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Influence of in-feed oxolinic acid therapy on the brain histoarchitecture and virulence factors of *Streptococcus agalactiae* infecting Nile tilapia *Oreochromis niloticus*

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Abstract

Streptococcosis and its management are major constraints to tilapia aquaculture. The present study assessed the effect of oral administration of 12 mg oxolinic acid (OA)/kg fish/day for 7 uninterrupted days against *Streptococcus agalactiae* LCR1 (Sa) infection and its influence on histopathological anomalies in the forebrain, optic tectum region of the mid-brain and granular cell layer of the cerebellum of *Oreochromis niloticus*. Besides, attempts were made to understand how the OA impacts the glycosyltransferases and CAMP factor of *S. agalactiae* through molecular docking. The LD_{50} of Sa was 1.26×10^8 cells/fish. Sa infection was apparent in OA-treated and untreated groups, and the brain tissues exhibited the progression and reversal of meningitis. The forebrain exhibited thickening of the *meninx primitiva*, vacuolation, and degeneration in the brain parenchyma and *meninx primitiva*. Inflammatory changes such as meningitis and mononuclear cell infiltration were documented. The midbrain had edematous optic tectum, loosening of connective tissue, and leukocyte infiltration. In the granular cell layer, cerebellum changes such as spongiform encephalopathy and necrosis indicate region-specific damage. However, the OA effectively reduced the severity of brain tissue damage, possibly by its binding affinities and upsetting the activities of glycosyltransferases and CAMP factor, as confirmed by molecular docking. These results confirmed that OA can cross the blood-brain barrier and interact with virulence proteins to reduce the Sa infection in the brain. Furthermore, the results underscore its responsible use in aquaculture, as OA is a critically important human medicine and ought to be used as a secondary treatment.

Keywords: Aquaculture, streptococcosis, virulence proteins, antibiotic therapy, meningitis, histoarchitecture.

Introduction

Aquaculture is swiftly intensifying, significantly contributing to global food production, economic development, and nutritional security. In 2022, aquaculture contributed about 59% of the total fish production of 185.4 million tonnes. Cichlids, including tilapias, ranked third in the major farmed fish group with a contribution of 10.6% [1]. Tilapias are highly favourable species for aquaculture due to their adaptability, rapid growth, disease resistance, and low production costs [2]. However, intensive tilapia aquaculture has led to a rise in diseases, particularly bacterial infections like streptococcosis, motile *Aeromonas* septicemia (MAS), vibriosis, and columnaris, which pose significant challenges to farming [3]. Streptococcosis, caused by *Streptococcus agalactiae* (Sa) and *S. iniae*, is one of the most important bacterial diseases in tilapia aquaculture, causing substantial economic losses globally [2; 4]. It can lead to meningoencephalitis in tilapia, characterised by several neurological disorders and brain lesions [5; 6; 7; 8]. Studies indicated that Sa causes extensive brain damage, particularly in regions controlling swimming activities, due to bacterial invasion and acute inflammatory responses [6; 8; 9]. Control measures for streptococcosis include proper farm location selection, good aquaculture practices, antibiotics, immunostimulants, and vaccines [2]. Antimicrobials like amphenicols, quinolones, and tetracyclines are widely used, but their use raises

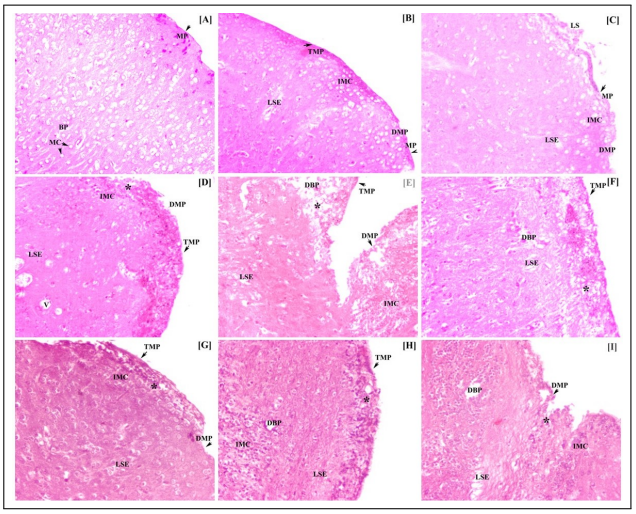

Figure 1. The histopathological changes indicating meningitis (*) in the forebrain of *Streptococcus agalactiae* LCR1 challenged and oxolinic acid (OA) treated and untreated *Oreochromis niloticus* juveniles. [A] Control, [B] Day post-injection (DPI) 1 OA treated and [C] untreated; [D] DPI 7 OA treated and [E] untreated; [F] DPI 14 OA treated and [G] untreated; and [H] DPI 21 OA treated and [I] untreated. DMP: Damage of meninx primitiva; FH: Focal haemorrhage; IMC: Infiltration of mononuclear cells; LS: Lifting of superficial layer; LSE: Localized spongiform encephalopathy; MP: *Meninx primitiva* (meningitis); TMP: Thickening of *meninx primitiva*; DBP: Degeneration of brain parenchyma; and V: Vacuolation x200, H&E staining.

