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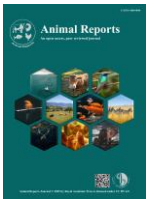

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Potential utilization of Streptomyces extract as an antibacterial, anti-inflammatory and immune stimulant in *Aeromonas hydrophila*-infected Nile tilapia, *Oreochromis niloticus*

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Abstract:
Frequent disease outbreaks in the aquaculture sector have increased with the widespread multiresistant bacterial strains as a result of overuse or misuse of antibiotics. Such diseases can lead to substantial economic losses and threaten the sustainability of aquaculture. *Streptomycetes sp.* is a genus of bacteria that produces numerous secondary metabolites with high potential as antimicrobial substances. Therefore, the present study aimed to evaluate the use of *Streptomyces coeruleorubidus* extract (SSM) on survival, immune, and inflammatory responses of Nile tilapia infected with *Aeromonas hydrophlia*. SSM was extracted from the bacterial media using ethyl acetate, then concentrated using a rotary evaporator, and injected into fish infected with or without *A. hydrophlia.* The results indicate that infected fish have a lower survival rate and experience low plasma levels of immune components such as IgM, lysozyme, and complement 3 levels. SSM treatment improved the survival rate of infected fish and stimulated immune responses in healthy or infected groups. The relative expression of pro-inflammatory cytokines was significantly increased by infection; meanwhile, anti-inflammatory cytokines were down-regulated. Treatment with SSM significantly regulated the expression of inflammatory cytokines. SSM revealed the potential to stimulate the immune response and improve the survival rate against pathogenic bacterial infection. It can be used and commercialized as an antibacterial agent and an alternative to antibiotics in aquaculture. Future studies could apply these metabolites in human applications; however, more studies are needed to study these metabolites and identify their composition.

Keywords: Streptomyces, novel antimicrobials, inflammation, immune stimulants, Nile tilapia.

1 Introduction

Antimicrobial resistance is a flourishing challenge with global attention and needs immediate action to stop the blooming of multidrug resistance , whereas, it led to more than one million global

deaths in 2019 and contributed to around five million deaths (Talebi et al., 2019; Gupta & Sharma, 2022). Anti-microbial resistance is the ability of bacteria and other micro-organisms to withstand antibiotics, particularly due to the overuse or misuse of antibiotics (Amábile-Cuevas, 2016).

Aquaculture sector, especially in intensive farming, antibiotics could be unsystematically used at sub-therapeutic levels for long periods that may lead to the development of emerging multi resistance bacteria and stimulates horizontal resistant gene transfer, which lead to increases of disease outbreaks (Santos & Ramos, 2018). These diseases can lead to substantial economic losses and threaten the sustainability of aquaculture, food security, and human health (Del Carratore et al., 2022). The uncontrolled use of antibiotics could also lead to the accumulation of antibiotic residues in fish and other aquaculture products (Okon et al., 2022). In aquatic environments, increasing concerns about antibiotic use and the rise of antibiotic resistance pathogens directed the attention towards the utilization of novel antimicrobials in aquaculture (Quinn et al., 2020; El-Houseiny et al., 2021).

The research for alternative antimicrobial agents included several microorganisms, animals, and plants as natural resources of pharmaceuticals (Tan et al., 2018). One of the most used microbes is Actinobacteria phylum as a supplier of medications (Bérdy, 2012), such as genus the streptomyces, which thought to produce 80% of the identified natural compounds (Subramani & Sipkema, 2019). Secondary metabolites are organic compounds formed by organisms that are not necessary for their growth or development but often play a pivotal role in ecological interactions, defense mechanisms, and other restricted purposes unlike primary metabolites essential for fundamental cellular functions. Secondary metabolites are generally produced in response to environmental stressors or to interact with other organisms (Tyc et al., 2017). Consequently, investigating actinobacteria strains from undiscovered environments could provide new biological active substances (Sivalingam et al., 2019).

Streptomyces coeruleorubidus is a soil that inhibiting bacterial strain (Kandula & Terli, 2013) that can produce large number of strong and unique active metabolites that were not previously discovered (Inglis et al., 1993). Some of the isolated products are cyclopeptides and anthraquinone dihydroxy derivatives (octasiloxane) which have complex architectures that enable it to have antiviral, antifungal, and antibacterial effects, and could be a potential therapeutic for resistant bacteria (Kumar et al., 2012; Wei et al., 2015). According to our knowledge, available information about the identification of new compounds from streptomyces with potential against multidrug resistant pathogens is still rare.

