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Efficacy of Injectable and Immersion Polyvalent Vaccine against Streptococcal Infections in Broodstock and Offspring of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

A vaccine against streptococcosis, lactococcosis and enterococcosis in tilapia was formulated, ME-VAC Aqua Strept, as a polyvalent inactivated vaccine containing *Streptococcus agalactiae*, *S. iniae*, *Lactococcus garvieae* and *Enterococcus faecalis* along with a nano-particulate adjuvant. Use of ME-VAC Aqua Strept by injection or immersion resulted in an improved non-specific and adaptive immunity of broodstock and offspring. Intra-peritoneal vaccination of tilapia broodstock increased the total leukocyte count, phagocytosis, lysozyme activity, antibody titer, number of seeds/vaccinated broodstock, seeds quality and survival rates. Also, immersion mass vaccination of tilapia larvae provided a long period of protection up to three months, with a relative percent of survivability (RPS) not less than 60% at this time. To our knowledge, this vaccine may be the first to offer a combined protection against streptococcosis, lactococcosis and enterococcosis in tilapia. The results support the use of this vaccine as an effective tool for disease control and well-being of fish.

1. Introduction

In September 2015, the world leaders agreed to implement the so-called sustainable development Agenda, which mainly vows to eradicate hunger by 2030 via innovative agricultural projects [1,2]. In line with this, intensive farming of Nile tilapia (*Oreochromis niloticus*); as the first popularly cultured fish in Egypt was started under health surveillance systems and control programs, including vaccines [3,4].

Streptococcosis, Lactococcosis and Enterococcosis are the most frequent diseases affecting Tilapia production in Egypt [5,6]. These infections are respectively caused by *S. agalactiae,S. iniae, L. garvieae,* and *E. faecalis* particularly during summer [5,6]. They may affect fish at all culturing stages [7] and are commonly associated with non-specific lesions, including hemorrhages, exophthalmia, congestion and ocular opacity, melanosis, nervous swimming behavior and rapid mortality, leading to high economic losses in marketable fish size and possible farm closure [5,8].

In Egypt, use of vaccines in fish farms has been hampered by many challenges, including the lack of vaccination programs, high cost of imported vaccines, small value of available tilapia farms, narrow spectrum of cross protection, high mortalities associated with stress during fish handling, cost of anesthetics and the need for trained manpower and equipment. However, the current trend of high scale tilapia production in many farms in Egypt supports the development of effective vaccines to control these circulating diseases.

Most previous research on fish vaccines against streptococcosis was focused on the production of monovalent vaccines that were administered parenterally (I/P) and consisted of formalin killed whole cultures of homologous bacterins against the above pathogens, with or without the extracellular products (ECP). These vaccines showed relative percent survival values (RPS) between 56 and 95% when challenged at 21 or 7 days post-vaccination, respectively [9–11]. Some vaccines contained mineral oil adjuvants to induce higher and longer protection, while others had non-oil adjuvants (e.g., Aquamun) and yielded good protection in rainbow trout, with RPS value up to 83.3% [11]. Limited research was done on polyvalent vaccines.

Three vaccine delivery systems have been used in fish with variable success, depending on the nature of each vaccine and production stage

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of fish; injection, immersion (dip or bath) and oral [12]. In general, vaccination of broodstock conferred protection of offspring against infection [9]. Tilapia offspring (approximately below 0.5 gm or less than 21 days age) rely on the delivered maternal immune response (innate and adaptive immunity) [9]. While parenteral vaccine delivery has been adopted for larger fish, a more feasible, practical and less hazardous vaccination strategy is highly required especially in fries, fingerlings and in grow out stages.

Recently, a nano-particulate delivery system (O/W adjuvant) has been optimized for mucosal immunity [13] and was found to improve the adsorption of antigens through gill tissues, skin and intestines, promoting their entrapment and retention in the lymphoid tissues. It also provided a slow continuous release of antigens, following the depot effect theory [14]. Soltani et al. [15] and Hwang et al. [16] recorded high protection levels in rainbow trout against *Yersinia ruckeri* when Montanide IMS 1312 VG was used with the antigen as an immersion adjuvant and similar protection was obtained in Olive flounder (*Paralichthys olivaceus*) when the same adjuvant was employed in an immersion vaccine against viral hemorrhagic septicemia. Such adjuvant was also reported to protect the antigens from hydrolytic enzymes and low pH of the gut and intestinal mucosal surfaces.

The present study was designed to develop and evaluate a locally prepared vaccine that protects tilapia broodstock and offspring against common infections in tilapia; streptococcal disease, lactococcosis and enterococcosis. The development of high quality bacterial vaccine seeds was the first approach to support the production of a multivalent tilapia vaccine at MEVAC laboratories. The vaccine, ME-VAC Aqua strept, was formulated as a polyvalent inactivated micro-emulsion containing antigens of *Streptococcus* spp. (*S. agalactiae* and *S. iniae*), *L. garvieae*, and *E. faecalis* along with Montanide IMS 1312 VG as a nano-adjuvant (Seppic, France). Efficacy and potency of the vaccine were evaluated in both broodstock and fries with a special emphasis on the number of seed/broodstock and mortality percent among tilapia fries at the end of the nursery period.