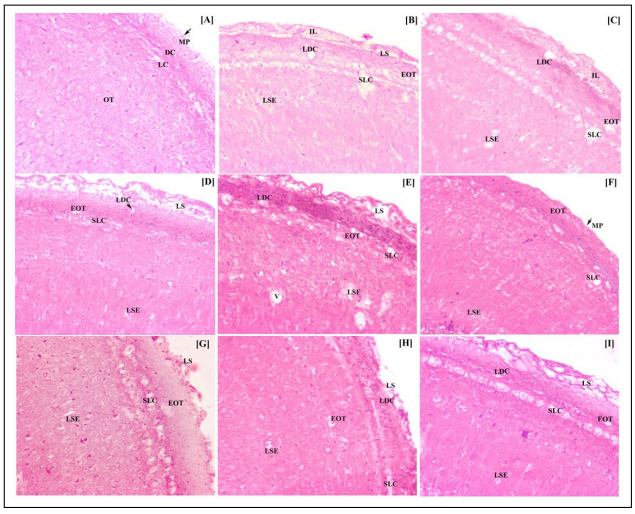

Figure 2. The histopathological changes in the optic tectum (OT) region of the brain of *Streptococcus agalactiae* LCR1 challenged and oxolinic acid (OA) treated and untreated *Oreochromis niloticus* juveniles. [A] Control, [B] Day post-injection (DPI) 1 OA treated and [C] untreated; [D] DPI 7 OA treated and [E] untreated; [F] DPI 14 OA treated and [G] untreated; and [H] DPI 21 OA treated and [I] untreated. EOT: Edematous optic tectum; IL: Infiltration of leucocytes; IMC: Infiltration of mononuclear cells; LDC: Loosened dense connective tissue; LS: Lifting of superficial layer; LSE: Localized spongiform encephalopathy; MP: *Meninx primitiva*; SLC: Spacing in loose connective tissue; TMP: Thickening of *meninx primitiva*; and V: Vacuolation x200, H&E staining.

concerns about antimicrobial resistance (AMR), particularly in regions like China and India [10; 11; 12; 13].

The quinolone antibiotic, oxolinic acid (OA) is not on the list of approved drugs for use in finfish production by the Food and Drug Administration in the United States [14]. It develops resistant bacterial strains that may threaten human health [10; 15]. However, the European Union, Japan, and other Asian countries have recommended it to prevent bacterial infections at 12 mg/kg fish/day for 7 days [11; 16]. The antibiotics of the quinolone family are categorized as critically important and the highest priority antimicrobial group for human use. Such medicines should not be used to treat infectious diseases in food-producing animals. Yet, these medicines are proposed for a second-line treatment in food-producing animals, complying with the national legislation in force, when no other alternatives are available [15; 17; 18].

Several studies reported the effectiveness of OA in controlling fish bacterial pathogens and the onset of diseases [19; 20; 21; 22]. However, more research is needed to evaluate its mode of action, safety in tropical fish species, and efficacy against several mesophilic fish pathogens to ensure sustainable aquaculture practices [23]. The infection of Sa relies on several virulence factors, which encompass adhesion, invasion, and infection. Numerous virulence factors contribute to invasion and colonisation [24; 25]. The relationship between antibiotics, virulence proteins, and the ability of bacteria to infect the brain is complex, involving factors like the blood-brain barrier (BBB) and host immune responses. For example, glycosyltransferases and the CAMP factor play crucial roles in pathogenesis, as highly pathogenic strains of Sa reportedly produce glycosylated serine-rich repeats and glycosyltransferases [26; 27]. The extracellular protein, such as CAMP factor, induces pore formation in target cells and heightens the pathogenicity of Sa [24; 25; 28].

However, there is a lack of scientific literature focusing on the specific effects of aquaculture antibiotics on brain-infecting bacteria, such as Sa, and their virulence proteins. Also, the misuse of antibiotics can lead to the emergence of AMR, which is a significant public health concern [10; 15]. Therefore, to combat Sa infections, understanding the roles of important virulent proteins and their interaction with antibiotics is essential. This study delved into the efficacy of OA, an antibiotic recommended for second-line treatment in food-producing animals [18], to curtail the effect of Sa-infection on the brain histoarchitecture of Nile tilapia *Oreochromis niloticus*, and to comprehend how OA impacts the invasion of Sa into the fish brain by molecular docking

using glycosyltransferases and CAMP factor, to plan for further course of research and specific mitigation strategies.

