Bulgarian One Health Journal, *2026, Volume 1, 7* 89-101

ISSN 3033-0408 https:// 10.59496/20261AH5 *Original article*

ISOLATION AND CHARACTERIZATION OF A POTENTIAL PROBIOTIC *Bacillus thuringiensis* FROM AN INTEGRATED AQUAPONIC SYSTEM FOR SUSTAINABLE AQUACULTURE

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Abstract

The increasing demand for sustainable aquaculture has accelerated the search for probiotic microorganisms capable of improving fish health and reducing dependence on antibiotics. This study aimed to isolate, identify, and characterize potential probiotic bacteria from an integrated aquaponic system used for Nile tilapia (*Oreochromis niloticus*) production. Bacterial isolates obtained from aquaponic water samples were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and their probiotic potential was evaluated through assessment of antagonistic activity against fish pathogens, hemolytic activity, antibiotic susceptibility, biofilm formation, pH tolerance, bile salt tolerance, and extracellular enzyme production. Four bacterial species, namely *Bacillus mycoides, Bacillus marisflavi, Bacillus thuringiensis,* and *Lactococcus lactis*, were identified. Among these isolates, *B. thuringiensis* exhibited the most promising probiotic characteristics, showing strong antibacterial activity against *Lactococcus garvieae* with an inhibition zone of 2.32 ± 0.012 cm, while no inhibitory effect was observed against *Aeromonas hydrophila* or *Aeromonas caviae*. The isolate displayed γ-hemolytic (non-hemolytic) activity was susceptible to all tested antibiotics except intermediate susceptibility to kanamycin and showed no biofilm formation. In addition, *B. thuringiensis* demonstrated optimal growth at pH 5.0 ($P < 0.05$), exhibited extracellular lipolytic and proteolytic activities, and maintained 47.3% viability after 4 h of exposure to 0.5% bile salts. Overall, the findings demonstrate that *B. thuringiensis* isolated from an aquaponic system possesses multiple desirable probiotic traits, including pathogen-specific antagonistic activity, enzymatic potential, gastrointestinal tolerance, and a favorable safety profile. These characteristics highlight its potential application as a probiotic candidate for sustainable aquaculture; however, further *in vivo* studies are required to confirm its efficacy and safety under practical farming conditions.

Key words: *Bacillus thuringiensis*; Antagonistic activity; Bile salt tolerance; Extracellular enzyme production; Antibiotic susceptibility; *Lactococcus garvieae*, aquaponic system

Introduction

Aquaculture has become one of the fastest-growing food production sectors worldwide due to increasing demand for aquatic animal protein and the decline of capture fisheries (Dewali et al., 2023). However, the intensification of aquaculture production has resulted in an

ISSN 3033-0408 https:// 10.59496/20261AH5 Original article

increase in the prevalence of infectious diseases, particularly bacterial infections, which represent a significant challenge to the sustainable farming of fish (Gundi et al., 2025). Among bacterial pathogens, *Lactococcus garvieae* is considered an important emerging pathogen affecting a wide range of cultured freshwater and marine fish species, causing severe septicemia, high mortality, and substantial economic losses (Meyburgh et al., 2017). Antibiotics have traditionally been used to treat bacterial diseases, however their extensive and inappropriate use has raised serious concerns regarding antimicrobial resistance development, environmental contamination, and potential risks to public health (Romero et al., 2012; Lulijwa et al., 2020). Therefore, the development of environmentally friendly and sustainable disease-control strategies has become a priority in modern aquaculture (Gadhiya et al., 2025).

Probiotics have attracted mounting interest as a promising alternative to antibiotics due to their ability to enhance host health through multiple mechanisms, including competitive exclusion of pathogens, production of antimicrobial compounds, enhancement of digestive efficiency, modulation of immune responses, and improvement of intestinal microbial balance. However, the successful application of probiotics is contingent upon the selection of bacterial strains that possess the desired functional and safety characteristics. Important criteria for probiotic selection include antimicrobial activity against pathogens, tolerance to gastrointestinal conditions, production of beneficial enzymes, absence of virulence-related traits, and lack of transferable antimicrobial resistance determinants (El-Saadony et al., 2021; Torres-Maravilla et al., 2024). Therefore, the identification and characterization of novel probiotic candidates from diverse ecological sources remain essential for advancing sustainable aquaculture practices (Kavitha et al., 2018; Kuebutornye et al., 2020; Amoah et al., 2021).

