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Occurrence of infectious *Streptococcus agalactiae* in the farmed Nile tilapia

Ahmed H. Sherif [1,*], Jehan I. Abdellatif [2], Mohamed M. Elsiefy [3], Mofeed Y. Gouda [4], and Abeer E. Mahmoud [5]

1. Department of Fish Diseases, Animal Health Research Institute, ARC, Kafrelsheikh, Egypt
2. Department of Fish Diseases, Animal Health Research Institute, ARC, Dokki, 12619, Egypt
3. Pathology Unit, Animal Health Research Institute, ARC, Kafrelsheikh, 12619, Egypt
4. Immunology Unit, Animal Health Research Institute, ARC, Kafrelsheikh, 12619, Egypt
5. Fish Diseases Unit, Animal Health Research Institute, ARC, Assiut, 12619, Egypt

*Corresponding Author:: ahsherif77@yahoo.com

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ABSTRACT

This study examined the Nile tilapia *(Oreochromis niloticus)* to determine the presence of *Streptococcus agalactiae* bacteria in three different sites. Isolates were identified, and gene sequences revealed four strains (accession numbers OL471406, OL471407, OL471408, and OL470978). The four strains harbored different virulence genes, and the most virulent strain was used in the treatment trial. The antibiotic of choice was florfenicol (FFC) for the isolate (minimum inhibitory concentration, 12 µg/g body weight, b.w.) and the median lethal dose of *S. agalactiae* was determined to be 0.3×10^5 CFU/mL. Experimental infection propagated the same clinical signs and post-mortem close to those obtained in the natural infection. *Spirulina platensis* at a concentration of 5 g/kg b.w. ameliorated the impact of FFC (12 and 1200 µg/k b.w.). A high dose of FFC could minimize the presence of carrier fish, but with deleterious effects on the immunity, which could be boosted by dietary *S. platensis.* Therefore, *S. agalactiae* could be confirmed to be associated with a high isolation rate in freshwater fish farms. A 100-fold of the minimum inhibitory concentration (MIC) in FFC could eliminate carriers and minimize coherent infection; thus, the addition of *S. platensis* to the medicated diet of *O. niloticus* is recommended to enhance the therapeutic efficacy by improving the immune responses of fish.

INTRODUCTION

Nile tilapia production largely dominated in Egypt; however, tilapia farming is becoming economically important in other African countries. The quantity and intensity of tilapia farming have increased dramatically, disease outbreaks that caused serious economic losses have been increasing.

Streptococcus infections have resulted in significant economic losses for the aquaculture industry in several parts of the world, especially in intensive fish farming **(Mishra *et al.*, 2018).** Various species from the Streptococcaceae family have been

identified as etiological agents of streptococcosis in fish; *Streptococcus iniae*, *S. agalactiae*, *S. parauberis*, and *S. dysgalactiae* are the prominent species worldwide **(Pereira *et al.*, 2010; Haines *et al.*, 2013).** *S. agalactiae* is a Gram-positive bacteria that causes septicemia and meningoencephalitis in freshwater and saltwater fish species, and is thus a severe danger to the aquaculture business, notably for tilapia *(Oreochromis spp.)* **(Leal *et al.*, 2019).** Since 2009, large-scale *S. agalactiae* infections have been breaking out in tilapia farms all over the world, leading in high mortality and significant economic losses **(Mian *et al.*, 2009; Zhang *et al.*, 2017)** in Zambia **(Bwalya *et al.*, 2020)** and Ghana **(Verner-Jeffreys *et al.*, 2018).**

Hence, using an antibiotic is the currently available practical therapeutic strategy **(Vendrell *et al.*, 2006).** However, immunosuppression may occur with antibiotic therapy **(Maklakova *et al.*, 2011; Guan *et al.*, 2011, Sherif *et al.*, 2021a).** Florfenicol (FFC) is one of the most extensively used antimicrobials for the treatment of streptococcosis **(Bowker *et al.*, 2010; Gaunt *et al.*, 2010).** FFC has a quick absorption rate (highest serum concentration after 12 hours) in Nile tilapia, and its wide dispersion in host tissues is temperature dependant **(Feng and Jia, 2009).**

However, concerns linked with the use of FFC include bacterial pathogen resistance to the medicine and dose-related toxicity, which causes unpleasant effects on aquatic animals **(Botelho *et al.*, 2015; Ren *et al.*, 2017; Wang *et al.*, 2017).** FFC has suppressed humoral and cellular immune responses accompanied by lymphoid organ damage **(Maklakova *et al.*, 2011).** However, another study suggested that FFC at 5 mg/kg body weight (b.w.) is safer in its effect on *O. niloticus* **(Reda *et al.*, 2013).**

Strengthening fish immunity with immunopotentiators such as herbal extracts, herbal compounds, bacterial components, and other natural substances is one potential strategy for reducing illnesses in aquaculture **(Lee and Gao, 2012; Talpur *et al.*, 2013; Yilmaz, 2019).** A recent study found that natural ingredients can boost the antibacterial medication action in combination treatment at lower dosages **(Zhao *et al.*, 2018).**

Therefore, this work was designed to clarify the importance of *S. agalactiae* in fresh fish farms and a treatment trial with antibiotic and immunostimulant therapy.

MATERIALS AND METHODS

2.1. Collection of fish samples

Nile tilapia was bacteriologically examined for the presence of *S. agalactiae* infection in three different sites (semi-extensive farm). Samples were collected three times at the same sites with a 2-month interval from March to October 2021. Moribund fish was euthanized with an overdose of tricaine methanesulfonate (MS222; Sigma, St. Louis, MO, USA) and then bacteriological sampling was aseptically performed on-site and collected in tightly closed plastic containers and preserved in an ice tank, and immediately transported to the bacteriology laboratory at Animal Health Research Institute, Kafrelsheikh Egypt.

2.2. Bacteriological examination

2.2.1. Primary isolation

Swabs were taken from internal organs, including the brain, spleen, kidney, and liver, and cultivated into tryptic soy broth for 48 h at 35°C, then streaked on tryptic soy agar with 5% sheep's blood for 24 h at 28°C. Bacterial swabs were also taken from the brain, liver, kidney, and spleen and were subcultured on blood agar plates to obtain pure cultures of predominant organisms. Phenotypic characterization of bacterial isolates was confirmed according to **Bergey (1994)**, **Elmer *et al.* (1997),** and **Madigan and Martinko (2005)**. Streptococcal spp. were identified biochemically using API 20E Strep **(Bio-merieux, 1984**).

2.2.2. Polymerase chain reaction (PCR), virulence, and sequences

DNA extraction

Extraction of genomic DNA was performed from purified isolates of *Streptococcus* spp. using the PrepMan® Ultra Sample Preparation Reagent protocol (Applied Biosystems, USA), following the manufacturer's instructions. The extracted DNA was eluted and placed in tightly sealed vials at −20°C for further molecular assays.

Virulence genes of the isolates

Specific PCR reactions were screened to detect the following virulence factors in four isolates as described by **Deng *et al.* (2019)**: A fbsA acts as an adhesin, fbsB as an adhesin, Lmb as an adhesin, cylE as immune evasin, scpB as immune evasin, Cfb as invasin, and cspA as invasion, the specific primers for each gene are presented in Table "1".

