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Evaluation of mosquitocidal and antibacterial activity of tilapia gut bacterial cell-free supernatant using *Culex quinquefasciatus* and pathogenic bacteria

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Abstract
Globally, mosquitoes are considered one of the deadliest animals, responsible for millions of human fatalities each year through the spread of infectious diseases. Initially controlled by synthetic pesticides that protected humans, these chemicals ultimately proved harmful due to health risks, environmental degradation, and emerging mosquito resistance. The present study investigated the isolation and characterization of bacterial strains from *Oreochromis niloticus* gut samples with potential antibacterial and larvicidal properties. Among 69 distinct bacterial isolates obtained, seven strains demonstrated significant larvicidal activity against *Culex quinquefasciatus* 3rd larval instar, with FG16 and FG61 showing the highest mortality rates of 70.2±3.5% and 62.2±4.6%, respectively. These two potent isolates were identified through 16S rRNA gene sequencing as *Bacillus velezensis* (NCBI accession: OP217139) and *Lysinibacillus capsici* (NCBI accession: MZ227505.1), sharing 99.9% and 99.5% sequence similarity, respectively. Both strains exhibited broad-spectrum antibacterial activity against human and fish pathogens, with notable inhibition zones against *Escherichia coli*, *Klebsiella pneumonia*, and *Aeromonas caviae*. Antibiotic susceptibility testing revealed complete resistance to Ampicillin in both strains, while showing varying sensitivity to other antibiotics. The cell-free supernatant of *B. velezensis* demonstrated superior larvicidal efficacy with LC50 values of 148.47, 64.22, and 42.19 mg/50mL at 12, 24, and 48 hours, respectively, compared to *L. capsici*. The findings suggest that these bacterial strains, particularly *B. velezensis*, hold promising potential as biological control agents of mosquito larvae and as sources of antibacterial compounds.

Keywords: *Oreochromis niloticus*, larvicidal, antibacterial, cell-free supernatant

Introduction

Vector-borne diseases transmitted by mosquitoes result in numerous human and animal fatalities annually. Mosquitoes (Diptera: Culicidae), among all blood-feeding insects, are recognized as the primary vectors transmitting pathogens that cause severe diseases like malaria, dengue fever, and yellow fever that significantly contribute to worldwide morbidity and mortality rates, thereby presenting substantial public health challenges and negatively impacting the global economy (Brugman *et al*., 2018; Samuel *et al*., 2023; Jones *et al*., 2021) [9, 18, 28]. Natural product-derived biological control methods and biopesticides represent the most effective control strategy (Egamberdieva *et al*., 2021) [12]. Bioactive compounds from epiphytic and endophytic bacteria are reported to possess larvicidal activity, *Bacillus thuringiensis* and *Lactobacillus sphaericus* based products lead the commercial market in mosquito larval control (Polanczyk *et al*., 2017) [25]. *Bacillus thuringiensis* and *Bacillus sphaericus* have shown powerful larvicidal capabilities when tested against Anopheles mosquito larvae (Hamza & El-Sanousi, 2022) [14]. Various *Bacillus species* produce cell-free supernatants (CFS) that exhibit significant antibacterial properties against human pathogens. *Lysinibacillus sphaericus* has been thoroughly researched for its mosquito larvicidal properties.

This study examines the combined mosquitocidal efficacy of bacterial endotoxins from *B. velezensis* and *L. capsici* against *Cx. quinquefasciatus* larvae, focusing on enhanced lethal concentrations. It aims to elucidate the biocontrol mechanisms of these Bacillus species through mortality assessments and toxicological analyses, contributing to novel biopesticide formulations and advancing bacterial endotoxin-based mosquito control strategies.

Materials and Methods

Sample collection and Isolation of fish gut bacteria

Tilapia *Oreochromis niloticus* (Linnaeus 1758) specimens averaging 30g were collected using drag net from Kuthukkal pond, Tirunelveli, Tamil Nadu, India (8.663828′N, 77.792085′E) and transported to the lab in oxygenated water-filled polythene bags. Water temperatures ranged from 27.3–30.1°C during collection. The fish gut tissue homogenates underwent serial dilution to 10−5 in sterilized saline. 100 µl from each dilution was spread on tryptic nutrient agar plates. The plates were incubated under aerobic conditions. Microbial colonies were counted using the plate counting method. Pure cultures were then developed using morphologically distinct colonies**.**

Preliminary antibacterial screening

A colony overlay technique was utilized to evaluate the antibacterial potential of gut-isolated bacteria. Overnight cultures of both indicator strains and isolated bacteria were prepared in Nutrient broth at 27°C. Indicator strain cultures (105 CFU/ml) were uniformly spread on Muller Hinton Agar plates using a sterile cotton swab (Alonso *et al*., 2019; Midhun *et al*., 2017) [3, 22]. Bacterial isolate droplets were then applied to the inoculated agar. After 24-hour incubation at 28°C, antibacterial activity was assessed by measuring inhibition zones against *Staphylococcus aureus*, *Aeromonas hydrophila*, *Vibrio cholerae*, and *Aeromonas caviae*. A predefined scoring system was employed to identify the most promising antagonistic bacterial isolates for subsequent research.