Among probiotic microorganisms, members of the genus Bacillus are of particular interest due to their ability to form resistant spores, survive under unfavorable environmental conditions, and produce a broad spectrum of extracellular enzymes and antimicrobial metabolites (Santos et al., 2021). It has been demonstrated that several Bacillus species have a beneficial effect on aquaculture by improving growth performance, enhancing immune responses, reducing pathogen load, and increasing resistance against infectious diseases (James et al., 2021). In particular, Bacillus thuringiensis has attracted interest because of its production of bioactive compounds with antibacterial and antiparasitic properties (Mendoza-Estrada et al., 2016). Previous studies have reported the inhibitory activity of *B. thuringiensis* against several aquatic pathogens, including *Aeromonas salmonicida*, *Vibrio mimicus*, and *Aeromonas hydrophila* (Reneshwary et al., 2011; Askarian et al., 2012; Anyanwu and Ariole, 2019). However, the probiotic potential of *B. thuringiensis* strains isolated from aquaponic systems remains largely unexplored.

Aquaponic systems are a sustainable food production technology that integrates aquaculture and hydroponic plant cultivation through nutrient recycling. (Palm et al., 2018) In addition to their economic and environmental advantages, these systems are home to complex microbial communities that are involved in the transformation of nutrient, the regulation of water quality, and host–microbe interactions. Despite the potential for microbial diversity in aquaponic environments, there has been limited research attention devoted to their utilization as sources for the isolation of beneficial bacteria with probiotic properties (Tunçelli et al. 2025).

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Exploration of these microbial reservoirs may yield novel opportunities for the identification of functional bacterial strains that could contribute to disease prevention and sustainable aquaculture development (Kasozi et al., 2024; Rudoy et al., 2025).

Therefore, the aim of the present study was to isolate and characterize potential probiotic bacteria from an aquaponic system and evaluate their functional and safety properties. The isolated strains were identified using MALDI-TOF mass spectrometry, and their probiotic potential was assessed through a range of antimicrobial activities, including those against important fish pathogens (*Aeromonas hydrophila*, *A. caviae*, and *Lactococcus garvieae*), hemolytic activity, antibiotic susceptibility, biofilm formation, acid and bile tolerance, catalase production, and extracellular enzyme activities. The characterization of *B. thuringiensis* as a potential probiotic candidate against *L. garvieae* was emphasised, providing new insights into the utilization of aquaponic microbial resources for sustainable disease management in aquaculture.

MATERIAL AND METHOD

Fish, Aquaponic and Aquaculture System Setup

The present study was conducted in a greenhouse structure specifically designed for the purpose of Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) aquaculture production in closed recirculating systems. These systems are integrated with plant cultivation through an organic closed loop aquaponic system, which comprises a total of six tanks with a combined working volume of 1136 L. The tanks of the system were stocked with 360 tilapia, with an average weight of 0.04 kg (total biomass 14,4 kg). The fish were fed with feed without nutritional additives, in an amount of about 2.3% of their body weight. The Aller Aqua (Tilapia Aller Bona Float, Germany) was floating pellets with a size of 3 and 4.5 mm and contained 42.0% crude protein; 28.2% NFE, 12.0% crude fat; 6.8% ash, 3.0% fiber and the content of heavy metals did not exceed for lead – 1 mg/kg, cadmium – 0.2 mg/kg, mercury – 0.5 mg/kg and arsenic – 5.0 mg/kg. The food ration was distributed in three feedings (9:00, 12:00 and 15:00).

The fish metabolic waste was biologically transformed through nitrification processes, thereby providing available nutrients for the cultivation of basil within the integrated aquaponic system.

Microbiological Analysis of Water Samples

Aquaponic system water samples were collected into sterile 250 mL Erlenmeyer flasks. The samples were then serially diluted (1:9, 1:99, and 1:999) using sterile distilled water. From each dilution, 200 µL aliquots were spread onto Tryptic Soy Agar (TSA) plates using sterile Drigalski spatulas, and all procedures were performed in triplicate. The inoculated plates were incubated at 22 °C for 24–48 hours. Following the incubation period, the bacterial colonies were evaluated based on their morphological characteristics, including color, shape, and margin. Subsequent subculturing of distinct colonies was taken to obtain pure isolates (Tunçelli et al. 2025).

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Identification of Potential Probiotic Candidates

For MALDI-TOF MS analysis, a single colony from each purified isolate was transferred onto a 96-spot polished steel UV-absorbing MALDI-TOF target plate. Each sample was overlaid with one drop of MALDI matrix solution consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA), which facilitates laser energy absorption. The spots were then permitted to air dry at room temperature. The prepared target plate was then introduced into a MALDI-TOF MS system equipped with a fixed, pulsed laser (Bruker Biotyper MALDI Automation Control software) for spectral analysis (Pečur Kazazić et al. 2019; Ürkü et al. 2024). All spectra obtained and identification results were recorded electronically in the system database.