2. Material and methods

2.1. Antigen preparation

2.1.1. Phenotypic and molecular characterization

Virulent strains of *S. agalactiae*, *S. iniae*, *L. garvieae* and *E. faecalis* were isolated from Nile tilapia during disease outbreaks [5,6]. They were cultured on MacConkey-Agar and Trypticase Soy Agar (TSA, Himedia, India), with or without 5% sheep blood. The obtained colonies were examined by Gram staining, colonial morphology on different growth media and hemolytic patterns on blood agar were determined. The phenotypic and molecular characterization attributes were determined prior to using them as antigens for vaccine preparation. Briefly, bacterial isolates were cultivated on TSA with blood and incubated at 30 °C for 48 h. API-Strep test kit (Biomerieux, France) was used for biochemical identification as per manufacturer's directions. All identified isolates were confirmed by PCR. Bacterial DNA was extracted by QIA gen DNA isolation kit, then amplified with universal bacterial primers F 5- AGAGTTTGATCMTGGCTCAG -3 and R 5- TACGGYTACCTTGTTACGACTT -3 [17]. After amplification, 10 µL of the PCR product was electrophoresed, showing a size of 1485 bp. Amplicons were purified with QIA quick PCR purification kit and directly sequenced with a 3500/3500xL genetic Analyzer (Applied Biosystems). Nucleotide sequence analysis and phylogenetic tree of 16-s rRNA were constructed.

2.1.2. Bacterial inactivation

Pure colonies of each *Streptococcus* spp., along with *L. garvieae* and *E. faecalis* were individually cultivated in brain heart infusion broth (BHIB, Himedia, India) and incubated at 30 °C for 24–48 h for growth. Serial dilutions (1/10) of the cultures were carried out, each plated on TSA culture medium and incubated at 30 °C for 24 h to estimate the corresponding number of colony forming units (CFU)/ml of each bacterial species present in the vaccine. CFU counts were compared to nephlometer McFaraland standard concentrations.

For inactivation, the cultures were suspended in 3% formalin solution (based on inactivation kinetics, European pharmacopeia 2013) under continuous agitation for 24 h at 25 °C. The prepared antigens were centrifuged at 1800 × *g* for 30min and supernatants were discarded. Pellets were re-suspended in PBS (pH 7.4). Safety testing was performed by plating 100 µl of inactivated bacterial suspensions on TSA and incubation at 30 °C for 72 h to monitor any growth. Sterility testing was also conducted by the incubation of inactivated cultures for 2 weeks (European pharmacopeia 2013).

2.1.3. Vaccine formulation and characterization

Montanide IMS 1312 VG was used as an adjuvant (provided by Seppic Co., France). To formulate the polyvalent vaccine, an equal volume (50/50) of the antigenic aqueous medium containing more than 10^9 CFU/ml of each bacterial species was mixed with the adjuvant at room temperature under moderate agitation. Different suspension concentrations were used to evaluate the dose response (data not shown). The product was delineated and authenticated using Transmission Electro n Microscopy TEM.

2.2. Study design and vaccine administration

2.2.1. Experiment I (IP vaccination of broodstock)

A total of 800 healthy broodstock female tilapias with an average body weight of 500 g ± 50 g were placed in concrete ponds (3 m × 8 m) and fed on commercial pellets containing 36% protein (Aler Aqua-Egypt). They were divided into two groups; group (G1) was vaccinated with ME-VAC Aqua Strept ® by IP route, while G2 served as a control and was injected with saline. Both groups were employed one-week post spawning as recommended by Nisaa et al. [18]. Blood sampling of broodstock was collected three weeks after vaccination. The haemato-immunological parameters observed in this study included red blood cell count, total leukocyte count, HB conc., total protein, albumin, globulin, A/G, phagocytic activity/index, lysozyme activity and serum antibody titers. Numbers of tilapia fry obtained from vaccinated broodstock and survival rates were recorded at the end of the nursery period and compared with the yield produced by non-vaccinated broodstock. Based on antibody titers of vaccinated fish, an arbitrary protective agglutination level of 4 log2 was determined based on Relative Percent of Survivability RPS values obtained from small scale infective trials using individual pathogens in special vaccine formulas (unpublished data).

2.2.2. Experiment II (immersion vaccination for tilapia fry)

One-month old tilapia fry produced by vaccinated broodstock were acquired from a hatchery. Upon arrival at the experimental station, they were randomly distributed in ten tanks (50 L capacity), each containing 100 fish. Water quality parameters were monitored daily to fall within the values described for species temperature 25 ± 2 °C, dissolved oxygen (D.O.) > 5 mg/L, and pH 7.5. Fish was acclimated in these conditions for 15 days, being fed to apparent satiation twice a day with a commercial diet (Aler-Aqua, Egypt) containing 30% protein; the fish was bacteriologically examined against *Streptococcus*, *Lactococcus* and *Enterococcus* infections to insure that it was free. All institutional and National guidelines for the care and use of fisheries were followed.