Sequencing

The whole 16S rRNA gene sequencing was amplified through the PCR technique using universal pair primers (FD1: 5′-AGAGTTTGATCCTGGCTCAG-3′) and (RD1: 5′-TAAGGAGGTGATCCAGCC-3′) described by **Batdorj *et al.* (2006)**. Briefly, PCR mixtures were composed of 12.5 µL EmeraldAmp® GT PCR Master Mix (Takara Bio Inc, Shiga, Japan), 4 µL genomic DNA, 1.0 µL of each primer, and 6.5 µL free nuclease water in a final volume of 25 µL. The conventional PCR consisted of 35 cycles in a 1.0 min denaturation at 94°C, 1.0 min annealing at 56°C, and 1.0 min extension at 72°C. These cycles followed the initial denaturation step at 94°C for 7 min. The cycles ended with a final extension step at 72°C for 10 min. PCR products were purified using the QIAqick Gel Extraction Kit (Qiagen, Hombrechtikon, Switzerland). The 16S rRNA genes of nine isolates of *Streptococcus* spp. were sequenced in both directions at the Macrogen sequencing company (Macrogen, Seol, South Korea) using the ABI 3730XL DNA sequencer. Raw data were checked and edited using Bio Edit version 7.0 **(Hall, 1999).** The bacterial isolates were identified by aligning assembled sequences against other sequences deposited in the GenBank database (National Center for Biotechnology Information, NCBI) using BLASTN search.

Phylogenetic tree

The phylogenetic tree was used to compare 16S rRNA sequences of four bacterial strains with 29 different accession numbers documented from *S. iniae, S. agalactiae, S. parauberis,* and *E. faecalis*. Multiple sequence alignment was carried out using the CLUSTALW program. The analysis of interspecies and interstrain similarities was accomplished using the maximum likelihood methods of MEGA X with 1000 bootstrap values **(Kumar *et al.*, 2018)**.

2.3. The median lethal dose (LD_{50}) determination

LD_{50} of *S. agalactiae* (OL471408) was estimated following **Reed & Muench's** procedure **(1938).** Briefly, *O. niloticus* (60 ± 5 g b.w.) was acclimated as the experimental fish above. Groups of 10 fish were intraperitoneally injected with serial tenfold dilutions of *S. agalactiae* cultured in the brain–heart infusion broth for 24 h at 30°C. First, 100 µL of *S. agalactiae* suspension was adjusted to 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, or 1×10^7 (CFU/mL) in normal saline (0.65%), and the suspension was injected into duplicate groups of five fish. Mortality rates were recorded for 14 days, and *S. agalactiae* was re-isolated from the dead fish and confirmed using API 20E Strep **(Bio-merieux, 1984**).

Antimicrobial Sensitivity Analyses

The activity of different antimicrobial drugs against the isolated *S. agalactiae* (OL471408) was analyzed following the procedures described by **Finegold & Martin (1982).** Pure cultures of the strains were cultivated in Tryptone Soy Broth (Oxoid) and incubated for 24 h at 26°C ± 1°C. Subcultures were spread with a sterile cotton stick onto Mueller–Hinton agar plates (Oxoid). Results were recorded after incubation at 26°C ± 1°C for 24 h, by disk diffusion, including FFC (KF 10 µg), ciprofloxacin (CIP 5 µg), clindamycin (DA 2 µg), amoxy + clavulanic AMC (30 µg), amoxicillin AML (10 µg), doxycycline (DO 30 µg), streptomycin (S 10 µg), spiramycin (SP 100 µg), sulfamethoxazole + trimethoprim (SXT 25 µg), lincomycin (MY 10 µg), cefotaxime (CTX 30 µg), and cephradine (CE 30 µg) (Oxoid, Waltham, MA, USA). According to the standards provided by the manufacturer and **NCCLS (1999)** guidelines**,** the isolated bacteria could be classified into three categories: resistant, intermediate, and sensitive depending on the diameters of inhibition zones.

2.4. Minimum inhibitory concentration (MIC) of FFC

MIC was performed following instructions from **Ravikumar *et al.* (2011).** Briefly, 50 µl of 24 h old *S. agalactiae* (OL471408) inoculum (corresponding to a concentration of 5×10^5 CFU were exposed to a dilution series of FFC ranging from 30 to 4 µg/mL (30, 28, 26, 24, 22, 20, 18, 16, 14, 12, 10, 8, 6, and 4 µg/mL). The culture was allowed to grow at 28°C for 48 h and the whole setup was triplicated, whereas the broth alone was considered as the negative control. The MIC of FFC was defined as the lowest concentration of the agent that restricted the bacterial growth in the culture media.

2.5. Experimental infection and treatment trial

A total number of 560 *O. niloticus* were purchased from a local fish farm without a record of diseases and/or antibiotic treatment. After 2-week acclimatization, the bacteriological examination was performed on 20 randomly selected fish to ensure they were disease-free. A 540 *O. niloticus* weighing 40 ± 5 g b.w. were divided into six treatments (T1–6) with three replicates for each, then each replicate was stocked in an aquarium (50 × 50 × 60 cm) with 90 L water volume. Water parameters were kept in normal range suitable for *O. niloticus*, and the rearing one-third of water was daily exchanged with fresh water to remove solid discharges, the water temperature of 27°C ± 0.5°C, dissolved oxygen of ≥5.5 ± 0.4 mg/L, pH of 8.1 ± 0.4, and salinity of ≤ 0.3 g/L **(Boyd, 1990).**

The *Spirulina platensis (S. platensis)* pellets used in this study were produced by Fresh-Life Pharma, Canada. Florfenicol Floricol® (Reg No. 2533/2015) was obtained from the local market, manufactured by Pharma Swede, Egypt. *S. platensis* and florfenicol were added to the fish feed by coating the surface of the pellets with capelin oil **(Samuelsen & Bergh, 2004).**

- **T1:** *O. niloticus* fed on a diet free of additives.
- **T2:** *O. niloticus* fed on a diet containing *S. platensis* at a concentration of 5 g/kg fish feed for 2 weeks before challenge and continued for another 2 weeks.
- **T3:** *O. niloticus* on diet containing MIC of FFC of 12 µg/g b.w.
- **T4:** *O. niloticus* containing the 100-fold MIC of FFC of 1200 µg/g b.w.
- **T5:** *O. niloticus* fed on a diet containing *S. platensis* at a concentration of 5g/kg fish feed for 2 weeks before challenge and continued plus MIC of FFC (12 µg/kg b.w.) for another 2 weeks.
- **T6:** *O. niloticus* fed on a diet containing *S. Platensis* at a concentration of 5g/kg fish feed for two weeks before challenge and continued plus MIC of FFC (1200 µg/g b.w.) for another two weeks.

A total of 40 *O. niloticus* (fish/group) was experimentally infected with highly virulent isolate *S. agalactiae* (OL471408). Fish was injected intraperitoneally of 10% of LD_{50} (3×10^5 CFU) following **Schaperclaus *et al.*** method **(1992)** and pure saline solution (0.65%) was injected similarly, in three fish, for negative control injection **(Boijink *et al.*, 2001).** The number of dead fish was recorded for 14 days, and the mortality rate during a specific period (MR) was measured using the following equation:

$$MR\ (\%) = \text{number of deaths} / \text{total fish number} \times 100$$

Meanwhile, the RLP was verified among the challenged fish according to **Ruangpan *et al.* (1986)'s** formula:

$$RLP\% = [1 - (\%\text{mortality in the treated group} / \%\text{mortality in the control group})] \times 100$$

O. niloticus was kept in the same experimental condition and under observation, and after 14 days, the fish that survived was bacteriologically examined for *S. agalactiae*. The fish abdomen was aseptically opened (sterilized with methyl alcohol 70%),

specimens were taken from internal organs **(Amlacher, 1970),** bacterial isolation was performed using randomly selected five fish from each treatment and anesthetized within 60 s using 50 mg/L MS222, and finally, isolates were confirmed for the presence of *S. agalactiae* using the API 20E Strep **(Bio-merieux, 1984**).