Antagonistic Test

The antibacterial activity of candidate probiotic bacteria isolated and identified from aquaponic system water was evaluated against pathogenic bacteria using the disc diffusion method (Zainuddin et al. 2026). Test pathogens previously isolated from diseased fish were retrieved from −80 °C storage and cultured on tryptic soy agar (TSA). Each pathogen suspension (100 µL, 10^8 CFU/mL) was inoculated onto Mueller-Hinton agar (MHA) plates and evenly spread over the surface of the agar using a sterile Drigalski spatula. The plates were permitted to desiccate for period of 10 min.

Candidate probiotic bacterial strains were inoculated into 6 mL of tryptic soy broth (TSB) and incubated at 24 °C for 72 h. Following incubation, cultures were centrifuged at 6000 rpm for 15 min to separate bacterial cells from the supernatant. The obtained supernatants were then filtered through a sterile 0.22 µm membrane filter to obtain cell-free supernatants. Sterile blank discs (6 mm diameter) were prepared by applying 50 µL of cell-free supernatant onto each paper disc and allowing them to dry, after which the discs were placed onto MHA plates previously inoculated with the fish bacterial pathogens *A. hydrophila*, *A. caviae*, and *L. garvieae*.

The positioning of the discs on the agar surface was conducted with equal distances maintained between each disc. An antibiotic disc (Oxytetracyline 30 µg (Oxoid, England)) was used as a positive control, while sterile distilled water was employed as a negative control. All experiments were performed in triplicate. The isolate demonstrating antagonistic activity against any of the tested pathogenic bacteria was selected as the candidate probiotic strain for further evaluation, and all subsequent *in vitro* assays were performed using this isolate.

Hemolytic and Catalase Activity

Candidate probiotic strain was subjected to a hemolytic test by streaking them onto agar plates supplemented with 7% sheep blood. The hemolytic zones were observed after incubation of plates at 37◦C for 48 h. The isolate was then classified as α-, β-, and γ-hemolysis. The isolate having a green zone around the colony was recorded as α-hemolysis, while those with a clear zone was designated as β-hemolysis. Also, those that did not produce any zone around the colony were referred to as no- or γ-hemolysis (Lee et al., 2017). Catalase activity was evaluated

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following the method of Barbosa et al. (2005). Overnight bacterial cultures were exposed to 3% hydrogen peroxide, and bubble formation was interpreted as a positive catalase reaction.

Antibiotic Susceptibility Test

The antibiotic susceptibility of the candidate probiotic strain was determined using the disc diffusion method. Commercially available antibiotic discs (Oxoid, England) were used, including chloramphenicol (30 µg/disc), kanamycin (30 µg/disc), flumequine (30 µg/disc), erythromycin (5 µg/disc), ciprofloxacin (1 µg/disc), sulphamethoxazole (25 µg/disc), ampicillin (10 µg/disc), florfenicol (30 µg/disc) and oxytetracycline (30 µg/disc). In summary, 100 µL of an overnight bacterial culture adjusted to the 0.5 McFarland standard (approximately 1.5×10^8 cfu/mL) was evenly spread onto Mueller–Hinton agar plates. The antibiotic discs were placed onto the agar surface in a sterile manner, and the plates were then incubated at 37°C for 24 h. Following this, incubation, the diameters of the inhibition zones were measured in millimeters (mm), and the antibiotic susceptibility profile of the candidate probiotic strain was determined (Patel et al., 2009). The classification of the strains as either susceptible or resistant was determined in accordance with the microbiological cut offs established by the European Food Safety Agency (EFSA) (Rychen et al., 2018)

Biofilm Formation

The capacity of the candidate probiotic strain under consideration to form biofilms was determined by means of the Congo red agar assay, as previously described by Amoah et al., (2021). Briefly, the bacterial culture was streaked onto Mueller–Hinton agar with the addition of Congo red dye (0.8 g/L, Merck, Darmstadt, Germany**)** and incubated at 37°C for 48 h. After incubation, colony morphology was evaluated in order to ascertain the production of biofilms. The development of black colonies with a dry crystalline appearance was considered indicative of biofilm formation, whereas red colonies were classified as non-biofilm-producing strains.