Nile Tilapia (*Oreachromis niloticus*) is a popular freshwater fish species that live in the tropics and subtropics regions and is known for its importance in aquaculture and fisheries around the world (El-Sayed, 2019). With more than 10 cultured tilapia species, Nile Tilapia contributes 74% of the total tilapia production in 2019, which reached six million mt (FAO, 2021). The success of Nile tilapia as an economic species could be due to its fast growth, acceptable conversion ratio, fed a low-price diet, easy to reproduce in captivity, and tolerate harsh water quality conditions (El‐Sayed & Fitzsimmons, 2023). However, under culture conditions the susceptibility to diseases of farmed tilapia increased particularly infectious diseases such as Aeromonas diseases. *A. hydrophila* is the main species of Aeromonas that causes septicaemic diseases and severe mortality in aquatic animals, it causes body ulceration, hemorrhages, tail and fin erosion, abdominal swelling, and bulging eyes (Dias et al., 2016; Hossain & Heo, 2021). Recent studies reported the high risk of *A. hydrophila* infection in aquaculture due to their diverse antibiotic

resistance and the presence of virulence genes that could increase their complications in aquaculture and public health (Ayoub et al., 2024). Therefore, the present study aimed to evaluate the use of *Streptomyces coeruleorubidus* extract (SSM) as a novel antimicrobial substance on survival, immune, and inflammatory responses of Nile tilapia infected with *A. hydrophlia*.

2 Materials and methods

2.1 Streptomyces coeruleorubidus culture

Streptomyces coeruleorubidus (GenBank accession number OP168352) was cultured in a starch nitrate broth media (28 ℃ and pH 7) for 4-6 days in 100 mL conical flask as a total culture. The broth media was then collected, melded, and the bacterial mycelium was removed from the via filtering with fabric using funnels to prepare cell-free culture broth. The broth media was centrifuged to segregate *S. coeruleorubidus* cell debris for 15 min at 3000 × g.

2.2 Extraction of metabolites

Ethyl acetate was used as a solvent in a 5:1 ratio to extract the suspension of metabolites acquired after filtration and centrifugation. For 24 hours, the extraction process was carried out in the refrigerator at 4 ℃. Using a separator funnel, the ethyl acetate layer was split apart and then filtered via filter paper. The obtained ethyl acetate fraction (crude extract of metabolites) was concentrated using a rotary evaporator to get the extract in powder form. The powder form of the metabolites extract was used as an antimicrobial agent in the fish experiment.

2.3 Experimental fish and culture technique

Apparently healthy 240 Nile tilapia fingerlings of 20.03 ± 0.2 g were obtained from a private fish farm. The fish were adapted to laboratory conditions for two weeks, while fed the control diet (protein content 30% and fat 12%). After adaptation, fish with similar initial size was randomly allotted to 12 fiberglass tanks (500 L) with triplicate tanks in each treatment at a stocking density of 20 fish/tank. During the trial, fish were fed the experimental diets three times a day at 8.00, 12.00 and 15.00 hrs at a rate of 3% of the weight. The water quality parameters were maintained in an acceptable range for tilapia [temperature (25 ℃), salinity (3 ppt), dissolved oxygen (5.5 mg/L), pH (7.8), total ammonia nitrogen (0.3 mg/L)]. The experimental protocol was approved by the ethics committee of King Faisal University with the approval number: KFU-2024-ETHICS2471.

Experimental design

The fish were divided into four experimental groups (Table 1), each in three replicates. The first group was the control without any treatment, the second group was treated with secondary metabolites of streptomyces (SSM) at a dose of 50 mg / kg of fish. The third group was infected with *Aeromonas hydrophila* 0.2 ml (1 × 107 CFU) by intraperitoneal injection (the 50% lethal dose (LD) was previously determined). The fourth group was treated with SSM and infected with *A. hydrophila* at the same levels. All groups were reared under the same conditions and monitored for two weeks.

Table 1: Experimental design.

