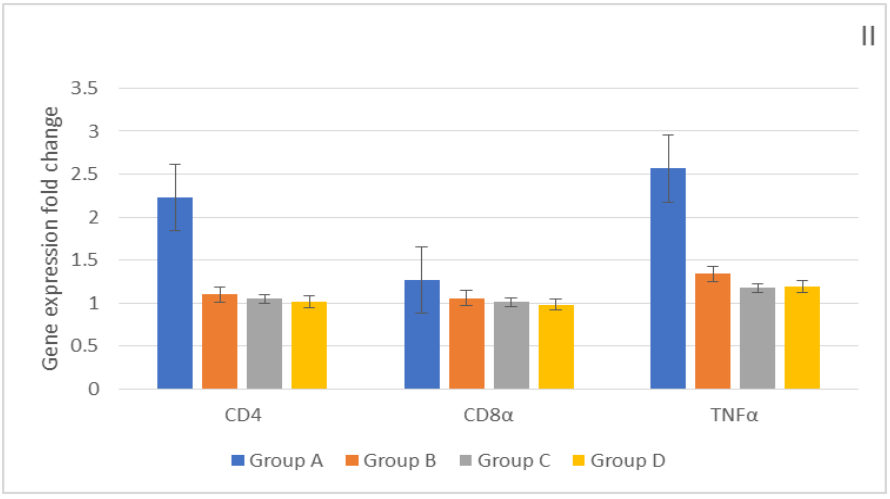
Figure 7 describes the upregulation of the tested genes (IgM, IgT, TCR β, CD4, CD8α, IL 1β, IL 8, IFN 1, and TNFα) at 29 dpv. One-way ANOVA analysis showed highly significant differences in genes expression between groups ($p<0.001$). Nevertheless, in comparison to the control group, group A was significantly higher ($p<0.001$) in all immune-related genes expression, but group B showed high significant differences ($p<0.001$) for only 5 genes (IgM, IgT, TCR β, IL 1β, and IL 8), in contrast, group C showed no significant difference in all genes expression ($p > 0.05$). Furthermore, to evaluate the effectiveness of FKC microencapsulation impact on immune-related genes expression, the analysis showed high significant differences ($p<0.001$) between the two groups for IgM, IgT, TCR β, IL 1β, and TNFα. Also, encapsulation significantly increased gene expression of CD4 and IFN 1 ($p<0.01$), as well

as CD8α and IL 8 ($p<0.05$). Overall, the results suggest that the alginate-covered FKC group elicited significantly higher and diverse immune responses compared to the non-covered FKC group.

In this regard, many studies report increasing in serum cluster of differentiation (CDs) rather than the recording of upregulation of CD4 and CD8α genes after immunization with microspheres encapsulated fish vaccine. The expression of CD4 and CD8α genes in group A was raised due to more sufficient vaccine delivery process since CD4 is surface marker for helper T-cells and CD8 for cytotoxic T-cells, which involve separately in antigen detection from APCs where CD4+T-cells (helper T-cells) are activated to produce different cytokines and CD8+T-cells (cytotoxic T-cells) are simulated to attack intracellular infected cells [64, 71]. Thus, can explain the high correlation coefficient between CD4 gene and tested cytokines genes (IL-1β, IL8, TNFα, and INF1), which are supposed to be secreted by CD4+ T cells after activation by APCs. While the relatively low expression of the CD4 and CD8α genes compared to other immune-related genes may be attributed to the late of sampling date (29 dpv), since CD4 and CD8 contribute as early immune reaction at the beginning of antigen administration, which peaked at earlier stage of vaccination period [32, 40].

In addition to the presence of CD4 and CD8 on the surface of helper and cytotoxic T-cells, respectively, T-cell receptors (TCRs) are key immunological protein located with CD3 complex molecules on the surface of all kinds of T-cells [20]. Which was confirmed in the current research by the obtained high correlation coefficient between gene expression of TCR β and CD4 and CD8 genes. TCR expression is lower upon initial antigen detection and rises gradually when T-cells are activated for proliferation in response to antigen detection by APCs [36]. TCR β gene transcription in group A was significantly greater than in group B and control group ($p<0.001$). Definitely, upregulation in TCR gene transcription reflects proliferation of all types of T-cells, which differentiate rapidly upon antigen delivery [31, 85]. The increase in TCR β expression in the alginate-covered FKC group compared to other groups reveals higher antigen delivery and more successful vaccination process.