Fig 1: *Cx. quinquefasciatus* larvae collection form sewage

Cross streak method

The cross-streaking method was used to shortlist the predominantly effective strains. Mueller-Hinton (MH) agar plates were prepared and bacterial species were inoculated as a single streak in the center of each petri dish. Following 1 day of incubation at 28°C, indicator bacteria such as *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Enterobacter aeruginosa, Salmonella typhi, Vibrio* species, *Staphylococcus aureus, Aeromonas hydrophila, Vibrio cholerae*, and *Aeromonas caviae*. were streaked at a 90° angle to the original species. The size of inhibition zones was measured to analyze microbial interactions, according to the method of (Lertcanawanichakul and Sawangnop 2008) [21].

Culturing of effective bacteria

Selected pure bacterial isolates (FG14 and FG60) were cultured in 250 ml of sterile LB broth (25 g L−1) in 500 ml conical flasks, with the media previously autoclaved at 121°C under 15 lbs pressure for 21 minutes. Cultures were maintained in an orbital shaker at 32 ± 1°C for 5 days. After incubation, the cultures underwent centrifugation at 10,000 rpm for 15 minutes, and supernatants were filtered through a 0.77 µm membrane filter to remove cellular debris.

Mosquito larvicidal assay

The insecticidal potential of bacterial isolates was evaluated through qualitative bioassay against larvae using a single dose, following (Palma's 2015) [23] method. Each bacterial isolate was collected from nutrient agar plates using a loop and homogenized in 1 ml sterile water at 25°C. For each treatment, 0.5 ml of the mixture was added to 50 ml water in plastic cups. Twenty-five 3rd larval instar were used per test with three replications, and larval mortality was assessed after 48 hours. Bacterial isolates causing over 50% larval mortality were identified as pathogenic and selected for further characterization.

Mosquito larvae collection

Mosquito larvae were collected from urban sewage water sites using standard 350ml dippers, following WHO vector surveillance guidelines (WHO, 2024). Third instar larvae were randomly selected as subsamples and identified according to (Becker *et al*., 2010) [8]. Environmental conditions were recorded, with water temperature at 27±2°C and pH ranging from 6.8-7.2. The Third instar larvae were transported to the laboratory under maintained field conditions, and species identification was performed using standard taxonomic keys by examining morphological features under a stereomicroscope.

Mosquito larvicidal effect of potent bacteria on *Culex* mosquito

A potent larvicidal actinobacterial strain was selected during preliminary screening for further investigation. Third instar larvae of *Cx. quinquefasciatus* were collected and tested in bioassays with three replicates of 25 larvae each at room temperature (28 ± 2°C) for 24 hours. Tests were conducted using different concentrations of active bacterial cell free supernatant (0, 125, 250, 500, and 1000 ppm) with larvae divided into three batches of 30 in ml of water. Dechlorinated tap water served as control. Larval mortality was recorded after 24 hours of exposure, and percentage mortality was calculated as an average from the three replicates.

16S rRNA genes in Bacillus isolates were amplified using universal primers fD1 and rD1 (Weisburg *et al*., 1991) [32] and Taq polymerase at a 52°C annealing temperature. The 1.5-kb products were purified using the QIAquick PCR purification kit and sequenced with rD1, fD1, and internal primers. Sequences were analyzed using Sequence Match software mega11 and BLAST against GenBank's nonredundant nucleotide database.

Antibacterial activity

Selected pathogens (four) were cultured overnight in nutrient broth at 35 ± 1°C. Fresh cultures (0.1 mL) were swabbed onto Mueller Hinton agar (35 g L−1) plates, and 6 mm diameter wells were created using cork borer. Cell-free supernatant samples were loaded into wells at varying volumes (10, 25, 50, 75, and 100 µL) to test against selected pathogens following well diffusion method (Balasubramani *et al*., 2015). After incubation at 37°C for 18-24 hours, pathogen susceptibility to the cell extracts (CEs) was determined by measuring inhibition zone diameters around the wells.