Group 1	Control (without any treatment)
Group 2	Injected with SSM (50 mg/kg fish)
Group 3	Injected with *A. hydrophila* (1.5×10^7 CFU / ml)

Group 4 Injected with both SSM and *A. hydrophila*

2.4 Measured parameters

2.4.1 Survival rate

At the end of the experiment the survival rate was determined according to the following formula:

Survival rate = 100 *(No. of fish at the end of the experiment/ No. of the fish at the beginning)

The relative protection level (RPL) was calculated using the formula described (Newman & Majnarich, 1982):

RPL = 100 - (% of treatment mortality ÷ % of infected group mortality) × 100.

The reason of mortality was confirmed by re-isolation of A. hydrophila from dead fish.

2.4.2 Immune response

At the end of the experiment, fish were anesthetized with 50 mg L^{-1} clove oil, then blood samples were collected from the caudal blood vessels, without anticoagulant. Blood samples were centrifuged at 3000 × g for 10 min to separate serum. Serum samples were stored in 1.5 mL Eppendorf type at -20 ℃ until used in the biochemical analysis.

Immunoglobulin M (IgM) was determined in serum using ELISA kits (Cusabio Biotech Co. Ltd., Wuhan, China) according to the manufacturer's instructions. Serum lysozyme activity was measured using a turbidimetric method by *Micrococcus lysodeikticus* (Sigma-Aldrich) at 450 nm using an ELISA reader (Swain et al., 2007). Complement 3 (C3) concentration was determined using diagnostic kits (Spinreact Company, Santa Coloma, Spain) at a wavelength of 340 nm.

2.4.3 Immune-related gene expression

The fish were killed with an overdose of anesthesia (200 mg L^{-1} clove oil) and dissected to get head kidney, which was subjected to molecular investigation. Total RNA was isolated from 0.2 g of tissue using TRIzol reagent (Chomczynski, 1993). The RNA concentration was calculated spectrophotometrically through the 260/280 nm ratio. cDNA synthesis was conducted using QuantiTect Reverse Transcription kit (Qiagen, Germany), according to the following thermal cycle: 35 cycles of 94 ℃ for 40 s, 60 ℃ for 30 s, and 72 ℃ for 60 s. The expression of selected genes (Table 2) was analyzed by real-time qPCR on an ABI PRISM 7500 (Applied Biosystems) using SYBR Green PCR Core Reagents. The relative fold expression was determined by $2^{-\Delta\Delta Ct}$ (Ct: cycle threshold) technique was applied to ascertain the relative fold alterations in the transcription of target genes (Livak & Schmittgen, 2001).

Table 2: Primer sequences of tested immune genes.

Gene name	Primer sequences		NCBI accession no.	References
β-actin	F	TGACCTCACAGACTACCTCATG	XM_003455949.2	(Abarike et al., 2020)
	R	TGATGTCACGCACGATTTCC		
TNF-α	F	CCAGAAGCACTAAAGGCGAAGA	NM_001279533.1	(Standen et al., 2016)
	R	CCTTGGCTTTGCTGCTGATC		
IFN-γ	F	GAAACTTCTGCAGGGATTGG	NM_001287402.1	(Velázquez et al., 2017)
	R	CTCTGGATCTTGATTTCGGG		
IL-1β	F	TGGTGACTCTCCTGGTCTGA	XM_005457887.3	(Standen et al., 2016)
	R	GCACAACTTTATCGGCTTCCA		
IL-8	F	GCACTGCCGCTGCATTAAG	NM_001279704.1	(Wangkaghart et al., 2021)
	R	GCAGTGGGAGTTGGGAAGAA		
IL-10	F	CTGCTAGATCAGTCCGTCGAA	XM_013269189.3	(Standen et al., 2016)
	R	GCAGAACCGTGTCCAGGTAA		
TGF- β	F	GTTTGAACTTCGGCGGTACTG	NM_001311325.1	(Standen et al., 2016)
	R	TCCTGCTCATAGTCCCAGAGA		

TNF-α: tumor necrosis factor-alpha, IFN-γ: interferon- gamma, IL1-β: interleukin 1 beta, IL-8: interleukin-8, IL-10: interleukin-10, TGF-β: transforming growth factor-beta.

2.5 Statistical analysis

The data was statistically analyzed using SPSS 21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) and expressed as mean ± standard error. Data were subjected to one-way ANOVA followed by Tukeys multiple comparison test to identify statistical differences between treatments at a P value of 0.05.