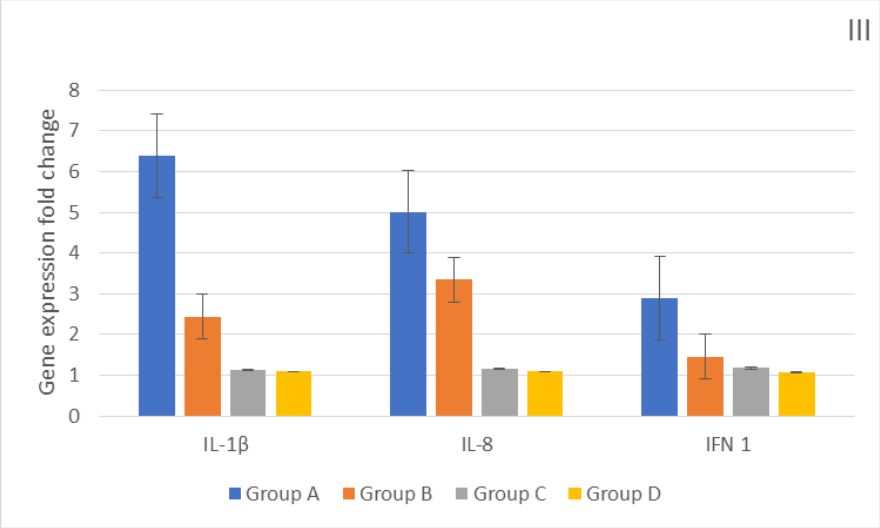


Figure 7. Immune related gene transcription analysis from Tilapia spleen which sampled at 4 weeks post vaccination in triplicate where Set A: fish vaccinated by alginate microcapsules covered FKC, Set B: fish vaccinated by non-covered FKC vaccine, Set C: fish feed by empty alginate microcapsules and Set D: fish feed by commercial pellets diet (negative control). The mRNA level of each immune-related gene was normalized to that of β-actin, and a fold change was calculated by dividing the values of the vaccinated tissues by those of the control. For each gene, the mRNA level of the control group was set as 1

Cytokines play a crucial role in fish immunity by regulating immune responses, coordinating inflammation, and promoting the effective functioning of immune cells [79, 86]. In the current research, genes expression of the cytokines IFN 1, TNFα, IL-1β and IL-8 were analyzed. Significant upregulation in all tested cytokines was noted in group A in comparison to control group ($p<0.001$). On the other hand, the encapsulation positively affects the upregulation of these cytokines at different levels. Likewise, similar immune-related genes synthesis increased after fish vaccination with microcapsules such as IFN-1 and Mx-1 [2], IFN-γ, TNF-α and MHC IIβ [79] and IL-6 [42]. Generally, secretion of cytokines is conjugated with vaccination as inflammatory response, also, cytokines act as essential mediators in fish immunity after vaccination by aiding the differentiation of B-cells into plasma cells and B-memory cells, ensuring an effective and targeted immune response against specific pathogens and contributing to the establishment of long-lasting immunity [18, 61, 77, 81]. Thus, the significant increase in cytokines genes expression in the alginate-covered FKC group compared to the non-covered FKC group is primarily the result of more sufficient activation of T helper cells as a consequence of extra vaccine delivery.

Immunoglobulin are important components of fish immunity, providing defense against pathogens and enhancing the fish's ability to fight infections [31]. The main three types of immunoglobulin in bony fish are IgM, IgD and IgT. Fish IgM and IgT are different lineages of B cells that play key roles in mucosal immune responses, as their numbers can be increased through oral vaccination. IgM is the primary immunoglobulin involved in the fish immune response, while IgT (mimicking the mammalian IgA) is more specific to mucosal surfaces and provides localized defense [83, 85]. In this research, IgM and IgT genes transcription in alginate-covered FKC group were significantly greater than that in the control group as well as in the non-covered FKC group ($p<0.001$).

Regarding our results, the upregulation in Ig genes is clearly correlated with administration of encapsulated FKC encapsulation, which enhanced the delivery of vaccine to MALT, and consequently activated B-cells to differentiate into plasma cells and memory B-cells [31, 71]. The higher transcription of IgM and IgT genes in group A resulted from the extra differentiation and proliferation of B-cells into plasma cells as Ig secretory cells [85, 87].