In a separate tank, 1 L of the vaccine was mixed with 9 L water to vaccinate one half of the fish number (Five hundred fish = 5 tanks) under aeration. Fish were immersed in the vaccine bath using a knotless hand net for 2 min then returned to the rearing tanks. They were daily monitored for two weeks post vaccination for eye irritation, skin affection, signs of abnormal swimming, asphyxia, food refusal or other safety markers. The other half of the fish (n = 500) were used as none vaccinated controls. Antibody titration was determined every two

weeks for three successive months. Challenge test was performed every month and RPS was recorded.

2.2.3. Haematoimmunological parameters

Fish were anesthetized with clove oil prior to blood sampling. Due to its incomplete solubility in water, pure clove oil (90–95% eugenol) was first dissolved in ethyl alcohol (92.8%) in 1:9 ratio (clove oil: ethyl alcohol) following Anderson et al. [19]. This solution was then diluted in water in order to obtain concentrations of 0.10 mL (100 mg) of clove oil per 500 mL of water [20].

Blood samples were collected from caudal vessels for examining the blood picture and serum biochemical parameters (Total protein, Albumin (A), Globulin (G) and A: G index), phagocytic activity/index and lysozyme activity in broodstock. All serum samples of broodstock and fingerlings were tested for agglutinating antibody levels in microtiter plates.

2.2.4. Phagocytic activity/Index

Sodium heparinized blood samples collected from broodstock were overlaid gradually on equal volumes of histopaque medium (1.077 g/ml, Sigma–Aldrich chemical St. Louis, MO, USA) in 15 cm falcon tubes. After centrifugation, the white blood cells were collected from the interface layer, washed and suspended in RPMI-1640 supplemented with tilapia serum, 100 IU ml−1 penicillin and 1 mg ml−1 streptomycin. The cell count was adjusted to be 10^7 cell/ml. *Saccharomyces cerevisiae* yeast was adjusted to 10^6 cell/ml. They were mixed with the white blood cells and incubated for 1 h. One drop of the mixture was smeared on a slide, soaked with methanol for 10 min, drained, then stained with Giemsa stain and observed under the microscope. The percentage of phagocytic activity was calculated as the number of ingested phagocytes over the total no. of phagocytes. The phagocytic index was expressed as the number of ingested yeast cells over the number of ingested phagocytes [21].

2.2.5. Lysozyme activity

One hundred µl of each serum was put in a flat-bottomed microplate and mixed with the bacteria *Micrococcus lysodeikyicus* (0.4 mg/ml in 0.1 M phosphate buffered saline, pH 6.2 at 25 C). The microplate was read using an ELISA reader at 450 nm wavelength. The activity of the lysozyme was measured as a decrease of 0.001/minute in the optical density of cell suspension [21].

2.2.6. Microtiter plate agglutination test

The agglutinating titer of serum antibodies was determined according to the method of Li et al. [22]. Briefly, 50 µl serum samples were serially diluted in PBS and placed in a 96-well microliter plate (rounded bottom). Then 50 µl of formalin-inactivated *Streptococcus* spp. (9×10^8 CFU/mL = McFarland 3) was mixed with the serum dilution in each well and incubated at 30 °C for 18 h, then for 4 h at 4 °C before reading. The agglutination reaction was observed visually, where the last serum dilution showing visible lumps (agglutination) like fog were regarded as the agglutination titer expressed as log2 **(x + 1)** of the reciprocal of the highest serum dilution showing visible agglutination compared to the positive control.

2.2.7. Challenge test

To evaluate the resistance of vaccinated fish to experimental infection with virulent *S. agalactiae*, *S. iniae, L. garvieae and E. faecalis*, challenge was performed in 4 different groups; each received a single homologous bacterial species by IP inoculation. However, each group was further divided into 3 subgroups for challenge after 1, 2 or 3 months post-vaccination (Fig. 3). Groups 5–8 served as non-vaccinated controls and each was challenged parallel to each vaccinated group. Group (9) was vaccinated received saline only by the IP rout. Group (10) was not vaccinated and received saline IP only.

All challenged fish groups were monitored daily for one week and the Relative Percentage Survival (RPS) was calculated at > 60% control mortality according to Amend [23] and the European Pharmacopeia (2013): $\mathbf{RPS} = 1\text{-}\left[\frac{\%\ mortality\ in\ vaccinated\ fish}{\%\ mortality\ in\ control\ fish}\right] x\ 100$

2.2.8. Re-isolation of challenge bacteria

Swabs were collected from the kidneys and brains of moribund and freshly dead fish after challenge with the virulent organisms. Each swab was streaked onto TSA and blood agar plates and incubated at 30 °C for 48hrs. Fish were considered protected if cultures showed no growth of involved *Streptococcus*, *Lactococcus* and *Enterococcus* species.