Materials and methods

Experimental fish and bacterial strain and its pathogenicity

Farm-raised young and healthy *Oreochromis niloticus* were chosen regardless of sex and adapted in circular tanks of 500 L capacity for 15 days before the experimentation [23]. *Streptococcus agalactiae* LCR1 (National Centre for Biotechnology Information (NCBI) accession number OP752129) was obtained from the State Referral Laboratory for Aquatic Animal Health, Madhavaram, Chennai, India. Following earlier descriptions, the bacterium was assessed for its pathogenicity against *O. niloticus* (n = 10 for each challenge dose from 10^5 to 10^9 and control, in triplicate) by intramuscular challenge [29; 30]. The mean lethal dose (LD_{50}) was calculated from the mortality data using the method established by Reed and Muench [31]. Using the broth dilution method, the minimum inhibitory concentration (MIC) of OA was calculated [32]. Before the challenge, inocula from the kidney of healthy tilapia (n = 2) were plated onto brain heart infusion agar (BHIA) to ascertain that the stocks were disease-free [5].

Efficacy of oxolinic acid against *Streptococcus agalactiae* infection

The European Medicines Evaluation Agency (EMEA) recommended a dose of 12 mg OA/kg fish/day for 7 uninterrupted days to control bacterial infection in fish [16]. The medicated feed to feed the experimental fish at 2% body weight (BW) was set by emulsifying 0.6 g of OA powder (O0877-25G; CAS-No: 14698-29-4; Sigma-Aldrich, India) in 5 mL vegetable oil. This emulsion was then top-coated onto the commercial floating pellet feed (1 kg) and admixed thoroughly. Correspondingly, a control feed with binder but without OA was prepared [21]. The analysis of OA in medicated feed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed 96 ± 1% of the incorporated dose [23]. The efficacy study utilized *O. niloticus* weighing 17.56 ± 0.89 g (n = 225) and distributed randomly in nine thoroughly cleaned polypropylene tanks (L58 × H45 × B45 cm). The rearing water (80 L) in each tank was conditioned for three days, stocked with 25 accustomed tilapias, and properly covered and marked. About half of the water was exchanged to remove feed and faecal wastes. The fish stocks were divided into three groups, in triplicate. The control, designated as group 1, received a 0.1 mL saline injection intramuscularly at the dorsal fin base. All fish of groups 2 and 3 were injected intramuscularly with *S. agalactiae* LCR1 at 1.32×10^7 cells/fish. Post-injection, the fish were placed in their respective tanks. Control feed was offered to groups 1 and 3 throughout the experimental period of 21 days. OA-medicated feed at 2% BW was served to group 2 continuously for 7 days post-injection (DPI) and then switched to a control feed [21; 23]. Water quality was continuously monitored, and any leftover feed, if any, was removed 1 hour after each feeding. Observations on mortality, infection symptoms, and behaviour were recorded daily. Inocula from the freshly dead Sa-challenged tilapia brain were plated onto BHIA to establish Sa-infection [5].

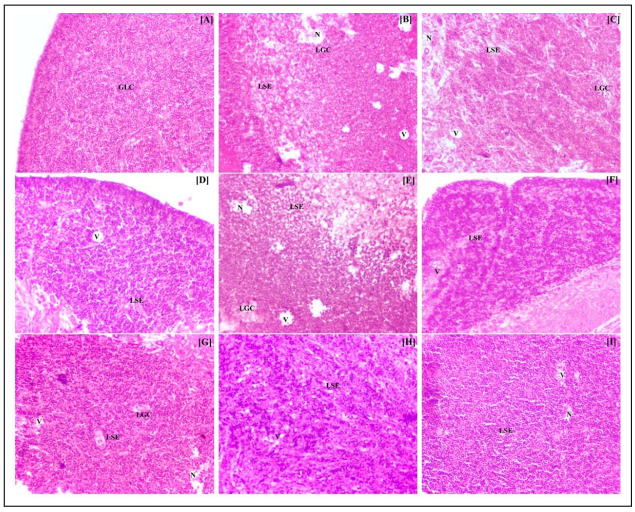

Figure 3. The histopathological changes in the granular cell layer of the cerebellum (GIC) of the brain of *Streptococcus agalactiae* LCR1 challenged and oxolinic acid (OA) treated and untreated *Oreochromis niloticus* juveniles. [A] Control, [B] Day post-injection (DPI) 1 OA treated and [C] untreated; [D] DPI 7 OA treated and [E] untreated; [F] DPI 14 OA treated and [G] untreated; and [H] DPI 21 OA treated and [I] untreated. LSE: Localized spongiform encephalopathy; V: Vacuolation; LGC: Lysis of granular cells of the cerebellum; and N: Necrotised area x200, H&E staining.

Histopathology and evaluation of pathological anomalies

On 0, 1, 7, 14, and 21 DPI, two fish were randomly sampled from each replicate tank, euthanized by adding clove oil at 100 μL/L, and dissected prudently. The cranial bone was removed carefully to expose the brain. The precisely collected whole-brain tissue samples were fixed for 24 hours in Bouin's fixative, processed, and embedded in paraffin wax. Sections of 5 μm thickness were sliced, processed, and double-stained using hematoxylin and eosin [33]. The brain sections were examined under an Olympus microscope (Model: BX51) at 20× magnification to identify abnormalities in tissue arrangement. For quantitative analysis, images were captured and processed using an SCOLUX camera (16 MP) and ToupTek ToupView software (Version x64).