Determination of Growth Under Different pH Conditions

The growth of the candidate probiotic strain under different pH conditions was evaluated as previously described by Kavitha et al. (2018), with minor modifications. Briefly, an overnight culture of the candidate probiotic strain was inoculated into Luria–Bertani (LB) broth adjusted to pH 3, 5, and 9 using glacial acetic acid (99%) or 5 N NaOH. The inoculated broths were then subjected to an incubation process at a temperature of 37°C for 24 h. The assessment of bacterial growth was conducted by measuring the optical density at a wavelength of 600 nm using a spectrophotometer. Uninoculated LB broth adjusted to the corresponding pH value served as the blank.

Bile Salt Tolerance

The bile salt tolerance of the candidate probiotic strain was evaluated by monitoring its survival in the presence of 0.5% (w/v) bile salts. Bacterial cultures were exposed to bile salts and incubated for 0, 1, 2, 3, and 4 h. At each designated time point, serial dilutions were prepared, and the number of viable cell was determined by the plate count method. Colony-

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forming units (CFU) were enumerated and the results were expressed as a percentage of survival relative to the initial bacterial concentration at 0 h (100% survival) (Kuebutornye et al., 2020).

Enzyme Production

The extracellular enzymatic activities of the bacterial isolates, including lipase, amylase, and protease production, were evaluated using agar plate assays. Lipolytic activity was assessed on Tween 80 agar following the method of Sierra (1957). The appearance of opaque crystalline precipitation or a distinct halo surrounding the bacterial colonies was considered indicative of lipase production. Amylolytic activity was determined on starch agar as described by Latorre et al. (2016). The evaluation of proteolytic activity was conducted in accordance with the method outlined by Zhou et al. (2009) using casein agar as a substrate. The presence of a transparent hydrolysis zone surrounding the bacterial colonies was colonies was indicative of protease production. All plates were incubated at 22 ± 0.5 °C for 48 h, after which enzymatic activities were recorded based on the presence or absence of characteristic hydrolysis halos.

Statistical Analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Data normality was evaluated using the Shapiro–Wilk test. The differences in bacterial growth under different pH conditions were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The effect of varying durations of bile salt exposure time on bacterial viability was evaluated using one-way repeated measures ANOVA followed by Tukey's multiple comparison test. Statistical significance was accepted at $P < 0.05$.

RESULT

Identification Results of Candidate Probiotic Bacteria

In MALDI-TOF MS analysis, identification was considered reliable based on the manufacturer's criteria, with confidence scores of ≥2.0 indicating species-level identification and ≥1.7 indicating genus-level identification. In the present study, the isolates were successfully identified using MALDI-TOF MS based on their score values. *Bacillus mycoides* was identified with scores ranging from 2.033 to 2.000 (++) indicating secure genus identification and probable species-level identification. *Lactococcus lactis* showed higher confidence scores ranging between 2.380 and 2.403 (+++), corresponding to highly reliable species-level identification. *Bacillus marisflavi* was identified within the score range of 1.916 to 2.003 (++), while *Bacillus thuringiensis* exhibited scores between 1.933 and 2.021, supporting reliable identification at the species level.

Antagonistic Activity Findings

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The antagonistic activity of candidate probiotic bacteria (*B. mycoides*, *B. marisflavi*, *B. thuringiensis*, and *L. lactis*) isolated from aquaponic system water was evaluated against fish pathogens using the disc diffusion method. The results showed that none of the probiotic candidates tested exhibited inhibition zones against *A. hydrophila* or *A. caviae*, suggesting an absence of antibacterial activity against these pathogens.

In contrast, *B. thuringiensis* exhibited a clear inhibition zone with a diameter of 2.32 ± 0.012 cm against *L. garvieae*, demonstrating a strong antagonistic effect. The absence of an inhibition zone was observed in the negative control, confirming that the antibacterial activity was associated with metabolites present in the cell-free supernatants (Figure 1).

Furthermore, the positive control, oxytetracycline, produced inhibition zones of 2.11 ± 0.015 cm, 1.81 ± 0.015 cm, and 1.70 ± 0.020 cm against *A. hydrophila*, *A. caviae*, and *L. garvieae*, respectively. Overall, these findings indicate that among the tested probiotic candidates, *B. thuringiensis* exhibits selective antibacterial activity with notable effectiveness against *L. garvieae*.