2.2.9. Statistical analyses

Data was analyzed with SPSS version 21. Results were expressed as mean values ± SE. Un-paired one-way ANOVA was used to test the significance of differences between fish groups at $p \leq 0.05$. Post hoc test (Tukey) was used for pairwise comparison.

3. Results

3.1. Phenotypic and molecular identification

Although *S. agalactiae* is known to belong to Lancefield group B streptococci as a β-hemolytic microorganism, it may be worth noting to report that all of our isolates recovered from fish were γ-hemolytic. *L. garvaiae* and *S. iniae* were α-β hemolytic. *E. faecalis* was γ-haemolytic. All isolates did not grow on Mac-Conky's agar except *Enterococcus faecalis*. All bacteria appeared on TSA as pin point to small size, translucent to white colonies and showed Gram positive cocci arranged in pairs or short to long chains under the microscope. Biochemical characterizations are summarized in Table 1. Nucleotide sequence analysis and Neighbor-joining phylogenetic tree of 16 S rRNA gene were performed to confirm the identity of vaccine seeds (Fig. 1).

3.2. Vaccine characterization

Transmission electron microscopy (TEM) was carried out to visualize the size and shape of the micro-emulsion particulate adjuvant with the embedded bacteria. In TEM, an electron beam is transmitted through the specimen (100 nm thick suspensions on a grid) and the developed image of the particle size of the adjuvant was about 100 nm (Fig. 2).

3.3. Vaccine efficacy and potency in broodstock

Haematological findings after administration of the vaccine by injection in broodstock are presented in Table 2. Results show no significant differences ($p^{>}0.05$) between the total counts of erythrocytes and concentrations of hemoglobin and albumin in vaccinated and non-vaccinated females. However, leukocyte counts, total proteins, globulins and A/G ratio were significantly higher in vaccinated groups ($p < 0.05$) versus controls. Antibody titers were also significantly higher (range = 9.38 ± 0.49 to 10.0 ± 0.51) than controls when evaluated three weeks post-vaccination ($p < 0.05$) using the micro agglutination test (Table 3). The parental vaccination program in broodstock increased the number of hatched fry/female from 500 ± 30 in non-vaccinated groups to 900 ± 34 in vaccinated groups and increased their survival rates at the end of the nursery stage to < 60% (depending on pathogen at one month of age).

3.4. Efficacy and potency of immersion vaccination

The vaccine provided protective antibody titers against the four different antigens starting from the second week (W2) post vaccination (Table 4). Titers were at least 3 log2 lower than those obtained by injection of broodstock, but remained much higher than the non-

Table 1
Biochemical profile of vaccine seeds.

Test	*S. agalactiae*	*S. iniae*	*L. garvieae*	*E. faecalis*
Cell morphology	Spherical cocci	Spherical cocci	Ovoid cocci	Ovoid cocci
Motility	–	–	–	–
Hemolysis	γ	**α\β**	**α\β**	γ
Growth on				
10% bile agar	+	+	+	+
40% bile agar	–	–	+	+
Growth in				
pH 9.6	–	–	+	+
6.5% NaCl	–	–	+	+
Growth at				
10 °C	–	–	+	+
45 °C	–	–	+	+
Hydrolysis of				
Arginine	+	+	+	+
Esculin	–	+	+	+
Hippurate	–	–	–	–
Starch	+	+	–	–
Decarboxylation				
Arginine	+	+	+	+
Lysine	–	–	–	–
Ornithine	–	–	–	–
Acid from				
Arabinose	–	–	–	–
Glucose	+	+	+	+
Glycerol	+	+	–	–
Lactose	–	–	+	+
Maltose	+	+	+	+
Raffinose	–	–	–	–
Sorbitol	–	–	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	–	–	–	–
Other tests				
Oxidase	–	–	–	–
Catalase	–	–	–	–
VP	+	–	+	+
Indol	–	–	–	–
H2S	–	–	–	–
Urease	–	–	–	–
Nitrate reduction	–	–	–	–
Citrate	–	–	–	–

vaccinated controls (2.43 ± 0.5). However, over 12 week's observation period after vaccination (Table 4), antibodies decreased gradually, but were still above the suggested protective level (4 log2).

Challenge results obtained monthly after immersion confirmed that Aqua Strept provided protection against the included pathogens up to three months post-vaccination (Fig. 3). Infected fish in the control groups showed skin darkness, eye opacity exophthalmia, nervous swimming behavior and hemorrhages. Necropsy findings included dark and enlarged spleen, pale liver, enlarged gall bladder and brain hemorrhages. All bacterial species used were successfully re-isolated from the brain, eyes and kidneys of all dead fish.