The histopathological abnormalities were evaluated based on circulatory, progressive, regressive, and inflammatory changes. Following the observations, reaction indices (RI) were calculated as a product of the score value and the importance factor. The score value, ranging from 0 to 6, was assigned based on the severity and degree of damage: no change (0), mild occurrence (2), moderate occurrence (4), and severe occurrence (6). The importance factor, ranging from 1 to 3, was assigned based on the pathological significance of each alteration, categorized 1: minimal pathological importance, easily reversible post-stressor

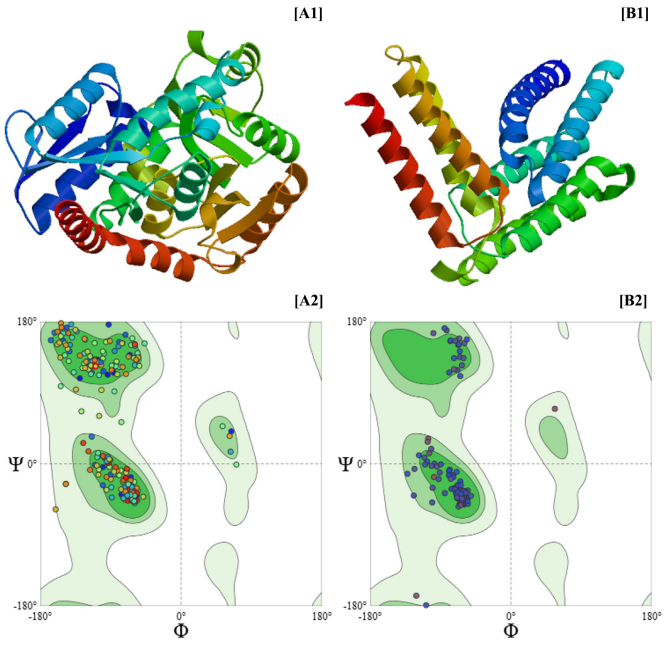

Figure 4. Homology modelled 3D structures [A1 and B1] and Ramachandran plots [A2 and B2] of glycosyltransferase (AAM99596) and CAMP factor (AKI96324) of *Streptococcus agalactiae*, respectively, computed using SWISS-MODEL.

administration, 2: moderate pathological importance, reversible if the stressor is neutralized, 3: marked pathological importance, generally irreversible with potential loss of organ function. For instance, for mild vacuolation, which has a pathological importance factor of 2, and an observed severity score of 1.65 depending on damage intensity, the RI would be 3.30 (i.e., 1.65×2). Alterations with a higher RI value signified greater severity [34]. The qualitative scores of the brain histological changes, expressed as mean ± standard deviation of six observations, were analysed by a non-parametric Kruskal–Wallis test. The Mann–Whitney U test was conducted for pairwise comparisons in IBM-SPSS Version 22.0 at a significance level of $P < 0.05$.

In silico and molecular docking of *Streptococcus agalactiae* virulence factors

Protein and ligand modelling for docking studies

The virulence proteins of *S. agalactiae*, such as the glycosyltransferase enzyme encoded by the *iagA* gene involved in BBB invasion, and an invasin protein, CAMP factor [24; 25; 27; 28], were retrieved from the NCBI database for further detailed analysis and understanding. The corresponding FASTA protein sequences were subsequently subjected to homology modelling using the ExPASy SWISS-MODEL server. Oxolinic acid (OA) was considered the ligand, and its 3D structure was obtained from the PubChem database in `.sdf` format and then conveniently converted to `.pdb` format using Open Babel software.

Molecular docking

The molecular docking and interactions between OA and the target virulence proteins of *S. agalactiae* were analysed using MGL Tools and AutoDock Tools 1.5.7 [35], employing a grid box size of 126 Å in the X, Y, and Z dimensions, and the Lamarckian Genetic Algorithm for docking simulations. The docking results and molecular interactions were visualised using PyMOL Edu version 2.5. The docking score, representing the interaction energy with appropriate scaling factors, was used to assess the binding affinity of the ligand. Furthermore, specific amino acid interactions between the ligand and target proteins were computed and outlined. Lower binding free energy may indicate stronger ligand-protein interaction, while hydrogen bond strength may categorise donoracceptor distances as strong (2.2–2.5 Å; mostly covalent), moderate (2.5–3.2 Å; mostly electrostatic), and weak (3.2–4.0 Å; electrostatic), as per Jeffrey [36].