2.6. Gene expression of cytokines and antioxidant enzymes

The gene expression of some cytokines (tumor necrosis factor alpha [*tnfa*], interleukin- 1beta (il-1b, and il-10) and antioxidant enzymes (superoxide dismutase [*sod*] and catalase [*cat*]) were determined pre-challenge with *S. agalactiae* then post-challenge (3 and 14 days) and treated with FFC and *S. platensis*. The primers used in this experiment are displayed in Table "1". Briefly, reverse transcription–polymerase chain reaction (RT-PCR) was performed following **Choi *et al.* (2004).** RNA was extracted from head kidney samples (100 µg) in triplicate (two fish/replicate) by applying the standard TRIzol extraction method (Invitrogen, Paisley, UK). To ensure the removal of genomic DNA, the eluted RNA was treated with DNase (Thermo Fisher Scientific Inc, USA). The obtained bands were examined with densitometric analysis using an ImageJ gel analysis program **(Abramoff *et al.*, 2004).** The density of each target gene band and the corresponding β-actin band were compared to estimate expression levels.

2.7. Histopathological examination

After the experimental trial, samples were collected from three tissues, the liver, spleen, kidney, and brain, after the bacterial challenge test. Formalin-fixed paraffin-embedded sections were processed routinely for H&E staining following the methods described by **Suvarna *et al.* (2012).**

2.8. Statistical analyses

The impacts of *S. agalactiae* on *O. niloticus* were statically analyzed with SPSS software for Windows (SPSS Inc., Chicago, IL, USA) **(SPSS, 2004)** using analysis of variance. All values are expressed as the mean ± SE (standard error). Duncan's multiple range test **(Duncan, 1955)** was used to determine differences among treatments at a significance level of 0.05.

2.9. The applied biosafety measures

This study followed the biosafety measures concerning the pathogen safety data sheets: Infectious substances were *S. agalactiae* (Pathogen Regulation Directorate, **Public Health Agency of Canada, 2010).**

Table 1. Primers used in this study

Gene	Sequence 5-3	Accession no. (GenBank)
tnfa	F: AGGGTGATCTGCGGGAATACT R: GgCCCAGGTAAATGGCGTTGT	NM_001279533.1
il−1b	F: TCTTCTACAAACGCGACACC R: TCTGGAGCTGGATGTTGAAG	KF747686.1
il−10	F: TTCAGGAACTCAAGCGGGATAT R: GCTGTTGACTTCAAAGGGATTTT	NM_0001020785
sod	F: CATGCCTTCGGAGACAACAC R: ACCTTCTCGTGGATCACCAT	AY491056.1
cat	F: AGCTCTTCATCCAGAAACGC R: GACGTCAGGCGTCACATCTT	JF801726.1
β−actin	F: CCACACAGTGCCCATCTACGA R: CCACGCTCTGTCAGGATCTTCA	EU887951.1
fbsA	F: 5'-AACCGCAGCGACTTGTTA-3' R: 5'-AAACAAGAGCCAAGTAGGTC-3'	**Syuhada *et al.* (2020)**
fbsB	F: 5'-TCTGTCCAACAGCCGGCTCC-3' R: 5'- TTCCGCAGTTGTTACACCGGC-3'	**Kayansamruaj *et al.* (2014)**
Lmb	F: 5'- TCAGTTAGTTGCTCTGCTTC-3' R: 5'- CTTTATGACCCACATACCTG-3'	**Syuhada *et al.* (2020)**
cylE	F: 5'- GTACATTAGGTGCCTTTGG-3' R: 5'- TACTCAGCCTTTCTCCATC-3'	**Syuhada *et al.* (2020)**
scpB	F: 5'-ACAACGGAAGGCGCTACTGTTC-3' R: 5'- ACCTGGTGTTTGACCTGAACTA-3'	**Dmitriev *et al.* (2004)**
cfb	F: 5'- GGATTCAACTGAACTCCAAC-3' R: 5'- GACAACTCCACAAGTGGTAA-3'	**Legario *et al.* (2020)**
cspA	F: 5'-CTGCTAAAGCACACCTAAAC-3' R: 5'-ATCAGTAGTGGTTCCTTTCC-3'	**Legario *et al.* (2020)**

RESULTS

3.1. Molecular identification of bacterial isolates

The whole sequences of 16S rRNA genes from streptococcal isolates were amplified with PCR using the universal pair primers. Four purified PCR products were directly sequenced to confirm the identity of these bacterial isolates. The four assembled sequences were submitted and deposited in the GenBank database under the following accession numbers: OL471406, OL471407, OL471408, and OL470978. Streptococcal identities were confirmed by comparing 16S rRNA sequences against relevant *Streptococcus* spp. deposited in the GenBank database. In this study, four accession numbers (OL471406, OL471407, OL471408, and OL470978) showed a high "99.77–100%" nucleotide homology with *S. agalactiae* (KT328464.1, KT328463.1, KT328461.1, KT328475.1, CP053027.1, CP036376.1, and MK517599.1). Therefore, the accession numbers (OL471406, OL471407, OL471408, and OL470978) were confirmed to be *S. agalactiae*. The intra-strains similarities of *S. agalactiae* four strains ranged from 99.65% to 99.86% with differences ranging from two to five nucleotides.

The phylogenetic analysis showed that four bacterial isolates belong to *S. agalactiae* (4 isolates) and grouped with other *S. agalactiae* isolates. The current isolates were separated from other streptococcal groups that belong to *E. faecalis*, *S. iniae*, and *S. parauberis* (Figure 1).