4. Discussion

Although huge investments are poured into the Egyptian aquaculture industry, there is a general demand of professional health management to account for disease outbreaks that cause high fish mortalities and economic losses. Streptococcosis, lactococcosis and enterococcosis are among the most common bacterial diseases affecting Nile tilapia and many other fish species, including Asian seabass (*Lates calcarifer*), grouper (*Epinephelus lanceolatus*), Japanese flounder (*Paralichthys olivaceus*), rabbit fish (*Siganus canaliculatus*), rainbow trout (*Oncorhynchus mykiss*), red drum (*Sciaenops ocellatus*), red tilapia, gilthead seabream (*Sparus aurata*), silver pomfret (*Pampus argenteus*), and wild mullet [24,25].,

Research carried out during the last decade was directed to develop protective monovalent vaccines against fish infection. Scanty efforts were attempted in the area of polyvalent vaccines for fear of incompetence. Monovalent vaccines were essentially injected intraperitoneally (I/P) and consisted of formalin killed whole cultures of homologous bacterins, with or without ECP. RPS values of vaccinated fish varied from 56% to 95% when challenged at 21 or 7 days post-vaccination, respectively [9–11]. Vaccines containing mineral oil adjuvants or non-oil adjuvants were also developed and yielded satisfactory protection levels [11].

However, the purpose of the present study was to develop and evaluate a locally prepared polyvalent vaccine that protects tilapia broodstock and offspring against common infections due to four pathogens; *S. iniae, S. agalactiae, L. garvieae,* and *E. faecalis* [5–7]. Combining antigens in an immersion polyvalent vaccine formula aimed at alleviating fish stress, vaccination cost and labor required for each single vaccination process. The involved bacterial species are potentially zoonotic and have been implicated in severe streptococcosis, lactococcosis and enterococcosis in cultured Egyptian Nile tilapia and elsewhere. Of these, *S. agalactiae* Lancefield group B is a part of the intestinal and genitourinary flora of man and may occasionally cause neonatal meningitis and sepsis. In fish, it has been associated with meningoencephalities, epicardities and choroiditis, compromising food security [26]. In cattle, it may cause mastitis. A correlation has been found between subpopulations of this species and target host species [27,28]. *S. iniae (*Synonym *S. shiloi)* is another infectious organism that was first identified from an Amazon freshwater dolphin in the 1970s and was zoonotically associated with acute cellulitis in man, by the virtue of a cluster of cases that presented in 1995–1996 with fever and lymphangitis after handling whole or live fish purchased in Toronto, Canada. The organism was cultured from their blood [29]. On the other hand, *L. garvieae*, though causes a hyper-acute, hemorrhagic septicemia in many freshwater and marine fish (lactococcosis), was also isolated from cattle mastitis and from an increasing number of human infections with complicated febrile illness, where handling and ingestion of raw fish were reported as a source of risk in the majority of clinical cases [30]. *Enterococcus faecalis* belongs to the gastrointestinal flora of man and animals and can cause a range of different infections in man, including urinary tract infections, sepsis, and endocarditis. Some strains are among the leading causes of multidrug-resistant, hospital-acquired infections [31].

The clinical signs of fish infected by any of these species are similar, showing unilateral or bilateral exophthalmia, opaque cornea, skin darkness, nervous swimming behavior, pale gills, external hemorrhages, enlarged spleen, ascites in abdominal cavities, and discolored liver. Infection was recorded in both hatcheries and ponds particularly when the temperature, pH, ammonia in the water are high and dissolved oxygen is low. Although Iregui et al. [26] claimed absence of vertical transmission among *Streptococcus* species in tilapia, Anshary et al. [24] and others [32] observed mortalities in cage cultures. This suggests that seeded tilapia raised in hatcheries may acquire the infection prior to stocking in the cage culture. Moreover, Pradeep et al. [25] confirmed the vertical transmission of *S. iniae* and *S. agalactiae* in less than 10-day old tilapia off-spring obtained from infected females, in agreement with our earlier observations.

Because the biochemical, morphological and hemolytic characteristics of organisms included in the polyvalent vaccine did not show definitive identification profiles, nucleotide sequence analysis and Neighbor-joining phylogenetic tree of 16 S rRNA gene were employed to confirm their identity (Table 1, Fig. 1).

When the adjuvant was mixed with the bacterins to form an O/W emulsion, the average maximum particle size was 100 nm by transmission electron microscopy (TEM), a size that supports the adhesion and diffusion of the emulsion particles into the skin and gills of the fish. TEM has been commonly used to characterize micro-emulsions and nano-particulate adjuvants [33]. The employed adjuvant has been optimized for mucosal immunity [13]. It improves the adsorption of