3 Results:

3.1 Survival rate

The survival rate was averaged at 96.67-100% in the control and SSM groups, meanwhile, survival was significantly decreased to 53.33% in the infected group, this percent was significantly increased in the infected and treated group to 72.33%. Relative protection levels increased in the SEM treated group by 48.13 compared to the infected group (Fig. 1).

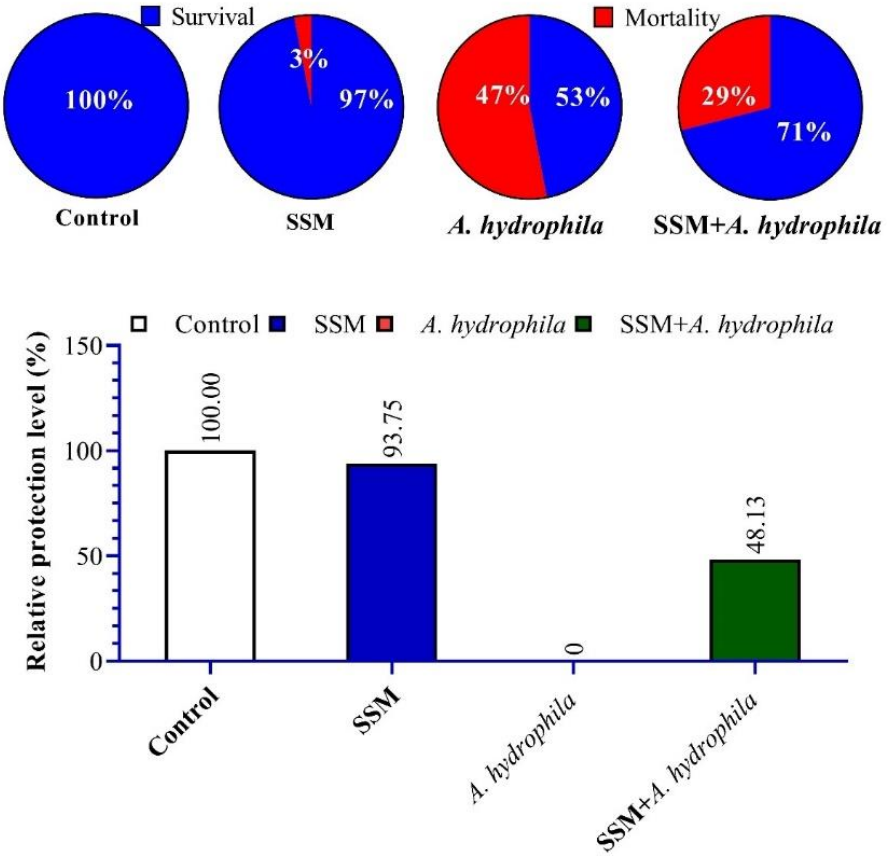

Fig. 1: Effect of *Streptomyces coeruleorubidus* extract (SSM) with or without *Aeromonas hydrphila* infection on survival rate and relative protection levels in Nile tilapia, *Oreochromis niloticus*, after two weeks of treatments.

3.2 Immune response

The humural immune response of Nile tilapia fingerlings treated with SSM with or without *A. hydrophila* infection is presented in Fig. 2. Treatment with SSM did not affect IgM levels compared to the control, while infection with *A. hydrophila* significantly decreased IgM levels. Co-treatment with SSM significantly improved IgM compared to the infected group. SSM treatment significantly improved lysozyme activity and complement c3 levels compared to other groups. Furthermore, SSM treatment alleviated the negative impact of *A. hydrophila* infection on lysozyme and complement.