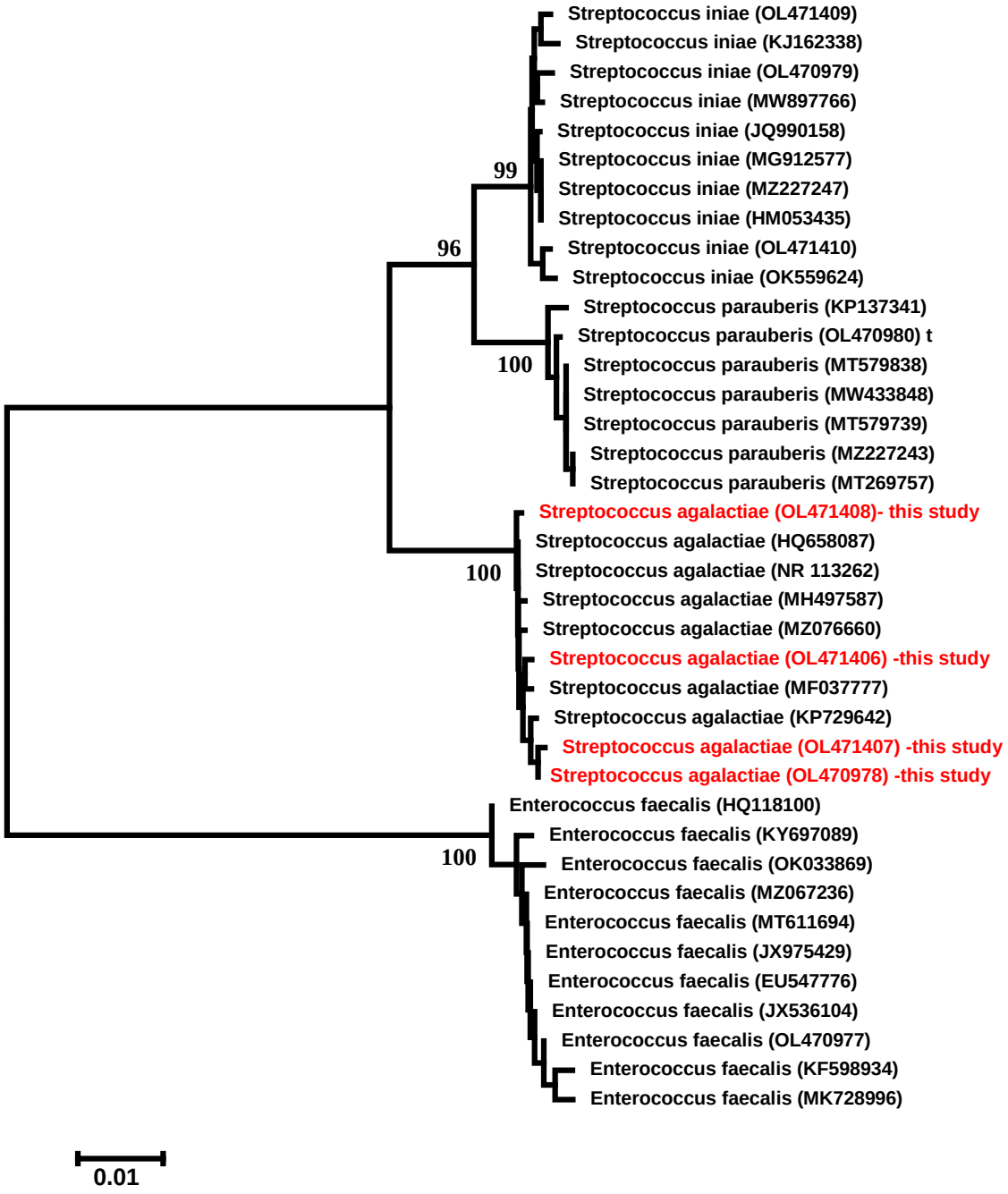

Figure 1. The phylogenetic tree shows the comparative analysis of 16S rRNA gene sequence of the current *S. agalactiae* isolates and other interrelated bacterial strains.

3.2. Clinical signs, post-mortem, and isolation rate of *Streptococcal* infection

In Figures 2, 3, and 4, the most prominent clinical signs were external hemorrhage and opaque eye lesions, pop-eyes, and pale gills in *O. niloticus* experimental infected

with *S. agalactiae*, whereas post-mortem changes were splenomegaly in an empty intestine and distended gall bladder. Farmed *O. niloticus*, which was naturally infected with *S. agalactiae*, showed the same clinical and post-mortem lesions as in experimentally infected ones; moreover, their infections were severe with fatty liver.

Figure 2. Clinical signs of the experimental *O. niloticus* infected with *S. agalactiae*, photo A: arrows 1, dentated tail fin and 2, hemorrhages on the body; photo B: arrows, pop-eyed; photo C: arrow, pop-opaque eye.

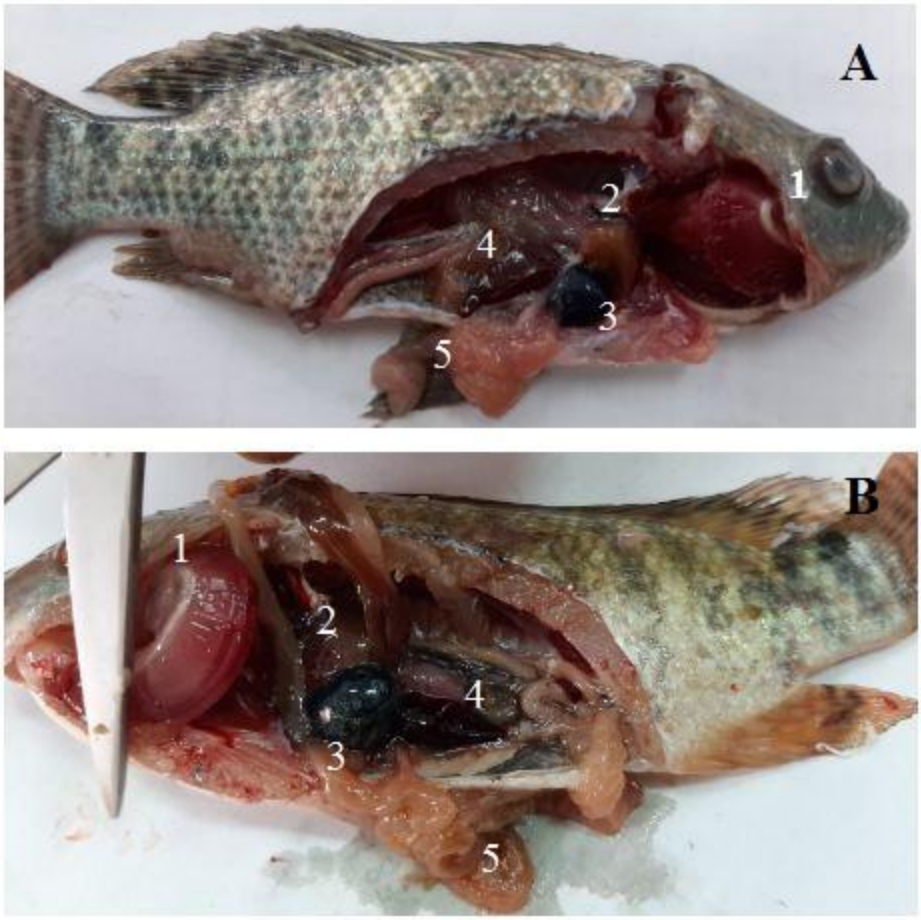

Figure 3. Post-mortem changes in the experimental *O. niloticus* infected with *S. agalactiae.* Photo A: 1, opaque eye; 2, friable liver; 3, distended gall bladder; 4, splenomegaly; and 5, empty intestinal tract. Photo B: 1, pale gills; 2, friable liver; 3, distended gall bladder; 4, splenomegaly; and 5, empty intestinal tract.

In Table "2", *S. agalactiae* prevailed in freshwater fish farms with different percentages influenced by the season and collection site. Regardless of the site, *S. agalactiae* was isolated with a high rate during Summer and Spring followed by Autumn, i.e., 48.46%, 43.12%, and 27.5%, respectively. All fish harbor clinical signs were infected with *S. agalactiae;* the severity of clinical signs was influenced by season as they were highly prominent during Summer (24.6%).

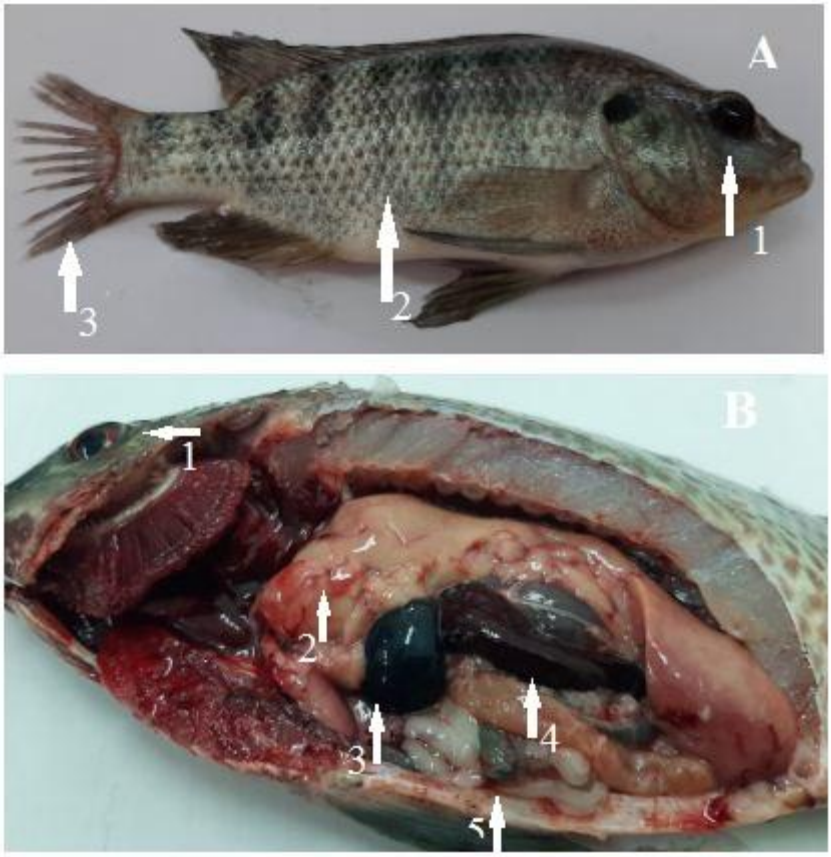

Figure 4. Clinical signs and post-mortem changes in farmed *O. niloticus* infected with *S. agalactiae*. Photo A: arrow 1, pop-darkened eye; 2, darken skin; and 3, fried tail. Photo B: 1, slightly pop-eye; 2, fatty liver; 3, gall bladder; 4, splenomegaly; and 5, full intestine.