Antibiotic sensitivity test

The sensitivity of isolated gut probiotic bacterial strains to selected antibiotics was evaluated using the disc diffusion method (Hi-Media, Mumbai). Antibiotic discs were placed on Mueller-Hinton agar plates inoculated with bacterial strains and incubated for 24 hours at 37°C. The antibiotics tested included Kanamycin (30 mcg/disc), Ampicillin (10 mcg/disc), Chloramphenicol (10 mcg/disc), Neomycin (30 mcg/disc), and Tetracycline (10 mcg/disc). After incubation, the zones of inhibition were measured to determine antibiotic sensitivity. The diameter of inhibition measured was in (mm) and the antibiotic sensitivity activity was recorded based on their activity (Kim and Austin, 2008; Kavitha *et al*., 2018) [19, 20]. The interpretations and zone sizes, respectively, were measured as aligned according to the table of the Kirby-Bauer test (Bauer *et al*., 1966) [6].

Statistical analysis

The CFS of SA5's larvicidal activity was expressed in terms of lethal concentrations LC_{50} the percentage of larval mortality data were subjected to profit analysis for calculating LC_{50} values (by converting into log base 10) and other statistics at 95% fiducial limits of upper and lower confidence and Chi square values were calculated using the software SPSS version. The statistically significant value was set $p<0.05$.

Results

The pour plate method yielded 69 distinct bacterial colonies from the *O. niloticus* gut sample when incubated at 32°C for 6 days on nutrient agar. Colony purification through subsequent streak plating revealed consistent morphological characteristics across isolates. Quadrant streak technique successfully isolated single colonies, confirming culture purity. The colonies displayed typical morphological features including circular shape, entire margins, and creamy-white coloration with convex elevation. Viable colony count indicated moderate bacterial load in the gut sample, with colonies showing optimal growth at the selected incubation parameters.

The study examined bacterial growth patterns across 15 samples (FG1-FG67) against both Gram-positive and Gram-negative bacteria. Samples FG16 and FG60 demonstrated exceptional antibacterial activity, showing strong inhibition (+++) against all tested bacterial strains. Moderate to strong activity (++ to +++) was observed in samples FG1, FG4, and FG21 against Gram-positive bacteria, particularly *Staphylococcus aureus* (Table 1). Among Gram-negative bacteria, Aeromonas species showed varying susceptibility patterns, with samples FG15, FG59, and FG67 exhibiting stronger activity (+++) against *Aeromonas Caviea*. Only sample FG3 showed complete resistance (-) to *Aeromonas hydrophila*, while maintaining activity against other tested organisms.

Table 1: Qualitative bioassays against *Cx. quinquefasciatus* larvae

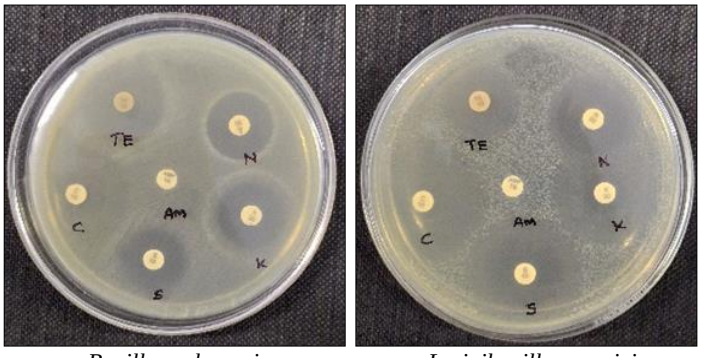
Bacterial code	Larvicidal activity against *Cx. quinquefasciatus*
FG4	50.7±2.7
FG16	70.2±3.5
FG19	56.2±4.5
FG21	52.7±6.2
FG25	55.4±3.4
FG42	50.2±3.6
FG61	62.2±4.6

Different bacterial isolates obtained from fish gut samples were evaluated for their larvicidal activity against *Cx quinquefasciatus* mosquito 3rd larval instar was used. Initial screening of 67 bacterial isolates revealed that only seven strains FG4, FG16, FG19, FG21, FG25, FG42, and FG61(Table 2) demonstrated significant larvicidal effects, with mortality rates ranging from 50.2% to 70.2%. Among these, strain FG16 exhibited the highest larvicidal activity (70.2±3.5% mortality), followed by FG61 (62.2±4.6% mortality), while the remaining five isolates showed moderate effects between 50.2% and 56.2%. The other 60 bacterial isolates showed less than 50% mortality against 4th instar *Cx. quinquefasciatus* larvae. Based on these preliminary screening results, FG16 and FG61, being the only isolates causing more than 60% mortality, were selected for further investigation as promising candidates for mosquito larval control.