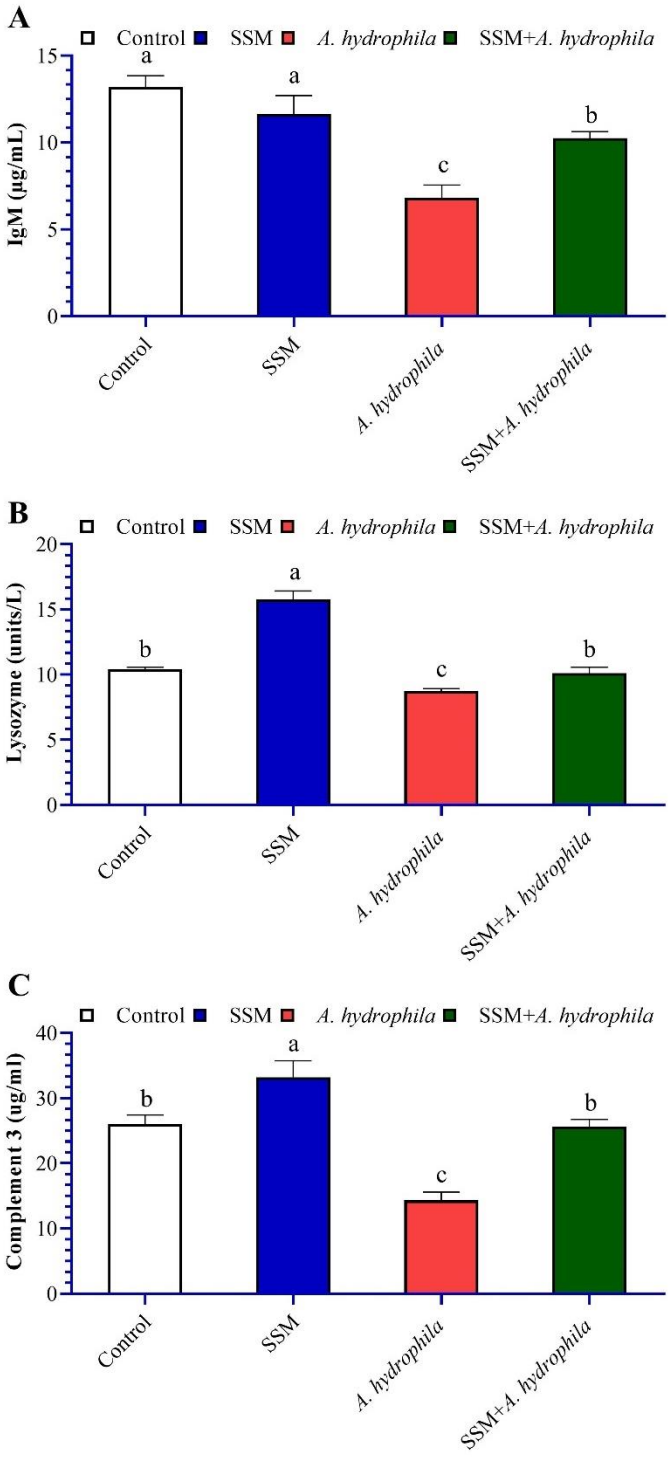

Fig. 2: Effect of *Streptomyces coeruleorubidus* extract (SSM) with or without *Aeromonas hydrphila* infection on immunoglobulin M (A), lysozyme activity (B), and complement 3 (C) of Nile tilapia, *Oreochromis niloticus*, after two weeks of treatments.

3.3 Relative expression of immune gene

The relative expression of the inflammatory gene in the head kidney of Nile tilapia is presented in Fig. 3. The expression of TNF-α, IFN-γ, and IL-1β were significantly up-regulated with *A. hydrophila* infection, while SSM treatment decreased pro-inflammatory cytokines. Anti-inflammatory cytokines (IL-8, IL-10, and TGF- β) were down-regulated with bacterial infection. SSM significantly alleviated the decrease in anti-inflammatory cytokines in the head kidney.