Table 2. Isolation rate of *S. agalactiae* in sites under investigation.

Item	**Spring**			**Total**	**Summer**			**Total**	**Autumn**			**Total**
Site (n=3)	**S1**	**S2**	**S3**		**S1**	**S2**	**S3**		**S1**	**S2**	**S3**	
Fish (no.)	60	50	50	160	40	50	40	130	40	40	40	120
Fish morb	12	3	10	25	18	2	12	32	8	1	18	27
%	20	6	20	15.6	45	4	30	24.6	20	2.5	45	22.5
Fish inf	25	20	24	69	21	30	12	63	10	9	14	33
%	41.67	40	48	43.12	52.5	60	30	48.46	25	22.5	35	27.5

Fish morb; fish harbor clinical signs.

3.3. Virulence genes, median lethal dose (LD_{50}), antibiotic sensitivity, and minimum inhibitory concentration for FFC and *S. platensis*

The *S. agalactiae* (OL471408) isolates harbor more virulence genes than the other isolates; therefore, it was used in the treatment trial (Table 3).

LD_{50} for bacterial isolates was determined to be 3×10^5 for *O. niloticus* weighing 40 ± 5 g b.w. at the water temperature of 27 ± 0.5°C, dissolved oxygen of ≥5.5 ± 0.4 mg/L, pH of 8.1 ± 0.4, and salinity of ≤0.3 g/L. Bacterial isolates were highly sensitive to FFC and ciprofloxacin, confirming that these antibiotics would be suitable for the treatment, whereas the bacteria exhibited intermediate resistance to clindamycin, amoxicillin clavulanate, and sulfamethoxazole-trimethoprim and full resistance to amoxicillin, lincomycin, cefotaxime, streptomycin, doxycycline, spiramycin, and cephradine. A 12 µg/mL concentration of FFC was determined as MIC of *S. agalactiae* at 28°C.

4. Table 3. Virulence genes of *S. agalactiae* isolated from *O. niloticus*

Items	OL471406*	OL471407	OL471408	OL470978
fbsA **	+	+	+	–
fbsB	+	+	+	–
Lmb	–	–	+	–
cylE	–	–	+	–
scpB	–	–	-	–
cfb	–	–	+	–
cspA	–	–	-	–

*Accession number of the isolate, ** virulence gene, + (presence), and – (absence).

3.4. Treatment trial

After the challenge with *S. agalactiae*, fish was treated with two different doses of MIC and 100-fold MIC to eliminate carrier cases. To mitigate the stress of FFC treatment, medicated diets were supplemented with *S. platensis* algae (Table 4).

Table 4. *O. niloticus* infected with *S. agalactiae* and treated with FFC and *S. platensis*

Item	Free T1	S.P. T2	FFC T3	FFC100 T4	FFC+S.P. T5	FFC10+S.P T6
UN Fish no.	40	40	40	40	40	40
Dead Fish no.	3	2	3	4	1	1
CMR %	7.5	5	7.5	10	2.5	2.5
CH Fish no.	40	40	40	40	40	40
Dead Fish no.	25	19	9	8	8	8
CMR %	62.5	47.5	22.5	20	20	20
RPL %	-	**24**	**64**	**68**	**68**	**68**
Live Fish no	15	21	31	32	32	32
Carrier Fish no.	13	17	16	6	9	4
Carrier %	**86.7**	**80.95**	**51.6**	**18.75**	**28.12**	**12.5**

UN, fish unchallenged; CH, fish challenged with *Streptococcus agalactiae*; no., number; CMR, cumulative mortality rate; RPL, relative protection level.

In Table 4, unchallenged fish received the same doses of FFC, *S. platensis*, and combination. *O. niloticus,* fed on 100-fold of FFC, showed higher MR (10%) compared with the control, whereas the addition of *S. platensis* to the fish feeds had a lower level (2.5%).

In challenged fish with *S. agalactiae, S. platensis* could decrease the MR% to 20% alone or plus both doses of FFC MIC (T5) and 100-fold (T6) providing RPL of 68% compared to the control of 62.5%. Fish treated with FFC had lower MR% of 47.5 and 22.5%.

The high dose of FFC could decrease carriers to 18.75% and the addition of *S. platensis* 12.5%. Although *S. platensis* (T2) could decrease carriers compared to the control (T1) by 80.95% and 86.7%, respectively, the MIC of FFC (T3) was higher by 51.6%, whereas the addition of *S. platensis* to the MIC of FFC (T5) lowered the carriers to 28.12%.

3.5. Gene expression of some cytokines of *O. niloticus.*

In Table 5, the mRNA expression of immune-related cytokines il-1b, *tnfa*, and il-10 revealed that FFC and *S. platensis* could modulate the immune response of *O. niloticus.* Gene expression of proinflammatory cytokines il-1b and *tnfa* was significantly lower in treatments receiving *S. platensis* in unchallenged *O. niloticus* even those administered higher doses of 100-fold of FFC (T6), whereas il-10 acts otherwise. Bacterial infection-induced inflammation and treatment with FFC and *S. platensis* had enhanced fish responses, il-1b and *tnfa*, were significantly increased in fish receiving *S. platensis* T6, T2, and T5 treatments compared with those received FFC and control. After 15 days of *S. agalactiae* challenge, inflammation of *O. niloticus* rapidly subsided as the gene expression of anti-inflammatory cytokine il-10 was significantly increased in *O. niloticus* fed on diets containing 100-fold FFC plus *S. platensis* T6 and *S. platensis* T2.

Table 5. Gene expression of some cytokines in the head kidney *O. niloticus.*

Item	Challenge	Free T1	S.P. T2	FFC T3	FFC100 T4	FFC+S.P. T5	FFC10+S.P T6
il-1b	Un	2.2^{A} ±0.03	0.47^{F} ±0.02	2.1^{A} ±0.01	1.91^{B} ±0.05	0.77^{E} ±0.05	1.42^{C} ±0.09
	post3	3.65^{E} ±.09	5.14^{B} ±0.1	4.2^{D} ±0.07	3.83^{E} ±0.05	4.76^{C} ±0.02	5.73^{A} ±0.17
	Post 14	1.63^{A} ±0.24	1.05^{B} ±0.03	0.9^{B} ±0.01	1.18^{B} ±0.03	0.47^{C} ±0.06	0.45^{C} ±0.02
tnfa	Un	1.87^{A} ±0.09	0.91^{D} ±0.03	1.52^{B} ±0.04	1.62^{B} ±0.05	1.13^{C} ±0.02	1.26^{C} ±0.03
	post3	4.19^{C} ±0.02	6.13^{A} ±0.2	4.34^{C} ±0.03	4.37^{C} ±0.09	5.71^{B} ±0.14	6^{A} ±0.16
	Post 14	2.98^{A} ±0.06	1.21^{DE} ±0.02	1.96^{C} ±0.06	2.4^{B} ±0.04	1.34^{D} ±0.02	1.14^{E} ±0.03
il-10	Un	4.16^{D} ±0.12	6.29^{A} ±0.19	4.71^{C} ±0.05	4.54^{C} ±0.67	5.85^{BC} ±0.03	5.77^{B} ±0.19
	post3	$2..08^{E}$ ±0.05	2.9^{C} ±0.06	2.33^{D} ±0.04	2.7^{C} ±0.12	3.11^{B} ±0.06	3.92^{A} ±0.05
	Post 14	2.99^{C} ±0.06	8.95^{A} ±0.1	5.83^{B} ±0.04	5.7^{B} ±0.12	5.79^{B} ±0.2	9.21^{A} ±0.05