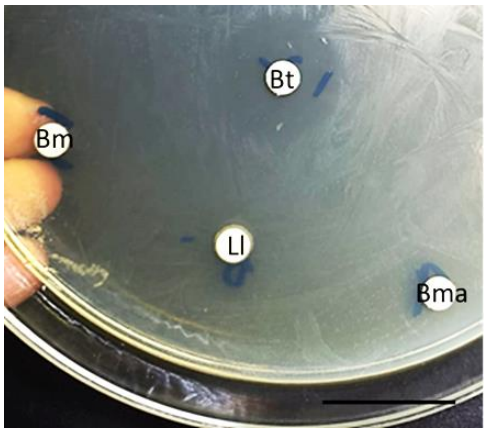


Figure 1. Antagonistic activity of candidate probiotic bacteria against *L. garvieae* determined by the disc diffusion method. No inhibition was observed *B. mycoides* (Bm), *B. marisflavi* (Bma), and *L. lactis* (Ll) while *B. thuringiensis* (Bt) showed an inhibition zone against *L. garvieae.* (Bar: 2.5 cm)

Hemolytic and Catalase Activity

B. thuringiensis demonstrated a positive catalase reaction, with immediate bubble formation observed after the addition of 3% hydrogen peroxide. The hemolytic activity analysis revealed that *B. thuringiensis* exhibited non-hemolysis, characterized by the absence of any zone around the colonies on 7% sheep blood agar after 48 h of incubation.

Antibiotic Susceptibility Test

The antibiotic susceptibility profile of *B. thuringiensis* is presented in Table 2. The isolate demonstrated susceptibility to chloramphenicol, flumequine, erythromycin, ciprofloxacin, sulphamethoxazole, ampicillin, florfenicol, and oxytetracycline. Conversely, the isolate exhibited moderate susceptibility (intermediate susceptibility) to kanamycin. The strain

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demonstrated susceptibility to the majority of the antibiotics tested, indicating the absence of broad-spectrum antibiotic resistance.

Table 2. Antibiotic susceptibility profile of the candidate probiotic strain *B. thuringiensis*.

Antibiotic	Disc content (µg/disc)	Susceptibility
Chloramphenicol	30	S
Kanamycin	30	MS
Flumequine	30	S
Erythromycin	5	S
Ciprofloxacin	1	S
Sulphamethoxazole	25	S
Ampicillin	10	S
Florfenicol	30	S
Oxytetracycline	30	S

MS: Moderatly Sensitive, S: Sensitive

Biofilm Formation Detection Using the Congo Red Agar Method

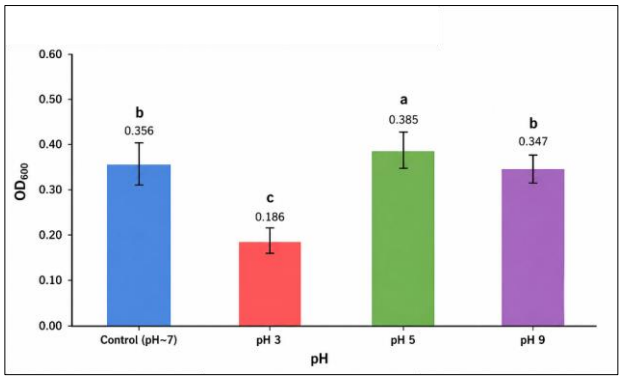
The assessment of biofilm formation was conducted utilising the Congo red agar method. The candidate probiotic strain showed no evidence of biofilm production, as evidenced by the absence of black colony formation on Congo red agar.

Determination of Growth Under Different pH Conditions

The growth of the candidate probiotic strain was significantly affected by the pH of the culture medium ($P < 0.05$). The highest optical density (OD_{600}) was recorded at pH 5 (0.385 ± 0.006), which was significantly higher than those observed at the other pH levels. In contrast, growth at pH 3 (0.186 ± 0.009) was significantly reduced compared with the control and the other treatment groups. No significant difference was detected between the control (0.356±0.006) and pH 9 (0.347 ± 0.006) groups ($P > 0.05$) (Figure 2).

Figure 2. Effect of different pH levels on the growth of *B. thuringiensis*. Growth was assessed by OD_{600} measurements and expressed as mean ± SD. Different letters indicate significant differences among treatments ($P < 0.05$).

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Bile Salt Tolerance

The viability of *B. thuringiensis* under 0.5% bile salt stress showed a progressive time-dependent decline. The initial bacterial count was 731.7 ± 27.5 CFU (100% survival). After 1 h of exposure, survival remained high at 92.6% ($P > 0.05$), followed by a marked decrease to 62.0% at 2 h ($P > 0.05$). Further significant reductions ($P < 0.05$) were observed at 3 h (58.9%) and 4 h (47.3%), indicating a gradual loss of viability under bile salt stress conditions.

Table 3. Viable cell counts (cfu) and survival percentages of the candidate probiotic strain during exposure to 0.5% (w/v) bile salts. Values are expressed as mean ± SD (n = 3). Survival (%) was calculated relative to the initial bacterial count at 0 h.