Results

Brain histopathology and evaluation of pathological anomalies

The LD_{50} of *S. agalactiae* and the MIC of oxolinic acid (OA) were 1.26×10^8 cells/fish and 12.50 μg/mL, respectively. In the forebrain of the control fish, the tissue structure was normal, with a well-organized epithelial layer, the *meninx primitiva*, and mononuclear cells present in the brain parenchyma (**Figure 1 A**). The histopathological analyses of OA-treated and untreated *S. agalactiae* challenged *O. niloticus* revealed three types of reaction patterns: progressive, regressive, and inflammatory (**Figure 1 B–I**; **Table 1**). The identified progressive change was thickening of the *meninx primitiva*, while regressive changes included superficial layer lifting, localized spongiform encephalopathy, vacuolation, and degeneration of the *meninx primitiva* and brain parenchyma. Inflammatory changes were characterized by meningitis and mononuclear cell infiltration. On DPI 1, both groups displayed mild progressive, regressive, and inflammatory changes, including *meninx primitiva* thickening with high RI values. The damage, particularly regressive and inflammatory changes, intensified on DPI 7. The OA-treated group showed reduced regressive changes and evidence of healing in the brain parenchyma, whereas the untreated group experienced significant damage on DPI 14. By DPI 21, the OA-treated group was nearly healed except for mild meningitis, while the untreated group continued to exhibit moderate brain damage.

In the midbrain, the normal structure consisted of an outer *meninx primitiva* and underlying connective tissue above the optic tectum (**Figure 2 A**). The histopathological changes included circulatory alterations such as edematous optic tectum, regressive changes like superficial layer lifting, loosening of connective tissue, and vacuolation, as well as inflammatory changes such as mononuclear cell infiltration (**Figure 2 B–I**; **Table 1**). On DPI 1, both groups exhibited mild circulatory, inflammatory, and regressive changes. On DPI 7, circulatory damage peaked in the OA-treated group, while regressive changes worsened in the untreated group. A gradual reduction in damage over time was noted in the

Table 1. Reaction indices* based on the major histopathological changes in the brain of *Streptococcus agalactiae* LCR1 challenged and oxolinic acid (OA)-fed *Oreochromis niloticus* juveniles.