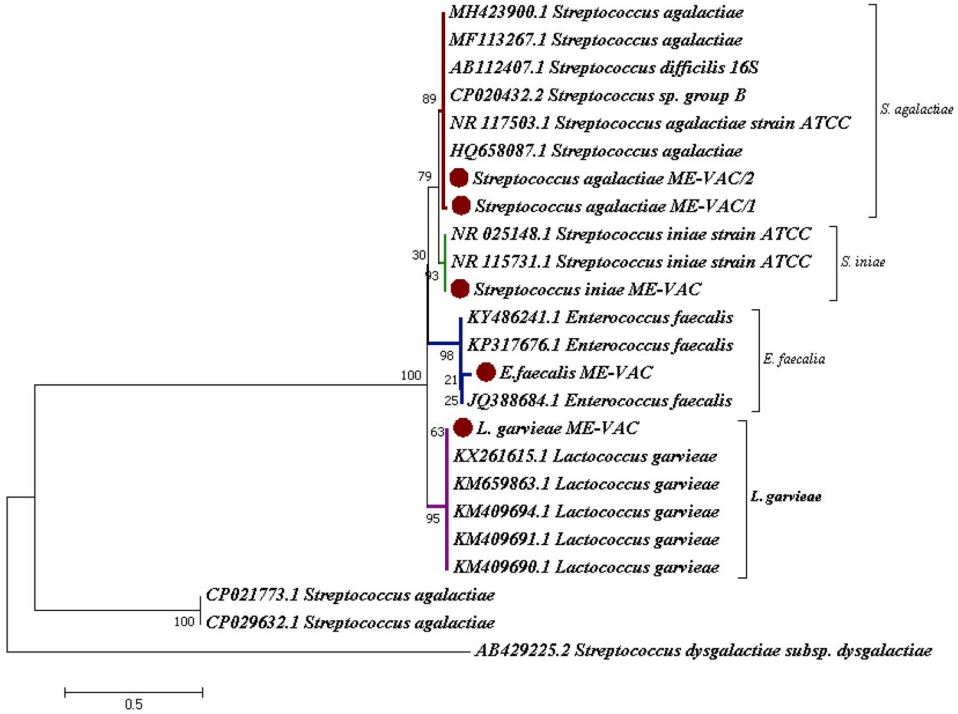

Fig. 1. Phylogentic tree of Egyptian *Streptococcus* species affecting Nile tilapia (*S. agalactiae, S. iniae, L. garvieae* and *E. faecalis*) and other related streptococci. Bootstrap percentages (based on 1000 replications) are shown at branch points.

antigens through gill tissues, skin and intestines and promotes their retention in the lymphoid tissues, providing a slow depot release [14]. In addition, it protects the antigens from hydrolytic enzymes and low pH of the gut and intestinal mucosal surfaces. Soltani et al. [15] reported high protection levels in rainbow trout against *Yersinia ruckeri* antigens mixed with Montanide IMS 1312 VG in an immersion vaccine formula. Also, an immersion vaccine containing hemorrhagic septicemia virus mixed with the same adjuvant elicited high protection in Olive flounder (*Paralichthys olivaceus*) [16].

The haematological parameters of broodstock vaccinated in this study are regarded as important indicators of fish health [34]. The haematological findings of leucocytes, total proteins, globulins, phagocytic index and lysozymes were significantly ($p < 0.05$) higher in vaccinated than non-vaccinated females three weeks post vaccination. Likewise, Silva et al. [34] established that increased leukocyte count was associated with higher antibody titers, phagocytic activity/index

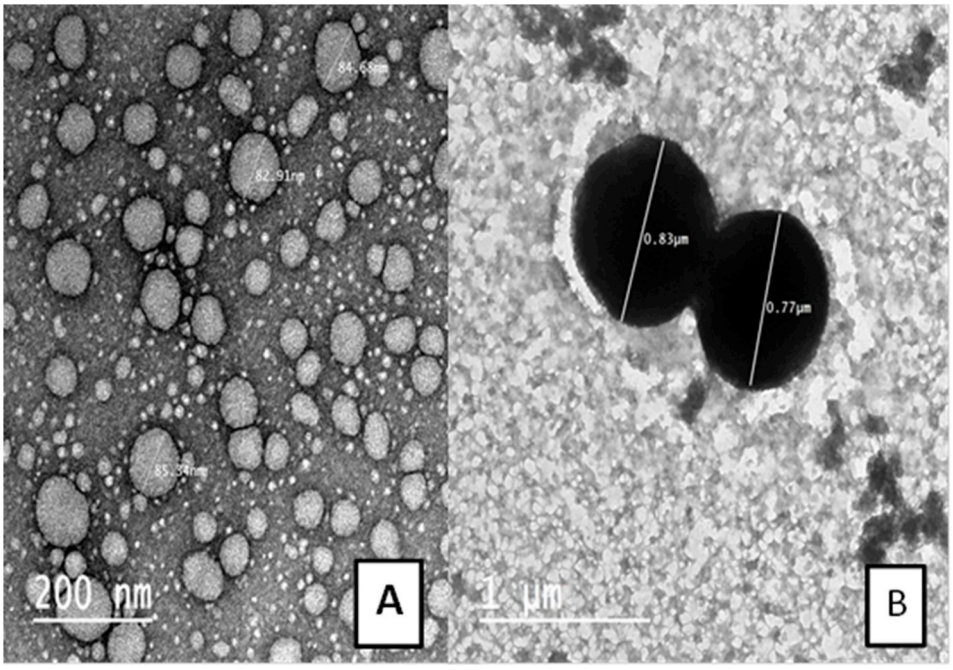

Fig. 2. TEM images showing (A) the size and shape of nano-emulsion particulate adjuvant (B) short chain streptococci embedded in nano-emulsion particulate adjuvant. The maximum average particle size of the adjuvant was about 100 nm.