In group C (treated with empty alginate microspheres), except for the fact that the group had significantly higher bactericidal activity than the control group ($p< 0.001$), there were no significant changes in lysozymal activity, antibody titer nor immune-related genes, compared to the control group ($p > 0.05$), which indicates the absence of inflammatory or stimulatory effect of alginate on Nile tilapia immune system and its safe application as a pharmacological microcarrier. Accordingly, most revised studies confirm the safety of biopolymers commonly used in the encapsulation of fish vaccines. Despite the fact that other biopolymers like chitosan showed an increase in fish immune parameters when administered

alone, like chitosan's inflammatory stimulatory effect in *Cyprinus carpio* [5]. The difference in stimulatory effects of these biopolymers can be explained by the type and concentration of polymers used, as well as their purity.

Relative Percent of Survival RPS after 14 Day of IP Challenge

Even though immune parameters can provide evidence of vaccination success, challenges with highly virulent pathogens are still the gold standard for assessing the efficacy of vaccines [1, 44]. After challenging the four experimental groups with 3.6×10^6 CFU mL^{-1} suspension of virulent *S. agalactiae*, daily deaths were recorded for 14 days. Figure 8 shows the mortality rates for treated groups during two weeks post challenge. At the first day post-challenge, no deaths were marked in group A, whereas massive mortalities were recorded in the other groups, where the dead fish per day were 6 ± 1, 11 ± 1 and 10 ± 1 in groups B, C and D respectively. During the next 3 days (2,3 and 4 days post-challenge), only one fish died per day in group A, while higher deaths rates in the same period were recorded for the remaining experiment groups in the rates 3 ± 1, 5 ± 1 and 7 ± 2 deaths per day for groups B, C and D respectively. By the fifth day post-challenge, all the fish in group D were dead, while mortalities stopped at the seventh day in group C, recording 96 ± 3.3% mortalities. Nevertheless, no deaths were marked in group A from the fifth day post-challenge until the last day of the experiment period, achieving only 8 ± 2% mortality. On the other hand, mortalities in group B stopped two days later, on the seventh day post-challenge, with a total mortality rate of 51 ± 5%.