Note: Data are shown as mean ± SE. Means with different letters in the same row are significantly different at $p \leq 0.05$. il-1b, interleukin-1beta; tnfa, tumor necrosis factor alpha; il-10, interleukin-10

3.6. Gene expression of some *O. niloticus* cytokines

In Table 6, the impact of *S. agalactiae* significantly induced oxidative stress in *O. niloticus* as gene expression of antioxidant enzymes (*sod* and *cat*) was increased. Oxidative A signs of oxidative stress in fish treated with antibiotic FFC (T4 and T3) and had significantly increased gene expression of *sod* and *cat* 3.68 and 3.53-fold change, 6.2-, and 6.11-fold changes, respectively. Although *S. platensis* could ameliorate the stress caused by FFC, they were still significantly higher than the control group.

Table 6. Gene expression of some antioxidant enzymes in the hepatic tissue *O. niloticus* treatment infected with *S. agalactiae* with FFC and *S. platensis.*

Item	Challenge	Free T1	S.P. T2	FFC T3	FFC100 T4	FFC+S.P. T5	FFC10+S.P T6
sod	**Un**	0.2^{D} ±0.01	0.97^{C} ±0.12	3.53^{A} ±0.12	3.68^{A} ±0.09	2.11^{B} ±0.02	2.26^{B} ±0..09
	Ch	2.57^{E} ±0.12	3.47^{D} ±0.18	5.43^{C} ±0.09	6^{B} ±0.1	6.33^{AB} ±0.05	6.63^{A} ±0.22
cat	**Un**	1.23^{E} ±0.04	1.56^{D} ±0.03	4.79^{B} ±0.02	5.81^{A} ±0.06	2.23^{C} ±0.04	2.43^{C} ±1.5
	Ch	4.11^{D} ±0.08	5.06^{C} ±0.06	6.11^{B} ±0.02	6.2^{B} ±0.1	7.6^{A} ±0.3	8^{A} ±0.1

Note: Data are shown as mean ±SE. Means with different letters in the same row are significantly different at $p \leq 0.05$. *sod*, superoxide dismutase; *cat*, catalase; Un, unchallenged; Ch, challenged.

3.7. Histopathology of the experimental *O. niloticus* challenged with *S. agalactiae*

The histopathological samples were collected after the *S. agalactiae* challenge to assess the impact of the challenge and the efficacy of the therapeutic agent. The hepatopancreatic tissues showed necrobiotic hepatocytes changes, vacuolar degeneration, hepatocytes with pyknotic nuclei, presence of hemorrhage, and mononuclear inflammatory cells between (Figure 5). In the splenic tissue, congested blood vessels with melanomacrophage aggregations surrounding it and depletion in the white pulp were observed (Figure 6). In kidney tissues, renal glomerular atrophy, and the presence of vacuolar degeneration in the renal tubular epithelium with pyknotic nuclei were observed (Figure 7), and the brain tissues showed intracellular edema, pre-vascular edema, and

congestion of minute blood capillaries in the brain (Figure 8). The fish lesions were repeated in different groups with various degrees of severity, indicating that dietary *S. platensis* could ameliorate the lesion severity.

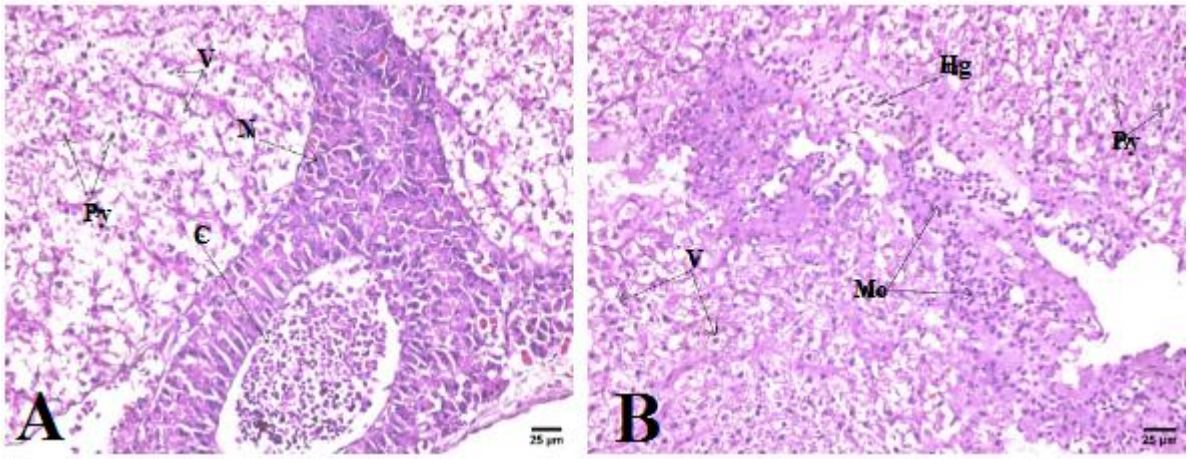

Figure 5. A. Hepatic tissue (T4) showing necrobiotic changes in the hepatopancreas (N) with congestion (C), in the hepatopancreatic blood vessels, hepatocytes showing severe vacuolar degeneration (V) with pyknotic nuclei (Py) (hematoxylin and eosin stain). B. Hepatic tissue (T1) showing vacuolar degeneration (V) in hepatocytes, hepatocytes with pyknotic nuclei (Py), presence of hemorrhage (Hg), and mononuclear inflammatory cells (Mo) between hepatocytes (hematoxylin and eosin stain).

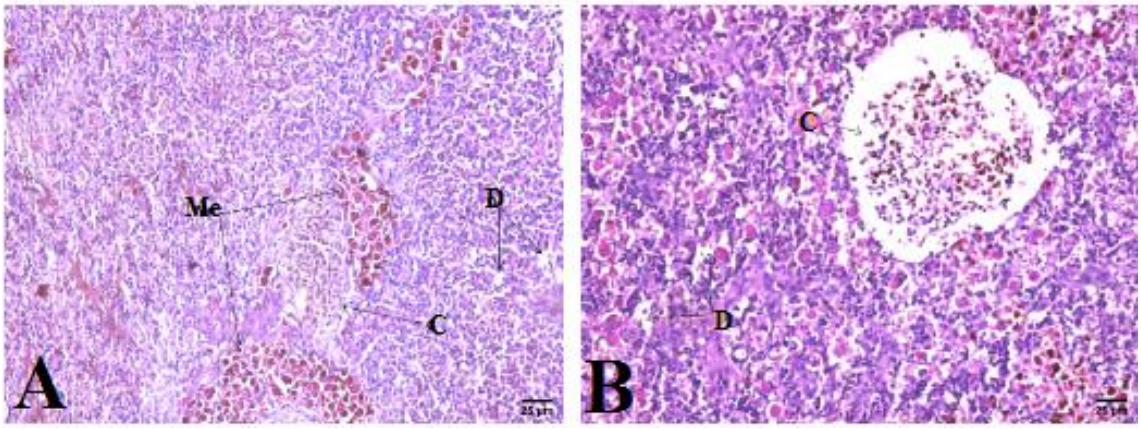

Figure 6. A. The splenic tissue (T1) shows congested (C) blood vessels with melanomacrophage aggregations (me) surrounding it with depletion (D) in the white pulp of the spleen (hematoxylin and eosin stain). B. The splenic tissue (T4) shows depletion (D) in the white pulp and congestion (c) in the splenic blood vessels (hematoxylin and eosin stain).