Time (h)	Mean ± SD	Survival (%)
0	731.7 ± 27.5	100.0
1	677.7 ± 16.5	92.6
2	453.7 ± 27.5	62.0
3	431.0 ± 9.00	58.9
4	346.3 ± 18.3	47.3

Enzyme production

B. thuringiensis showed positive extracellular lipolytic and proteolytic activities. Evidence of lipolytic activity was indicated by the formation of a distinct opaque crystalline halo surrounding the colonies on tween 80 agar. In contrast, the presence of a clear hydrolysis zone on casein agar after 48 h of incubation at 22 ± 0.5 °C confirmed the occurrence of proteolytic activity. Following the incubation period for amylolytic activity, Lugol's iodine solution was poured onto the surface of the agar. However, no clear zone was observed around the colonies and therefore the amylolytic activity was deemed to be negative.

DISCUSSION

The provenance of probiotic microorganisms exhibits significant variation, with candidate probiotic strains being isolated from the intestines of healthy fish, aquaculture water, sediment from culture systems, other animals, and fermented food products (Simón et al., 2021; Thepnarong et al., 2024). Among the probiotic genera previously isolated from fish,

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Lactobacillus, Bacillus, and Enterococcus have been the most extensively documented (Santos et al., 2021). In contrast to the findings of previous studies, the present study identified *B. mycoides*, *B. marisflavi*, *B. thuringiensis*, and *L. lactis* as potential probiotic bacteria isolated from an aquaponic system. Previous studies have reported the isolation of *B. mycoides* (Andriani et al., 2017), *B. marisflavi* (Akayli et al., 2016; Nguyen et al., 2023), and *L. lactis* (Cano-Lozano et al., 2022) as probiotic candidates from different ecological niches. The findings, when considered as a whole, demonstrate that aquaponic systems constitute a previously underexplored source of probiotic bacteria. Furthermore, they provide new evidence in support of the exploitation of aquaponic microbiota for the development of sustainable biocontrol strategies in aquaculture.

Bacillus species are among the most extensively investigated probiotic bacteria because of their ability to produce a broad spectrum of extracellular enzymes and bioactive metabolites with antibacterial, antiviral, and antifungal properties (Prieto et al., 2012). The antimicrobial activity of these organism is primarily attributed to the production of various secondary metabolites, including bacteriocins, lipopeptides, and hydrolytic enzymes. These metabolites that inhibit pathogen colonization through mechanisms such as competitive exclusion and growth suppression. In the field of aquaculture, *B. thuringiensis* has garnered mounting interest not only for its insecticidal cry proteins but also for its potential utilisation as a biological control agent against bacterial pathogens and aquatic parasites (Saha et al., 2025). As demonstrated in previous studies *B. thuringiensis* has been shown to possess inhibitory activity against several important fish pathogens, including *Aeromonas salmonicida* subsp. *salmonicida*, *Listonella anguillarum*, *Moritella viscosa*, and *Carnobacterium maltaromaticum* (Askarian et al., 2012). In addition, isolates derived from shrimp and African catfish have also been reported to inhibit *Vibrio mimicus* and *A. hydrophila*, respectively (Reneshwary et al., 2011; Anyanwu and Ariole, 2019). Moreover, *B. thuringiensis* has been successfully employed in aquaponic systems for the management of pest (Kasozi et al., 2021) and has demonstrated efficacy against the fish gill trematode *Centrocestus formosanus* (Mendoza-Estrada et al., 2016), underscoring its broad applicability in the field of aquatic animal health.

In contrast to the findings of previous reports on anti-Aeromonas activity, the *B. thuringiensis* characterized in the present study did not inhibit *A. hydrophila* but exhibited pronounced antagonistic activity against *L. garvieae*, one of the most economically important Gram-positive bacterial pathogens in aquaculture. This selective antimicrobial spectrum may be indicative of strain-specific differences in the production of antimicrobial metabolites, given the established variance in the composition and expression of bacteriocins and other bioactive compounds are known to vary considerably among Bacillus isolates. The present findings are consistent with those reported by Shahverdi et al. (2023), who suggested that probiotic bacteria often display greater inhibitory activity against Gram-positive pathogens than Gram-negative bacteria because the outer membrane of Gram-negative bacteria provides an additional permeability barrier against many antimicrobial compounds. Therefore, the selective inhibition of *L. garvieae* observed in the present study suggests that the isolated *B. thuringiensis* could represent a promising biological control agent for the prevention of lactococcosis in aquaculture, although its efficacy should be confirmed under *in vivo* conditions. In view of the

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mounting constraints antibiotic usage in aquaculture, probiotic strains that are able to selectively inhibit *L. garvieae* may offer an ecologically viable option for the management of lactococcosis.