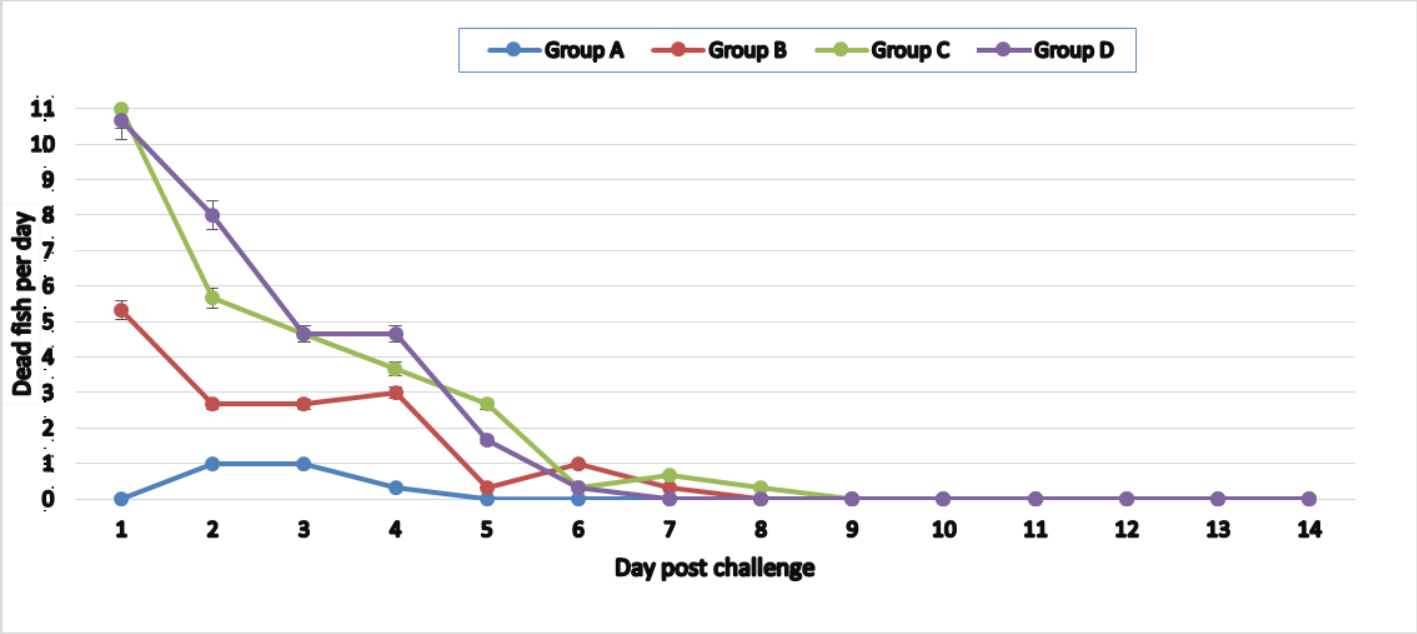


Figure 8. Cumulative mortality during 14 day post injection with viable *S.agalactiae* for Nile tilapia where Set A: fish vaccinated by alginate microcapsules covered FKC, Set B: fish vaccinated by non-covered FKC vaccine, Set C: fish feed by empty alginate microcapsules and Set D: fish feed by commercial pellets diet (negative control)

The relative percent of survival (RPS) reflect survival rate comparing to control group. Figure 9 shows the RPS values for groups A, B and C. The appeared RPS value in group A (vaccinated with microcapsulated FKC) was 92± 2%, which is twice the RPS obtained in group B (vaccinated with non-covered FKC). While the RPS in group C (supplied with empty alginate microsphere) gives rise to a very low survival rate (4± 3%),. Clearly, the tilapia that were immunized with alginate-covered FKC showed a stronger immune response compared to the control group, which resulted in better protection against the experimental infection. Similarly, our outcomes sympathize with previous vaccination studies of different fish species with encapsulated antigens [25, 42, 79].

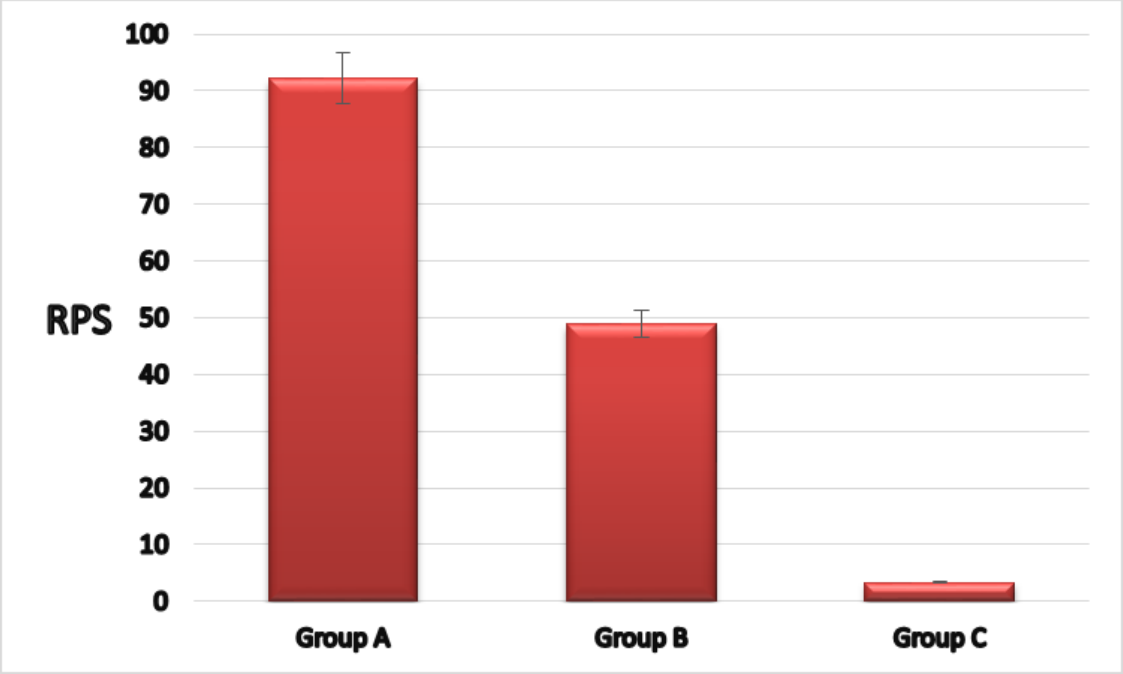


Figure 9. Relative percent of survival (RPS) for Nile tilapia after14 days post injection with viable *S.agalactiae* where Set A: fish vaccinated by alginate microcapsules covered FKC, Set B: fish vaccinated by non-covered FKC vaccine and Set C: fish feed by empty alginate microcapsules

The higher survival rate in the current study compared to previous studies [2, 25, 42, 43, 48, 79] may be attributed to different circumstances like capsule size, applied biopolymers, type of vaccine and vaccine concentration within the capsules, capsules fabrication method, fish species, duration of immunization, pathogen type in addition to dose and timing of challenge after vaccination.