Table 2
Haematological and immunological findings of vaccinated and non-vaccinated broodstock three weeks post vaccination*.

Items	Vaccinated female	Non-vaccinated
Red blood cell/ml	$2.53 \times 10^6 \pm 0.31^a$	$1.72 \times 10^6 \pm 0.31^b$
White blood cell/ml	**$758.33 \times 10^3 \pm 60.09^a$**	**$550 \times 10^3 \pm 52.04^b$**
Hemoglobin (g/dl)	13.30 ± 0.46	11.67 ± 0.33
Total protein (g/dl)	5.07 ± 0.28^a	3.67 ± 0.12^b
Albumin (g/dl)	1.57 ± 0.3	1.5 ± 0.58
Globulin (g/dl)	**3.5 ± 0.25^a**	**2.2 ± 1.7^b**
Phagocytic activity %	67% ± 1.45	70% ± 1.5
Phagocytic Index	**2.73 ± 0.67^a**	**1.99 ± 0.06^b**
Lysozyme activity (unit/ml)	**34 ± 2.3^a**	**20 ± 2.0^b**

*Data was represented as a mean ± SE (n = 10) within raw, values with different subscripts a, b indicate that their corresponding means were significantly different ($p < 0.05$) according to one-way ANOVA and Tukey tests.

Table 3
Microagglutination test ($\log_2 (x+1)$) of vaccinated broodstock versus controls.

Antigens in ME-VAC Aqua Strept*	Titers three weeks post vaccination in Vaccinated females	Titers three weeks post vaccination in non-vaccinated controls
S. agalactiae	10 ± 0.51^a	4.75 ± 0.25^b
S. iniae	9.8 ± 0.56^a	4.75 ± 0.25^b
L. garvieae	10.88 ± 0.23^a	4.25 ± 0.75^b
E. faecalis	9.38 ± 0.49^a	4.25 ± 0.25^b

*Data was represented as a mean ± SE (n = 10) within raw, values with different subscripts a, b indicate that their corresponding means are significantly different ($p < 0.05$) according one way ANOVA followed by Tukey test.

and lysozyme function. Antibody titers against *Streptococcus* spp., *L. garviae and E. faecalis* ($\log_2 (x+1)$) were higher in vaccinated broodstock (range = 9.38 ± 0.49 to 10.88 ± 0.23) than the controls (4.25 ± 0.25) ($p < 0.05$) using the micro agglutination test (Table 3). The production of fish larvae is often hampered by mortality rates that increase in summer season due to higher temperature, pH, ammonia and lower dissolved oxygen [35]. The parental vaccination program in broodstock reduced the mortality percent among fry at the end of the nursery period by more than 60% (depending on pathogen) and increased the number of seeds/female from 500 ± 30 in non-vaccinated groups to 900 ± 34 in vaccinated groups. This could be attributed to the large amount of antibodies and lysozymes transferred by the broodstock during the process of vitellogensis [9]. Nur et al. [36] and Nissa et al. [18] observed that vaccination of broodstock against *S. iniae* and *S. agalactiae* had a potential to improve the seed quality at the age of less than one month. The antibody levels in fish larvae gradually decreased 14 days post-hatching because the specific antibodies transferred by the mother are naturally metabolized together with the egg yolk [9]. Results show that *S. iniae* vaccine failed to protect tilapia against *S. agalactiae* infection, suggesting absence of cross-protection, in consent with Evans et al., [10]. Likewise, *L. garvieae* and *E. faecalis* produced specific immunity that may protect from within species differences only [37,38].

The size of fish suitable for vaccination has been an issue in many vaccination studies and method of vaccine delivery. Generally, younger or smaller fish are not believed to be immuno-competent to acquire immunity following vaccination with any type of vaccine or by any method of vaccination. However, it is generally believed that tilapia could be immuno-competent when more than 21 days of age [10]. Thus, mass vaccination of tilapia fry (after the nursery period) by immersion using Aqua Strept vaccine has increased the resistance against *S. agalactiae, S. iniae, L. garvieae, and E. faecalis* for a minimum of three months and provided the required protection levels (RPS 62–80%, depending on pathogen). Immersion vaccination works on the ability of mucosal surfaces to recognize pathogens they get in contact with. When fish are immersed in water containing the diluted vaccine, the suspended antigens from the vaccine may be adsorbed by the skin and gills. Then, specialized cells, such as antibody-secreting cells, present in the skin and gill epithelium will be activated to protect the fish from exposure to the live pathogen at a later stage. Other cells located in the skin and gill epithelium such as the antigen presenting cells (macrophages), absorb the vaccine antigens and transport them to specialized tissues where the systemic immune response builds up. Protective antibodies against the four different antigens were detected at high titers by the second week post vaccination ($\geq$ 7 log2) and lasted beyond the 12th week challenge time (> 4 log2). Advantages of Aqua Strept are that it contains a combination of highly concentrated antigens dispersed in a liquid nanoparticle micro-emulsion, which improves the immunogenicity and efficacy of the vaccine. The adjuvant facilitates the early onset of immune response, modulates adaptive and nonspecific immunity and improves the uptake of antigen by mucosal surfaces to induce a prolonged immunity. Similarly, previous reports show that nano-particulate adjuvants had improved the efficacy of *Yersinia ruckeri* and viral hemorrhagic septicemia vaccines in rainbow trout and Olive flounder [15,16].