Reaction pattern	Histopathological changes	IF	DPI 1		DPI 7		DPI 14		DPI 21	
			OA treated	Untreated	OA treated	Untreated	OA treated	Untreated	OA treated	Untreated
Forebrain										
Progressive	Thickening of MP	1	0.80 ± 0.17^{1ab}	1.02 ± 0.55^{1ab}	1.06 ± 0.27^{1a}	1.12 ± 0.21^{1A}	0.57 ± 0.25^{1b}	0.72 ± 0.30^{1AB}	0.27 ± 0.12^{1c}	0.57 ± 0.10^{2B}
Regressive	Lifting of SL	1	0.52 ± 0.45^{1ab}	1.07 ± 0.53^{1A}	0.62 ± 0.24^{1a}	0.98 ± 0.37^{1A}	0.25 ± 0.12^{1ab}	1.05 ± 0.43^{2A}	0.23 ± 0.05^{1b}	0.47 ± 0.19^{2B}
	LSE	2	2.10 ± 1.18^{1ab}	1.67 ± 1.54^{1A}	2.60 ± 0.49^{1a}	3.80 ± 1.21^{1B}	1.07 ± 0.21^{1bc}	1.63 ± 0.39^{2A}	0.83 ± 0.46^{1c}	1.27 ± 0.39^{1A}
	Vacuolation	2	1.93 ± 0.64^{1ab}	1.13 ± 0.63^{1A}	1.93 ± 0.65^{1a}	3.47 ± 1.04^{2B}	1.03 ± 0.59^{1ab}	1.73 ± 0.62^{1A}	0.67 ± 0.33^{1b}	1.33 ± 0.55^{2A}
	Damaged MP	3	3.05 ± 1.26^{1ab}	2.95 ± 1.22^{1AB}	3.05 ± 0.48^{1a}	5.55 ± 2.54^{1A}	1.70 ± 0.92^{1ab}	3.55 ± 1.72^{1AB}	1.00 ± 0.49^{1b}	2.20 ± 0.62^{2B}
	Degeneration of BP	3	3.00 ± 1.00^{1ab}	1.90 ± 1.38^{1A}	3.00 ± 0.80^{1b}	5.70 ± 1.61^{2B}	1.05 ± 1.32^{1a}	3.15 ± 0.73^{2C}	0.75 ± 0.37^{1a}	1.90 ± 0.82^{2A}
Inflammatory	Meningitis	2	3.57 ± 0.41^{1a}	1.70 ± 1.27^{1A}	3.57 ± 1.24^{1b}	3.90 ± 1.05^{1B}	1.13 ± 0.39^{1a}	2.03 ± 0.96^{1A}	0.70 ± 0.35^{1a}	1.43 ± 0.37^{2A}
	Infiltration of MNC	2	2.17 ± 0.35^{1a}	1.23 ± 0.73^{1A}	2.17 ± 0.60^{1b}	3.10 ± 1.16^{1B}	0.67 ± 0.43^{1c}	1.50 ± 0.90^{1A}	0.43 ± 0.39^{1c}	1.47 ± 0.352^{A}
Mid-brain (Optic tectum)										
Circulatory	Edematous OT	1	0.70 ± 0.17^{1a}	0.72 ± 0.17^{1A}	0.73 ± 0.21^{1a}	1.42 ± 0.18^{2B}	0.73 ± 0.16^{1a}	1.00 ± 0.24^{1C}	0.37 ± 0.14^{1b}	0.55 ± 0.18^{1A}
Regressive	Lifting of SL	1	0.80 ± 0.13^{1a}	0.70 ± 0.23^{1A}	0.68 ± 0.17^{1a}	1.50 ± 0.37^{2B}	0.43 ± 0.23^{1b}	1.02 ± 0.28^{2C}	0.38 ± 0.12^{1b}	0.75 ± 0.10^{2A}
	Loosening of DCT	1	0.83 ± 0.08^{1a}	0.72 ± 0.12^{1A}	0.67 ± 0.15^{1a}	1.18 ± 0.33^{2B}	0.75 ± 0.21^{1a}	1.10 ± 0.32^{1AB}	0.47 ± 0.10^{1b}	0.72 ± 0.18^{2A}
	Spacing of LCT	1	0.85 ± 0.21^{1a}	0.85 ± 0.14^{1A}	0.73 ± 0.26^{1ab}	1.43 ± 0.59^{2B}	0.65 ± 0.14^{1ab}	1.15 ± 0.34^{2B}	0.47 ± 0.18^{1b}	0.83 ± 0.18^{2A}
	Vacuolation	2	1.30 ± 0.58^{1a}	1.60 ± 0.28^{1A}	1.50 ± 0.45^{1a}	3.13 ± 0.70^{2B}	1.30 ± 0.45^{1a}	2.50 ± 1.062^{AB}	0.97 ± 0.23^{1a}	1.13 ± 0.24^{1C}
Inflammatory	Infiltration of LC	2	1.73 ± 0.24^{1a}	1.63 ± 0.29^{1ab}	1.27 ± 0.45^{1ab}	1.30 ± 0.68^{1AB}	0.83 ± 0.61^{1b}	1.73 ± 0.33^{2A}	0.57 ± 0.15^{1b}	1.03 ± 0.23^{2B}
Cerebellum (Granular cell layer)										
Regressive	LSE	2	2.57 ± 0.23^{1a}	2.40 ± 0.18^{1A}	2.20 ± 0.33^{1a}	4.20 ± 0.33^{2B}	0.47 ± 0.10^{1b}	1.93 ± 0.53^{2A}	0.90 ± 0.28^{1c}	1.30 ± 0.28^{2C}
	Lysis of GC	3	3.75 ± 0.31^{1a}	3.70 ± 0.24^{1A}	2.30 ± 0.59^{1b}	5.25 ± 0.68^{2B}	0.65 ± 0.12^{1c}	3.10 ± 1.21^{2A}	1.25 ± 0.35^{1d}	1.80 ± 0.66^{1C}
	Necrotized area	3	5.45 ± 0.58^{1a}	5.30 ± 0.41^{1A}	1.85 ± 0.58^{1b}	5.45 ± 0.96^{2A}	1.35 ± 0.16^{1b}	3.25 ± 1.36^{2B}	1.35 ± 0.31^{1b}	1.80 ± 0.42^{1C}

* As per the descriptions of Bernet et al. (1999). No changes were noted in the control group. 1-2: Values sharing a common numerical superscript for a specific row among the treatment groups for a specific day differed insignificantly (P>0.05). a-d: Values sharing a common alphabetical superscript within a row for the treated group on different DPI differed insignificantly (P>0.05). A-C: Values sharing a common alphabetical superscript within a row for the untreated group on different DPI differed insignificantly (P>0.05). DPI: Day post-injection. IF: Importance factor; MP: *Meninx primitiva*; SL: Superficial layer; LSE: Localized spongiform encephalopathy; BP: Brain parenchyma; MNC: Mononuclear cells; OT: Optic tectum; DCT: Loosened dense connective tissue; LCT: Loose connective tissue; LC: Leucocytes; GC: Granular cells of the cerebellum.

OA-treated group, whereas the untreated group presented more severe and persistent damage. By DPI 21, both groups showed decreasing damage, with near-normalization of architecture in the OA-treated group. In the granular cell layer of the cerebellum, both groups experienced regressive changes, including spongiform encephalopathy with vacuolation and necrosis, which decreased with time. On DPI 21, the treated group displayed minimal vacuolation, while the untreated group showed moderate damage (**Figure 3 B–I**; **Table 1**). Overall, the OA-treated group exhibited less damage, improved recovery, and reduced mortalities (26%; $P > 0.05$) compared to the untreated group (32%).