The antagonistic potential of bacterial isolates FG16 and FG61 was evaluated against various human and fish pathogens using the cross-streak method. Both isolates demonstrated strong antagonistic activity (>10 mm inhibition zone) against *Escherichia coli*, *Klebsiella pneumonia*, and *Aeromonas caviae*. FG16 showed particularly strong inhibition against *Staphylococcus aureus* and *Aeromonas hydrophilia*, while FG61 exhibited strong activity against *Vibrio spp*. However, neither isolate showed any inhibitory effect against *Pseudomonas aeruginosa* and *Vibrio cholera*, indicating selective antibacterial activity. The results suggest that both FG16 and FG61 possess broad-spectrum antibacterial properties against most tested pathogens, with varying degrees of effectiveness.

Molecular phylogeny of the selected strains

Bacterial isolates, FG16, and FG61 were identified with enhanced antibacterial activities from diverse samples. They were classified into two distinct genera *Bacillus velezensis* and *Lysinibacillus capsici* based on 16S rRNA gene sequence analysis and phylogenetic analysis with neighbor-joining tree. Their sequences were submitted to NCBI GenBank with accession numbers OP217139 and MZ227505.1 *Bacillus velezensis* CARE 1.1 shares 99.9% and *Lysinibacillus capsici* shares 99.5% sequence similarity.



Bacillus velezensis *Lysinibacillus capsici*

Fig 2: Table Zone inhibition of six antibiotic sensitivity tests against the 12hour fresh culture of isolated gut strain in the disc diffusion method

Table 2: Antibiotic sensitivity test of the selected bacteria against the selective antibiotics

Antibiotics	Zone of inhibition in (mm)	
	Bacillus velezensis	***Lycinibacillus capsici***
Tetracycline (10 mcg)	20	19
Neomycin (10 mcg)	16	21
Chloramphenicol (10 mcg)	10	25
Ampicillin (10 mcg)	0	0
Kanamycin (10 mcg)	18	29
Streptomycin (10 mcg)	16	30

The antibiotic susceptibility patterns of *Bacillus velezensis* and *Lycinibacillus capsici* were evaluated using six different antibiotics at 10 mcg concentration (Table 2). In this study, both bacterial strains showed 100% resistance to Ampicillin (10 mcg), while demonstrating varying levels of susceptibility to other antibiotics. *B. velezensis* exhibited highest sensitivity to Tetracycline (20 mm), followed by Kanamycin (18 mm), while showing moderate sensitivity to Neomycin and Streptomycin (16 mm each) and lowest sensitivity to Chloramphenicol (10 mm). *L. capsici* demonstrated highest susceptibility to Streptomycin (30 mm) and Kanamycin (29 mm), followed by Chloramphenicol (25 mm), while showing moderate sensitivity to Neomycin (21 mm) and Tetracycline (19 mm).

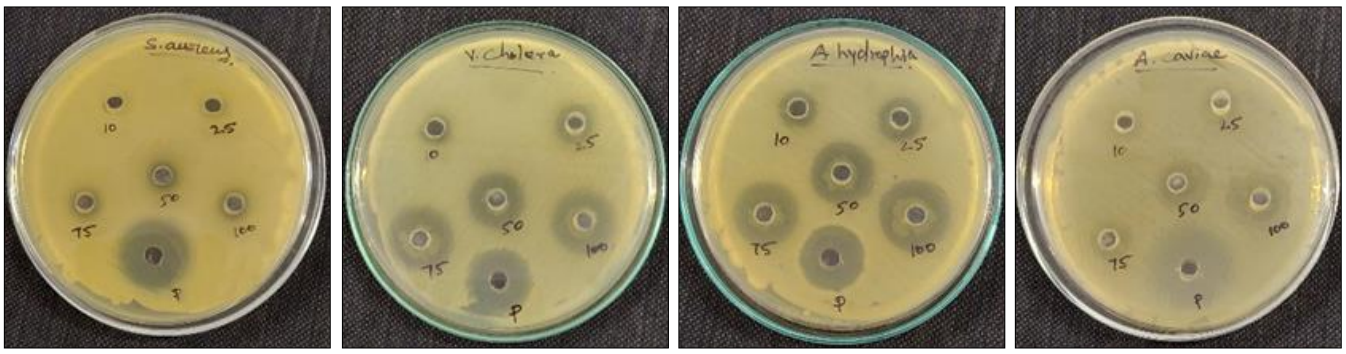


Fig 3: Antibacterial activity of *B. velezensis* against two human and fish pathogens