The paramount consideration in selection of probiotic microorganisms intended for aquaculture applications is safety. Among the safety-related characteristics, the absence of hemolytic activity is considered particularly important because hemolysins are recognized virulence factors that facilitate bacterial invasion by damaging host cell membranes and surrounding tissues (Nandi et al., 2017). Consequently, probiotic strains exhibiting α- or β-hemolytic activity are generally regarded as unsuitable for commercial application, whereas γ-hemolytic (non-hemolytic) strains are considered safer alternatives (Amoah et al., 2021). In the present study, the *B. thuringiensis* exhibited γ-hemolytic activity, indicating the absence of detectable hemolytic potential and supporting its favorable safety profile. This observation is consistent with the findings of previous studies reporting non-hemolytic *Bacillus* spp. isolated from *Labeo calbasu* and other aquatic environments (Kavitha et al., 2018). The absence of hemolytic activity, in conjunction with the antimicrobial properties exhibited by the isolate, suggests that this strain possesses favourable characteristics for probiotic application without manifesting one of the predominant virulence-associated phenotypes commonly evaluated during probiotic screening. While the absence of hemolytic activity in itself does not ensure the overall safety of a probiotic candidate, it is one of the fundamental screening criteria.

Catalase production is widely regarded as a significant functional characteristic of probiotic bacteria. This is due to the ability of catalase to detoxify hydrogen peroxide and other reactive oxygen species (ROS), thus protecting both bacterial cells and the host from oxidative damage (Nishikawa et al., 2009). Oxidative stress is a prevalent physiological response in fish exposed to environmental stressors, intensive farming conditions, and infectious diseases. Therefore, probiotic bacteria that possess antioxidant enzyme systems may contribute to the maintenance of intestinal homeostasis and the enhancement of the antioxidant status of the host. Members of the genus Bacillus are renowned for their capacity to produce catalase and other antioxidant enzymes that enhance their survival under oxidative conditions while concomitantly reducing ROS-mediated cellular damage (Thankappan et al., 2015). In the present study, *B. thuringiensis* exhibited positive catalase activity, indicating an intrinsic antioxidant defense mechanism that may enhance its persistence under stressful gastrointestinal conditions.

Antibiotic susceptibility is widely regarded as one of the most significant safety criteria in the selection of probiotic bacteria. This is due to the potential of probiotic strains to act as reservoirs of antimicrobial resistance genes. These genes can be transferred to pathogenic microorganisms through horizontal gene transfer (Lastauskienė et al., 2021; Daliri et al., 2023). Such transfer has the potential to compromise the therapeutic efficacy of antibiotics used in both aquaculture and human medicine, thus rendering the assessment of antibiotic susceptibility an essential component of probiotic safety evaluation. In the present study, *B. thuringiensis* demonstrated susceptibility to chloramphenicol, flumequine, erythromycin, ciprofloxacin, sulphamethoxazole, ampicillin, florfenicol, and oxytetracycline, all of which are commonly utilised for the treatment of bacterial diseases in aquaculture (Romero et al., 2012).

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The absence of phenotypic resistance to these antimicrobial agents suggests that the isolate is less likely to serve as a reservoir for transferable antibiotic resistance determinants, thereby supporting its suitability as a probiotic candidate. As indicated by Kuebutornye et al. (2020), analogous findings have been reported for *Bacillus* spp. isolated from the intestinal microbiota of Nile tilapia. This exhibited susceptibility to a broad range of clinically relevant antibiotics with no evidence of acquired resistance. Collectively, these findings indicate that the isolated *B. thuringiensis* strain satisfies an important safety requirement for probiotic application and may represent a safer alternative to extensive antibiotic use in sustainable aquaculture. However, further strengthening of its safety assessment would be achieved by molecular screening for transferable resistance genes.

The formation of biofilms is a complex trait that can influence both the persistence and safety of probiotic microorganisms. On the one hand, the formation of biofilm may facilitate bacterial colonization of the gastrointestinal tract and enhance competitive exclusion of pathogens. On the other hand, biofilm-producing bacteria frequently demonstrate elevated levels of tolerance to antimicrobial agents and environmental stress, a factor which has the potential to give rise to biosafety concerns, especially in instances where strains are intended for large-scale application in aquaculture (Donlan, 2001; Morikawa, 2006). Therefore, the significance of biofilm formation should be interpreted in conjunction with the intended application and overall safety profile of the probiotic strain. In the present study, *B. thuringiensis* was found not to produce biofilm. It has been reported that a similar phenomenon has been observed in relation to *Bacillus* spp. isolated from the gastrointestinal tract of *Labeo calbasu* (Kavitha et al., 2018) and Nile tilapia (*Oreochromis niloticus)* (Kuebutornye et al., 2020). This observation suggests that the absence of biofilm formation is not an uncommon occurrence among fish-associated probiotic *Bacillus* spp.