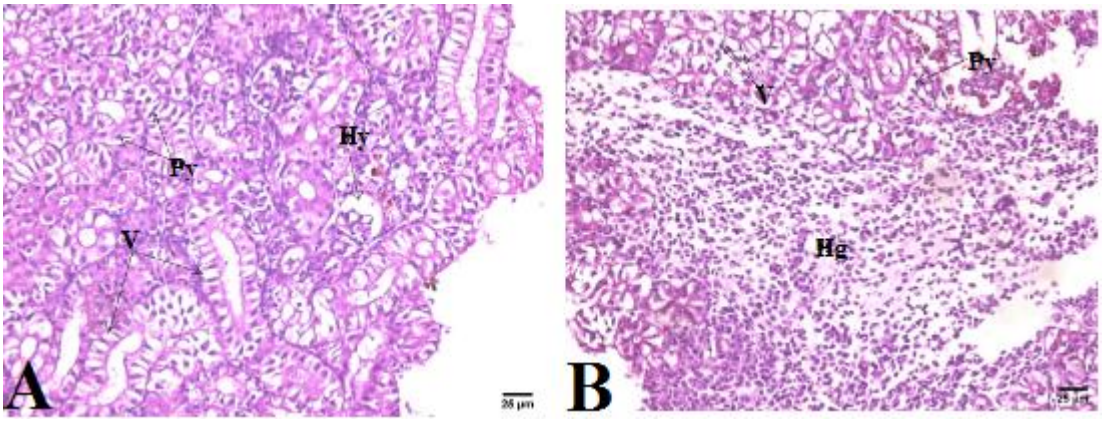

Figure 7. A. The renal tissue (T1) shows renal glomerular atrophy (Hy), the presence of vacuolar degeneration (V) in the renal tubular epithelium with pyknotic nuclei (Py) (hematoxylin and eosin stain). B. The splenic tissue (T4) shows hemorrhage (Hg) between renal tubules and the presence of vacuolar degeneration (V) in renal tubular epithelium with pyknotic nuclei (Py) (hematoxylin and eosin stain).

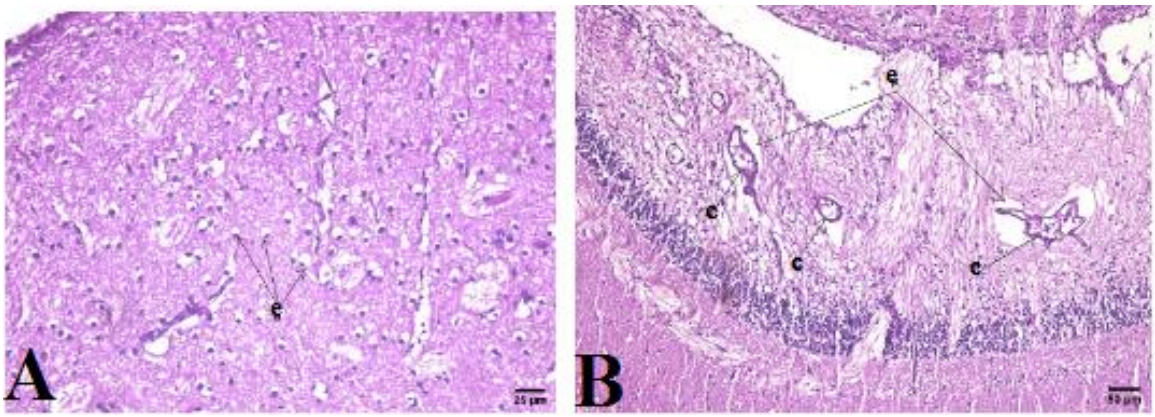

Figure 8. A. The brain tissue (T1) showing intracellular edema (e) (hematoxylin and eosin stain. B. The brain tissue (T4) shows pre-vascular edema (e) and congestion (C) of the minute blood capillaries in the brain (hematoxylin and eosin stain).

DISCUSSION

In this study, clinical signs of streptococcosis in both farmed and experimental *O. niloticus* were pop-opaque eye, pale gills, splenomegaly, friable liver, distended gall bladder, and empty intestine, which are close to the systemic bacterial disease findings in North Africa outbreaks that showed clinical signs such as exophthalmia, anorexia, and skin darkness, and inflammatory fluid accumulation in the body tissues of *O. niloticus* **(Ye *et al.*, 2011; Tavares *et al.*, 2016; Delannoy *et al.*, 2021).** The severity of streptococcosis signs of the farmed *O. niloticus* was significantly affected by the seasons (water temperature) during Summer and Spring followed by Autumn of 48.46%, 43.12%, and 27.5%, respectively. While high water temperatures have been described as a

predisposing factor for the diseases of tilapia, irrespective of the serotype or genotype involved **(Mian *et al.*, 2009; Rodkhum *et al.*, 2011).** The *S. agalactiae* CC2/IV outbreaks were associated with high water temperatures of >30°C of all fish sizes and lasted 9–14 weeks, with a total death rate in adult fish representing 6–14% of *O. niloticus* **(Delannoy *et al.*, 2021)**. Moreover, *S. agalactiae* CC283/III caused in MR between 25 and 35% of *O. niloticus* cultured in the floating-cage culture in Brazil **(Leal *et al.*, 2019),** whereas *S. agalactiae* CC7/Ia has MR of 20–30% in farmed *O. niloticus* in China **(Ye *et al.*, 2011).**

LD_{50} for *S. agalactiae* (OL471408) was determined to be 3×10^5 for *O. niloticus* and was highly sensitive to FFC. Accordingly, the sensitivity of Gram-positive bacteria (such as Streptococcus) to FFC is at least four times higher than Gram-negatives **(Treves-Brown, 2000),** whereas some strains of *S. agalactiae* that infect fish were resistant to gentamycin, kanamycin, trimethoprim, nitrofurantoin, ampicillin, spiramycin, oleandomycin, sulfamethoxazole, oxolinic acid, penicillin, erythromycin, and oxytetracycline **(Soto *et al.*, 2015).** Recently, the complete genome of *S. agalactiae* (CP019812.1) was the lack of resistance genes associated with FFC resistance, like the floR genes or orthologs **(Barony *et al.*, 2017).**

The doses used in the fish treatment were near to the MIC obtained in this study (12 µg/mL) at the water temperature of 28°C. FFC doses of 20 and 40 µg/g of live b.w. of *O. niloticus* for 10 days could completely prevent the mortality caused by *S. agalactiae* **(de Oliveira *et al.*, 2018),** whereas at 10 µg/g dose of the plasma FFC concentration reached 4.46 µg/mL after 12 h *(Oreochromis niloticus* × *O. aureus)* **(Feng & Jia, 2009)**.