Overall, the current polyvalent vaccine developed for tilapia showed high protection and efficacy (RPS), in all susceptible ages, whether administered by IP injection or immersion. To our knowledge, this vaccine may be the first to offer a combination of antigens that protect tilapia from streptococcosis, lactococcosis and enterococcosis. The results support the use of this vaccine as an effective tool for disease control and well-being of fish. Though immersion vaccination is faster and easier, further attempts are needed to produce effective oral vaccines to protect tilapia in the second stage of production cycle. Finally, it's important to realize that it's only the combination of vaccination with several factors such as high quality seed, good nutrition, good biosecurity and sanitary practices that can assure the highest possible survival rates and best profit margins.

Table 4
Agglutination titers ($\log_2 (x+1)$) of Nile tilapia during 12 weeks after immersion vaccination with MEVAC Aqua Strept*.

Weeks post-vaccination	Agglutination titers log2				Control non-vaccinated fish
	S. agalactiae	*S. iniae*	*L. garvieae*	*E. faecalis*	
2	7.8 ± 0.4^a	7.4 ± 0.3^a	7.3 ± 0.29^a	7.9 ± 0.26^a	2.43 ± 0.5^b
4	7.5 ± 0.12^a	7.3 ± 0.29^a	7.0 ± 0.22^a	7.8 ± 0.29^a	2.43 ± 0.5^b
6	7.0 ± 0.3^a	7.0 ± 0.2^a	6.9 ± 0.28^a	6.7 ± 0.31^a	2.43 ± 0.5^b
8	6.8 ± 0.11^a	6.0 ± 0.3^a	6.3 ± 0.29^a	6.4 ± 0.2^a	2.43 ± 0.5^b
10	6.0 ± 0.2^a	5.7 ± 0.7^a	5.6 ± 0.30^a	6.0 ± 0.3^a	2.43 ± 0.5^b
12	5.2 ± 0.3^a	5.3 ± 0.8^a	5.1 ± 0.2^a	5.6 ± 0.2^a	2.43 ± 0.5^b

*Data was represented as a mean ± SE (n = 10) within raw, values with different subscripts a, b indicate that their corresponding means are significantly different ($p < 0.05$) according one way ANOVA followed by Tukey test.

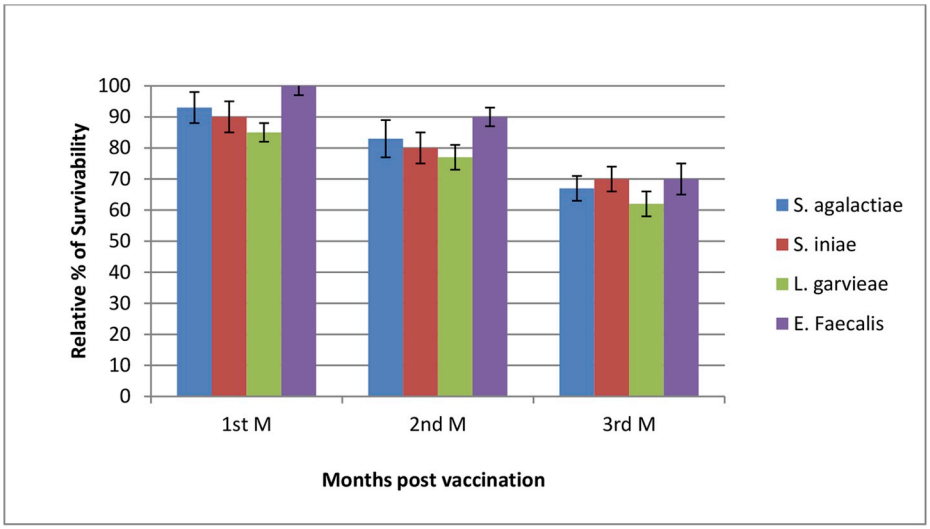

Fig. 3. Relative percent of survivability of tilapia administered ME-VAC Aqua Strept by immersion and challenged with virulent *S. agalactiae*, *S. iniae*, *L. garvieae* and *E. faecalis* (0.2 ml of 6×10^8 CFU/ml, injected IP). All non-vaccinated groups challenged with *S. agalactiae, S. iniae, L. garvieae,* and *E. faecalis* showed more than 60% mortality **(European Pharmacopeia 2013).**
IP: Intra-peritoneal.
CFU/ml: Colony Forming Unit.
RPS: relative percent of survivability $\mathbf{RPS} = 1 - \left[\frac{\%\ mortality\ in\ vaccinated\ fish}{\%\ mortality\ in\ control\ fish}\right] x\ 100.$

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