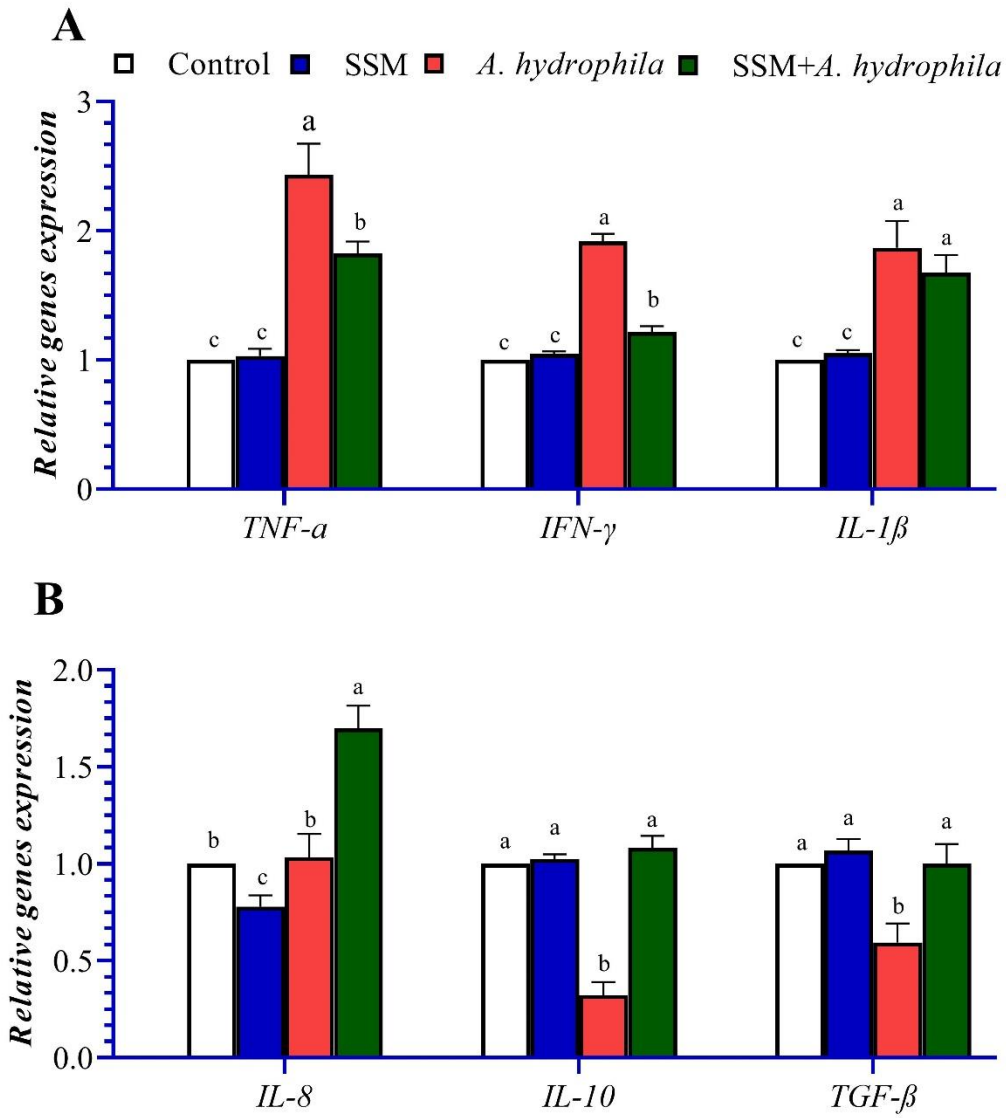

Fig. 3: Effect of *Streptomyces coeruleorubidus* extract (SSM) with or without *Aeromonas hydrphila* infection on relative inflammatory gene expression in head kidney of Nile tilapia, *Oreochromis niloticus*, after two weeks of treatments.

4 Discussion

The use of an antimicrobial substance in aquaculture as a preventive measure in high stocking fish farming systems triggers an increase in the bacterial resistance phenomenon and subsequent diseases outbreaks (Santos & Ramos, 2018). Recently, Ayoub et al. (2024) reported that 63.10%

of *A. hydrophilia* isolated from fresh water tilapia samples were considered multiple drug resistant and 28.9% were considered extensive drug-resistant to the common used antibiotics in aquaculture. Even in Nile tilapia broodstock, *A. hydrophilia* was isolated from moribund broodstock and recorded high resistant and multi-resistant strains to antibiotics with the presence of virulent genes, which could affect the vertical transition of this bacteria to geographically wide fish farms (Sherif & Kassab, 2023). The screening of natural antimicrobial agents is a continuous process of identifying novel substances of nature with high potential application in the field of aquaculture.