Homology modelling and molecular docking

Using the SWISS-MODEL server, three-dimensional (3D) models of glycosyltransferase and CAMP factor from *S. agalactiae* were constructed based on sequence similarity with identified structural templates. It is generally important to note, in a rather typical and somewhat broadly consistent and commonly recognised manner, that this modelling approach essentially follows a broadly conventional and widely accepted procedure overall. The models generated from templates with the highest sequence identity are presented in **(Figure 4 A1, B1)**. This approach, in many ways, essentially leverages the inherent structural similarity among proteins of the same family, which usually tend to share broadly comparable and functionally related three-dimensional architectures. The outcomes of the homology modelling are, more or less, conveniently summarised in **(Table 2)**. The Global Model Quality Estimation (GMQE) and Qualitative Model Energy Analysis (QMEAN) scores were high, corresponding to higher sequence identity with templates. The generated models exhibited optimal quality, as validated by SWISS-MODEL, with MolProbity scores above 0.66, and were considered excellent. Nearly 98% of residues were located in the favoured regions of the Ramachandran plot (**Figure 4 A2, B2**). The ligand OA exhibited notable binding affinities toward both glycosyltransferase and CAMP factor, with docking scores of –4.99 and –4.31 kcal/mol, respectively. These interactions were supported, in a fairly straightforward and somewhat broadly descriptive and methodologically consistent manner, by multiple hydrogen bonds and close-contact residues, suggesting generally stable and favourable binding conformations (**Table 3**; **Figure 5 A, B**).

Table 2. Homology modelling data of glycosyltransferase AAM99596 and CAMP factor AKI96324 of *Streptococcus agalactiae* computed using SWISS MODEL.

Characteristics	Glycosyltransferase AAM99596	CAMP factor AKI96324
Template ID	A0A4V0HA53.1.A	5h6i.1.A
Sequence identity (%)	71.69	100
QMEAN	0.90	0.86
GMQE	0.96	0.80
MolProbity score	0.91	0.70
Ramachandran plot – Favoured region (%)	96.97	98.12
Ramachandran plot – Outlier region (%)	0.00	0.00

QMEAN: Qualitative Model Energy Analysis; GMQE: Global Model Quality Estimation.

Discussions

In terms of virulence, the Sa strain, isolated from the kidney of Asian seabass *Lates calcarifer*, was borderline avirulent to weakly virulent [7; 37; 38] with a moderately high MIC for OA. Sa affects the brain using several virulence factors to allow the infection process [4; 38; 39]. In this study, Sa resulted in systemic pathological alterations, including inflammation, haemorrhages, and meningitis in challenged fish, as in previous research [5; 6; 7; 8; 9]. The Sa-infected fish also exhibited aberrant swimming, marked by circular movement and C-shaped body bending. It could be due to the possible neurological problem, as the midbrain reportedly controls fish swimming by transmitting axons to innervate the spinal cord's primary and secondary motor neurons [9; 40]. Further, abnormal swimming behaviours due to streptococcal infections were linked to brain fluid accumulation [7]. In this work, we isolated Sa from the brain tissues of challenged tilapias on BHIA, indicating a breach of the BBB. Sa employ various strategies to survive in the bloodstream, colonize brain vasculature, and cross the BBB [41]. The results indicated that the Sa can enter the brain parenchyma by destroying the host BBB and causing localized spongiform encephalopathy. Several earlier studies confirmed the establishment of Sa [6; 8; 42] or biofilm formation [43] in fish brains. When the bacteria enter the circulatory system, they survive within macrophages, allowing them to evade the immune system and spread to the central nervous system (CNS) [8; 44]. Such macrophages reportedly act as a vehicle for Sa, allowing it to cross the BBB and gain access to the CNS [6; 8].

The current investigation revealed progressive alterations such as thickening of the *meninx primitiva*, suggesting bacterial meningitis similar to earlier studies [5; 6; 7; 9]. Regressive changes in the forebrain, midbrain, and cerebellum of the current study were in line with the earlier findings. They reported that bacteria can permeate brain parenchymal regions and cause localised spongiform encephalopathy, characterised by focal haemorrhages and vacuolization, mostly in the midbrain and hindbrain. Also, Gram-positive cocci in the granular cell layer were observed, causing lysis of granular cells, and brain lesions, including haemorrhages, neuronal necrosis, and inflammation in regions governing swimming in tilapia [9]. Sa infection also disrupted cerebral bioenergetics by impairing mitochondrial complex IV and creatine kinase activities, leading to energy imbalance [45]. All these investigations pointed out that Sa is neurotropic, seriously harming neurological function and influencing swimming behaviour and survival of fish. The inflammatory responses in the forebrain and mid-brain of Sa-infected tilapias corroborated earlier studies [6; 7; 8; 9; 46], which documented meningitis and several significant brain alterations. Sa reportedly triggers an inflammatory response, which impairs phagocytosis, cytokine and chemokine levels, contributing to oxidative stress and disruption of the BBB [45; 47] and interfering with the immune system [48]. The resulting neuroinflammation can be characterized by immune cell migration and the production of pro-inflammatory cytokines, which drive the immune response [6].