Fish, like other vertebrates, develop adaptive immunity, memory B-cells are essential part of the adaptive immune system, they aid in the development of immunological memory, enabling a quicker and more effective immune reaction upon re-exposure to a previously encountered pathogen [27, 37, 66]. Overall, during the primary immune response (vaccination or infection) memory B -cells generated after stimulation by helper T cells, this long-lived memory B -cells memorize the characteristics of the antigen encountered during the primary immune response by undergone affinity maturation, a process in which B cells with higher-affinity B-cell receptors (BCRs) are selected and expanded, this BCRs developed to be specific to the antigen encountered during primary immune response, if the fish attacked by the same pathogen again, memory B cells recognize it through their BCRs, enabling a targeted immune response by production of antibodies with improved binding affinity for the target pathogen, enhancing their effectiveness in neutralizing the pathogen [21, 41, 72]. Additionally, the adaptive immune response is considered faster and stronger compared to the primary immune response, where naïve B cells are involved [27, 31].

In the current study, the production of specific antibodies against *S. agalactiae* have been elevated in vaccinated groups at four different sampling point post vaccination process (primary immune response), thus, this elevation indicate the sufficient activation of memory B-cells during primary immune response which responsible for specific antibodies secretion, on other hand, the high RPS in group A indicate the rapid and robust antibodies response triggered by memory B-cells upon re-infection (second immune response) allowing vaccinated fish to ensure effective invaders clearance, without deaths or severe illness, also the higher protection rate in group A when challenged with the vital pathogen after 30 days post vaccination reflect a better initiation of adaptive immune system comparing to group B, this take-shape by higher production of specific antibodies against *S. agalactiae* making quicker elimination of the infection and save fish lives. The superior RPS in group vaccinated with alginate-covered FKC compared to the non-covered FKC group confirms the advantage of encapsulation with alginate, which leads to successful protection of antigens from degradation by gastric juice and delivers the vaccine to MALT, where antigens react efficiently with the immune system.

Conclusions

In this study, the results clearly demonstrate the enhanced immunogenicity achieved through the utilization of alginate-covered FKC in fish vaccination. Comparing the alginate-covered FKC to their non-covered counterparts, it is evident that the former exhibited significantly higher bactericidal activity, lysozymal activity, and specific antibody titers against *S. agalactiae* in the serum of tilapia. Furthermore, fish vaccinated with alginate-covered FKC displayed a notable upregulation in all tested immune-related genes, indicating a robust and comprehensive immune response. Clearly, these elevations resulted from an increase in delivered antigens to the intestine as advantage of vaccine encapsulation. Of particular significance is the observation that fish treated with alginate-covered FKC exhibited a considerably higher survival rate when compared to the non-covered FKC group. This underscores the efficacy of the alginate microcapsules in enhancing the protective response elicited by the vaccine. The obtained higher protection in the covered vaccine group is predicted due to the increased antigenicity that results from the protection of the vaccine by alginate microcapsules from acidic gastric juice, which partially degrade the uncovered vaccine, leading to a decrease in the antigenicity, which results in a less immune response and consequently a lower survival rate in fish that treated with non-covered vaccine. This highlights the importance of vaccine antigen encapsulation within alginate microcapsules in inducing a comprehensive and specific immune response. Additionally, in fish treated with alginate-covered empty microspheres (without vaccine), despite, there were no significant increases in lysozymal activity or antibody production, and no substantial changes in immune-related gene synthesis were observed, it is noteworthy that this group showed a significant increase in bactericidal activity. Thus, confirmed that alginate itself doesn't have a significant role in immune protection and can be safely applied as encapsulation agent for fish vaccine delivery.

In conclusion, the results of this study provide compelling evidence that the encapsulation of FKC within alginate microcapsules significantly enhances vaccine antigenicity, leading to the induction of robust and effective immunity. These findings hold promise for the development of biotechnological vaccination strategies in the field of fish immunology, with potential applications in aquaculture disease management.

Conflicts of Interest

The authors declares that there is no conflict of interest regarding the publication of this paper.

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