The current study investigated the use of *S. coeruleorubidus* extract as an antibacterial treatment for Nile tilapia infected with *A. hydrophilia*. Actinobacteria, such as *S. coeruleorubidus* is known to produces several novel biologically active compounds, enzymes, and potent antimicrobial substances that have antibacterial and anticancer properties (Kandula & Terli, 2013; Amarasinghe et al., 2018). Streptomyces was used for production of half of the discovered bioactive secondary metabolites, such as antibiotics, antitumor and other pharmaceutical agents, so it is considered the highest economically important prokaryotes (Berdy, 2005). In the present study, *A. hydrophilia* infection induced a significant mortality of 47% compared to the control. *A. hydrophila* was the dominant bacteria in infected tilapia fish in freshwater farms during the summer disease outbreaks and was identified as the etiological agent of the summer mortality phenomenon (Elsheshtawy et al., 2019; Abdel‐Latif & Khafaga, 2020). *Aeromonas spp.* is an opportunistic gram negative bacteria ubiquitous in the aquatic environment (Dahdouh et al., 2016). The pathogenicity of *A. hydrophila* rely on the secretion of several toxins such as aerolysin, hemolysin, lipases, enterotoxic, cytotoxic, and hemolytic (Ørmen et al., 2003; Abdel‐Latif & Khafaga, 2020). Nile tilapia challenged with *A. hydrophila* showed anemia, decreased of IGM, lysozyme, C3 and increased of reactive oxygen species levels (Neamat‐Allah et al., 2021).

The IP treatment with SSM extract improved the survival rate of infected fish to 72.33% and reported a 48.13% relative protection levels rate higher than the infected and untreated group in the present study. This improvement was associated with the improvement of humoral immune components (lysozyme activity and complement c3 levels) in the SSM treated groups or in infected and treated groups. Whereas SSM injection alleviated the immune suppression effect of bacterial infection in Nile tilapia. In the same line, *S. coeruleorubidus* metabolites revealed a suppression effect against *Streptococcus agalactiae* by inducing ultrastructural cell changes, including organelles and cell wall (Abdelaziz et al., 2024). Furthermore, aqueous treatment with octasiloxane-hexadecamethyl, one of *S. coeruleorubidus* metabolites, enhanced hematological, hepatorenal functions, antioxidant, and innate immunity of Nile tilapia infected with *S. agalactiae* (Abdelaziz et al., 2024). Nile tilapia fed a supplemented diet with bacteriocin producing species *Paenibacillus ehimensis* showed higher survival rate, immune response, and antioxidant status than fish infected with *A. hydrophila* (Chen et al., 2019). The crude extract of some probiotics bacterial species as known as bacteriocin could be used as alternative to broad-spectrum classical antibiotics with several advantages, such as immune stimulation effects, low toxicity, and can be produced in situ with high potential of biotechnological production (Cochrane et al., 2015).

The regulation of the immune system was caried out by numerous cytokines (Holloway et al., 2002). The cytokine genes transcription are expressed after exposure of the cell to antigens or a specific set of signals (Reyes-Cerpa et al., 2012). In the present study, infection with *A. hydrophila* upregulated expression of TNF-α, IFN-γ, and IL-1β and downregulated IL-8, IL-10, and TGF- β.

Meanwhile, these effects were alleviated by injection of metabolites of *S. coeruleorubidus* metabolites injection. The waterborne octasiloxane-hexadecamethyl modulated the negative impact of bacterial infection with *S. agalactiae* on the expression of the immunity, inflammatory, and apoptosis gene (Abdelaziz et al., 2024). The expression of cytokines TNF-α and IL-1β were increased in Nile tilapia fed bacteriocin producing bacteria *Paenibacillus ehimensis* than the fish fed the control diet (Chen et al., 2019).

5 Conclusion:

Intraperitoneal injection of crude extract of *S. coeruleorubidus* metabolites increased survival rate and relative protection level in Nile tilapia-infected fish with *Aeromonas hydrophila*. Depression of humoral immune components and disruption of immune-related cytokine genes induced by bacterial infection were alleviated by the metabolites of *S. coeruleorubidus*. It could be concluded that *S. coeruleorubidus* metabolites could be used as an alternative to antibiotics in aquaculture. However, extensive studies could be conducted to identify the major active metabolites secreted by *S. coeruleorubidus* and if they have pharmaceutical properties.

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Authors Contribution:

Loujaina Farhan Abdalmalk: Conceptualization, Investigation, Software, Methodology, Validation, Formal analysis, Writing-Original Draft, Writing-Review & Editing

Ethical approval:

Not applicable.

Informed consent:

Not applicable.

Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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