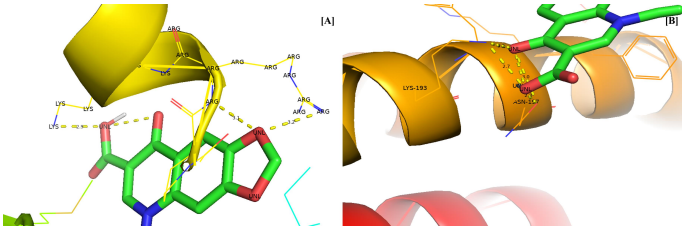

Figure 5. Protein–ligand interaction of *Streptococcus agalactiae* virulence factors: [A] glycosyltransferase and [B] CAMP factor against oxolinic acid, visualised using the PyMOL tool. Note: green structures represent ligands; coloured ribbons depict the target protein; yellow dotted lines indicate interaction sites. Distances between the ligand and target at the interaction sites are measured in angstroms (Å).

Antibiotics like ampicillin, colistin sulfate, sulfadimethoxine-ormetoprim, and florfenicol are effectively used against *Streptococcus* spp. Moreover, the use of β-lactams, fluoroquinolones and macrolides has been highlighted as effective against meningitis-causing *S. pneumoniae* [49; 50; 51; 46; 52]. In this study, the RI values increased during the initial period of infection and

Table 3. Molecular docking summary of glycosyltransferase AAM99596 and CAMP factor AKI96324 of *Streptococcus agalactiae* against the ligand oxolinic acid using the AutoDock tool.

Proteins	Affinity	Ligand–Protein interaction	Distance (Å)
Glycosyltransferase	-4.99	A:ASNÌ97:OD1–:UNLÌ:H	2.3
		A:LYSÌ93:NZ–:UNLÌ:O	2.7
		A:ASNÌ97:OD1–:UNLÌ:O	3.0
		A:LYSÌ93:NZ–:UNLÌ:O	3.2
CAMP factor	-4.31	A:LYSȊ05:NZ–:UNLÌ:O	2.9
		A:ARGȊ04:N–:UNLÌ:O	3.1
		A:ARGȊ04:NH1–:UNLÌ:O	3.2

gradually reduced with time, indicating the fish's adaptive and immune responses to healing the brain lesions. While the effects were almost similar, the OA-treated tilapia showed lower RI. The OA-treatment facilitated quicker recovery, indicating that OA can cross the BBB and lessen the Sa-infection. The glycosyltransferase and the CAMP factor play crucial roles in the pathogenesis of Sa. The infection may influence bacterial interactions with the host, create specific pores in host membranes, modify proteins and carbohydrates, and disrupt cell integrity, leading to cell lysis [24; 53]. The lesser impact of Sa-infection in the brain suggested that the OA can exhibit binding affinities with virulent proteins, as confirmed by interactions with glycosyltransferases and the CAMP factor, and to prevent the activities of Sa. In the molecular docking study, our observations on the shorter bond distances and number of interactions with multiple hydrogen bonds and close-contact residues suggested stable and favourable binding conformations. These results supported the potential of OA as a strong inhibitor of the virulent proteins of Sa. Our study further noted an insignificant difference in mortalities due to the tested strains moderately high MIC for OA. Strikingly, the 7 days of treatment at 12 mg OA/kg fish/day inhibited the Sa strain, mitigated the severity of brain damage, and improved fish survival. Thus, these biologically meaningful results proved the protective effect of OA against the progression of Sa-infection and ensuing brain histopathological changes. However, future studies are warranted on the expression of glycosyltransferase, CAMP factor and other virulence factors using qRT-PCR or Western blotting to provide direct evidence of their involvement in Sa pathogenesis and OAs mechanism of action.

Conclusions

Our results demonstrated that understanding the role of virulence proteins of *S. agalactiae* in pathogenesis and interactions with drugs can offer scope for new therapeutic strategies, such as targeting such factors or blocking interactions with host cells and developing targeted therapies that can disrupt the bacterial toxin's activity. In this study, oxolinic acid (OA) therapy in *S. agalactiae*-infected tilapia revealed a protective effect by reducing the mortalities, brain damage and histopathological alterations. The treated fish unveiled less damage in the forebrain, midbrain, and cerebellum, with tissue architecture almost normalized on DPI 21. The results implied that OA is efficacious and can be used in treating tilapia against *S. agalactiae* infection. However, the World Health Organisation stated that quinolones, including OA, are critically important human medicines, and they should at no time be used in animals produced for food as a primary treatment agent. Based on the results, we advocate the use of OA as a therapeutic agent for second-line treatment in food-producing animals following the national and international criteria. Yet, a prudent and responsible use of antibiotics, targeted therapeutic applications for treating *S. agalactiae*-induced meningitis in fish and implementing long-term AMR and residue surveillance to safeguard public health are obligatory.

Data Availability Statement

All relevant data are within the paper and supplementary materials.

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Competing interests

The authors declare that they have no competing interests.

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