It is widely recognised that tolerance to acidic conditions and bile salts is widely recognized as one of the fundamental criteria for probiotic selection (Lazado et al., 2010). The ability of microorganisms to survive gastrointestinal transit, remain metabolically active, and transiently colonize the host intestine is determined by these characteristics. A substantial body of research has previously demonstrated significant variation in acid tolerance among probiotic bacteria. While optimal growth has generally been reported under neutral or slightly acidic conditions (Rwezawula et al., 2025; Ganguly et al., 2019), certain probiotic species, such as Enterococcus spp., have been shown to maintain growth even at pH 2–3 (Hussain et al., 2025). In accordance with these observations, the *B. thuringiensis* isolate evaluated in the present study demonstrated viability at both pH 3.0 and pH 5.0, with bacterial growth at pH 5.0 exceeding that of the control culture. This finding indicates that the isolate possesses remarkable acid tolerance and may be well adapted to survive transient exposure to acidic gastrointestinal environments.

Bile salts represent a significant physiological barrier that limits the survival of ingested probiotic bacteria. In the present study, the viability of the isolate was found to decrease during exposure to 0.5% bile salts; however, approximately 47.3% of the bacterial population remained viable after 4 h of incubation. Despite the finding that prolonged bile exposure led to a reduction in bacterial survival, the observed tolerance suggests that a significant proportion

ISSN 3033-0408 https:// 10.59496/20261AH5 *Original article*

of the population possesses the capacity to withstand physiologically relevant bile concentrations.

The synthesis of extracellular digestive enzymes is widely regarded as one of the most valuable functional characteristics of probiotic bacteria employed in aquaculture. These enzymes have been demonstrated to enhance nutrient digestion, improve feed utilization, and consequently promote to fish growth and health (Yi et al., 2019; Lin et al., 2019). Members of the genus Bacillus distinguished by their capacity to secrete a diverse array of hydrolytic enzymes, including proteases, amylases, and lipases. These enzymes play a pivotal role in the decomposition of proteins, carbohydrates, and lipids, which are integral components of formulated feeds (Prazdnova et al., 2025). In addition to their nutritional role, the presence of extracellular proteases may contribute to host defence mechanisms by the degradation of bacterial adhesion proteins or receptor-binding sites on the intestinal epithelium, thereby limiting the colonisation of the intestinal tract by pathogens (Hosoi and Kiuchi, 2003). In the present study, the isolated *B. thuringiensis* strain exhibited clear proteolytic and lipolytic activities, indicating its ability to produce enzymes involved in nutrient hydrolysis. The observed protease activity is consistent with previous reports describing strong proteolytic activity in *B. thuringiensis* isolated from the intestinal microbiota of wild cyprinid fish (*Systomus saranus)* (Dhayalan et al., 2022), as well as in another fish-associated Bacillus spp. (Prazdnova et al., 2025). It is noteworthy that, despite the prevalence of reports indicating that probiotic Bacillus strains isolated from sediment typically demonstrate low lipase activity (Mazanko et al., 2025), the isolate examined in the present study exhibited substantial lipolytic activity, indicating a potential strain-dependent functional variability. Such enzymatic diversity is expected because extracellular enzyme production is influenced by both the genetic background of the strain and the environmental conditions from which it originates.

CONCLUSION

The present study demonstrated that aquaponic systems have the potential to serve as promising sources for the isolation of potential probiotic microorganisms with beneficial functional characteristics for aquaculture applications. Among the isolated bacterial strains, *Bacillus thuringiensis* demonstrated several desirable probiotic properties, including significant antagonistic activity against *L. garvieae,* absence of hemolytic activity and biofilm formation, susceptibility to commonly used aquaculture antibiotics, catalase production, tolerance to acidic pH and bile salt conditions, and extracellular enzyme production. These characteristics indicate that *B. thuringiensis* has a favorable safety profile and functional potential as a probiotic candidate for improving fish health and reducing reliance on antibiotics in aquaculture. However, further in vivo investigations are required to evaluate its colonization ability, immunomodulatory effects, growth-promoting capacity, and effectiveness in controlling bacterial diseases under practical aquaculture conditions.

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