The identification of carrier fish is one of the main issues affecting the control and prevention of infectious diseases at the farm level **(Altinok & Kurt, 2003; Sherif *et al.*, 2021b).** In this study, to eliminate carrier cases, 100-fold of MIC was used in the treatment trial, and to mitigate the FFC stress, *S. platensis* algae were added to fish feed. Abused antibiotic treatment resulting in the emergence of a carrier state for *Streptococcus* bacteria has been found under natural conditions **(Evans *et al.*, 2002; Faria *et al.*, 2014)** and a recurrent infection was observed in the treatment of fish-pathogenic *S. agalactiae* with oxytetracycline **(Faria *et al.*, 2013).** As different findings, *S. agalactiae* (SA95-10) remained viable for 10 days when exposed to a 100-fold MIC value of FFC **(de Oliveira *et al.*, 2018).**

A need for different treatment trends motivates many researchers to use combinations with antibiotics and natural products to fight bacterial disease in aquatic animals. Therefore, a high dose of FFC alone or combined with *S. platensis* could decrease carriers to 18.75% and 12.5%, respectively, whereas the combination of *S. platensis* and MIC of FFC and the comparison with the control showed 28.12% and, 86.7%, respectively. Several authors combined FFC with natural products to enhance treatment and eliminate carriers. The combination of antibiotics with immune stimulators improved their efficacy, and no mortality occurred for 30 days before the bacterial

challenge in all groups fed with Rutin/FFC and control basal diets **(Deepika *et al.*, 2019)**. Consistent with the observations of **Zhao *et al.* (2018),** our findings suggest that co-treatment of FFC with myo-inositol significantly decreases the cumulative mortality. The combination of oxytetracycline and *Moringa oleifera* enhanced the efficacy of tetracycline for the treatment of aeromoniasis in *O. niloticus* **(Sherif *et al.*, 2021a).** Similarly, **Cao *et al.* (2018)** found that Gibel carp juveniles fed diets supplemented with *A. platensis* (3.38 g/kg fish feed) had low MR after a 7-day *A. hydrophila* challenge compared to the control fish. In *O. niloticus* challenged with *A. hydrophila* fed on diets containing *A. platensis* at 5 and 10 g/kg fish feed for 28 days were 6.7% and 3.3%, respectively, compared with that of the control, 13.3%. Moreover, *O. niloticus* receiving dietary *A. platensis* at 5 g/kg and 10 g/kg fish feed had lower MR than the control by approximately 5% **(Sherif *et al.*, 2020).**

Streptococcosis induced gene expression of immune-related cytokines in the experimental *O. niloticus* compared with unchallenged control. Similarly, previous studies illustrated that bacterial infection naturally and significantly stimulates alterations in the il-8, interferon gamma (inf-γ), *tnfa*, and immunoglobulin (Ig) M levels of rainbow trout *(Oncorhynchus mykiss)* **(Raida & Buchmann, 2008; Evenhius & Cleveland, 2012),** and in *O. niloticus* **(Sherif *et al.*, 2019, 2020, 2021c).**

The natural additives more likely stimulated the immune system rather than directly affecting the pathogen. In this study, dietary *S. platensis* stimulated the gene expression of il-1b and *tnfa* in *O. niloticus* to fight against streptococcosis. Similarly, the expression of the *tnfa* gene was elevated in *O. mykiss* fed with diets supplemented with *S. platensis* **(Sheikhzadeh *et al.*, 2019),** up-regulation of *tnfa* gene in *O. niloticus* **(Mahmoud *et al.*, 2018)** in response to *S. platensis.*

The addition of MIC of FFC 12 µg/g b.w. to the diet of unchallenged fish did not affect the gene expression of il-1b, *tnfa*, and il-10. The gene expression of IL−1β and *tnfa* were increased by the 100-fold MIC of FFC 1200 µg/g b.w. in the unchallenged and challenged fish. Accordingly, this study showed that *O. niloticus* infected with pathogenic *S. agalactiae* and treated with FFC has significantly decreased gene expression of immune-related cytokines, which could be explained by the reduced FFC in the bacterial load and by subsequently decreasing the need for protective protein synthesis **(Na-Phatthalung *et al.*, 2018).** However, **Shiry *et al.* (2019)** found that *O. mykiss* (55 ± 7.6 g) challenged with the *Lactococcus garvieae* and *S. iniae* was treated with FFC (15 µg/g b.w. for ten consecutive days), revealing a significantly elevated expression of *tnfa* and il-1b genes in the FFC treated/infected fish compared to untreated diseased fish. Moreover, the immunosuppressive impacts of FFC could be noticed. Similarly, *tnfa* and il-1b genes expressions in FFC treated healthy and infected *O. mykiss* were significantly higher than those in the control group **(Shiry *et al.*, 2019)**. Furthermore, some studies accentuated the inducing effects of FFC on il-1b, il-8, and *tnfa* gene expressions in Nile tilapia and Atlantic cod *(Gadus morhua)* **(Yilmaz, 2019;**

Caipang *et al.*, 2009). Meanwhile, in brown trouts *(Salmo trutta)*, no changes were observed on the *tnfa* following the application of FFC at 40 µg/g b.w. **(Er & Dik, 2014).** Cytokine il-10 was significantly and rapidly increased in *O. niloticus* fed on diets containing 100-fold FFC plus *S. platensis* compared to the other treatments, indicating the elimination of bacterial infection. Moreover, **Xinxin *et al.* (2011)** confirmed the inflammatory/down-regulatory effects of FFC on the interleukins.

Accretion of *sod* and *cat* in response to oxidative stress caused by pathogens is one of the main defense pathways of antioxidants. In this study, the gene expression of antioxidant enzymes *sod* and *cat* were induced by the response to *S. agalactiae* infection and/or FFC treatment in *O. niloticus*, although *S. platensis* could ameliorate the stress caused by FFC and were still significantly higher than that of the control group. Consistent with our findings, **Deepika *et al.* (2019)** observed that the antioxidant-mediated defense was induced by feeding Rutin/FF in tilapia infected with *A. hydrophila.* Other observations, decreased *sod* and *cat* activities, may indicate the susceptibility of cells to pathogens **(Mohankumar & Ramasamy, 2006),** an important finding for the protection of fish against potential pathogens **(Lorenzon *et al.*, 2002).** Natural antioxidants have the ability for reactive oxygen species (ROS) scavenging on the intrinsic antioxidant system and clean them to prevent oxidative stress **(Hassaan *et al.*, 2021)**. Our results are parallel with those of the previous studies **(Lee *et al.*, 2010; Kim *et al.*, 2013; Gora *et al.*, 2019)** that indicated adding *S. platensis* significantly improved the antioxidant enzyme activities of fish by inhibiting the formation of ROS.

In this study, histopathological analyses revealed that *S. agalactiae* infection resulted in severe inflammatory changes in the tissues of experimental *O. niloticus*, mainly characterized by the presence of hemorrhage, mononuclear inflammatory cells, intracellular edema, and congested blood vessels in the liver and brain, and by melanomacrophage aggregations and the presence of vacuolar degeneration in the kidney tissues in the splenic tissue. Lesions developed due to the septicemia induced by the bacterium, which was similar to the streptococcosis in tilapia **(Suanyuk *et al.*, 2008; Mian *et al.*, 2009; Abdullah *et al.*, 2013).** Consistent with our findings, **Amal *et al.* (2019)** found that the main histopathological diagnoses of Javanese medaka infected by *S. agalactiae* were moderate brain meningeal congestion, moderate kidney tubular necrosis, mild glomerular atrophy, mild hepatic necrosis, spleen congestion, and hyper-aggregation of the melanomacrophage center. In Javanese medaka, the most notable histopathological findings were generalized congestion of internal organs particularly in the brains and livers in *S. agalactiae* infection **(Zamri-Saad *et al.*, 2010; Abdullah *et al.*, 2013).**

CONCLUSION

This study concluded that *S. agalactiae* had a high isolation rate in *O. niloticus.* FFC could also eliminate carriers and minimize coherent infection; however, its high dose suppressed immune responses that could be restored by adding dietary *S. platensis.*

Histopathological lesions were related to septicemic bacteria and the dietary *S. platensis* at a dose of 5g/kg b.w. of fish.

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