Fig 4: Antibacterial activity of *L. capsici* against two human and fish pathogens

Table 3: Antibacterial activity of *B. velezensis* and *L. capsici* against two human and fish pathogens

Pathogens	***Bacillus velezensis***						***Lycinibacillus capcisi***					
	10 µl	25 µl	50 µl	75 µl	100 µl	P	10 µl	25 µl	50 µl	75 µl	100 µl	P
Staphylococcus aureus	0	0	7	8	10	20	0	0	8	12	15	20
Vibrio cholerae	7	10	12	15	17	22	0	0	10	12	15	21
Aeromonas hydrophila	10	12	15	17	20	23	0	0	0	0	0	22
Aeromonas caviae	0	0	10	12	15	25	0	0	13	15	17	32

The antibacterial activity assessment revealed varying inhibition patterns for *B. velezensis* and *L. capcisi* against four pathogenic bacteria (Table 3). At concentrations ranging from 10-100 µl, *B. velezensis* demonstrated notable inhibition zones against *V. cholerae* (7-17 mm) and *A. hydrophila* (10-20 mm), while showing moderate activity against *S. aureus* (0-10 mm) and *A. caviae* (0-15 mm). *L. capcisi* exhibited stronger inhibition against *A. caviae* (0-17 mm) and S. aureus (0-15 mm), moderate activity against *V. cholerae* (0-15 mm), but showed no inhibition against *A. hydrophila*. Both bacterial strains showed concentration-dependent antibacterial activity, with maximum inhibition observed at higher concentrations of 75-100 µl. The results suggest these bacterial strains possess promising antibacterial properties that could be potentially utilized for therapeutic applications.

Table 4: Impact of different bacterial CFS make different concentration against fourth instars larvae of *Cx. Quinquefasciatus*

Bacterial CFS	Concentration (ul/50mL)	Mortality percentage		
		Cell Free Supernatant (Mean± SE)		
		12 hrs	24 hrs	48 hrs
Control	0	0.00±0.00	4.43±0.00	11.00±0.00
B. velezensis	25	15.36±3.33	27.15±3.33	35.73±3.33
	50	27.60±3.33	45.56±3.33	58.46±3.33
	100	38.54±3.33	67.63±6.66	90.00±0.00
L. capsici	25	7.66±3.33	21.10±0.00	32.20±0.00
	50	17.45±3.33	36.00±0.00	49.00±0.00
	100	26.66±3.33	58.00±0.00	82.00±0.00

The study investigated the impact of *B. velezensis* and *L. capsica* cell free supernatant from various concentrations of 25, 50, and 100 ul/50mL against fourth instar larvae of *Cx quinquefasciatus* over 12, 24, and 48-hour intervals (Table 4). Consistently, mortality percentages increased with higher concentrations across. In the 12-hour analysis, the highest mortality percentage 38.54% was observed in *B. velezensis* of supernatant (df= 3,8; F= 28.23 p= 0.000) followed by *L. capsici* supernatant 26.66% (df= 3,8; F= 17.56; p= 0.000) at higher concentrations compared to the control. *B. velezensis* supernatant exhibited the best larvicidal activity with an LC_{50} value of 148.47mg/50mL, followed by *L. capsica* supernatant 159.86 mg/50mL and others. In the 24-hour assessment, *B. Velezensis* supernatant (67.63%) displayed the highest mortality percentages (df= 3,8; F= 19.83; p= 0.000), followed by 58.00% of *L. capsici* supernatant (df= 3,8; F= 15.33; p= 0.000) at higher concentrations compared to the control. Notably, *B. velezensis* supernatant showed the best larvicidal activity with an LC50 value of 64.22 mg/50mL, followed by *L. capsici* supernatant 77.68 mg/50mL. In the 48-hour analysis, the highest mortality percentages were recorded in *B. velezensis* 90% (df= 3,8; F= 80.00; p= 0.000), followed by *L. capsici 82%* (df= 3,8; F= 77.50; p= 0.000) at higher concentrations compared to the control. *B. velezensis* showed the best larvicidal activity with an LC50 value of 42.19 mg/50mL, followed by *L. capsici* 47.67 mg/50mL and others (Table 5).

Table 5: Probit analysis of different bacterial CFS against forth instar larvae of *Culex quinquefasciatus*

Bacterial CFS	LC_{50}		
	12 hrs	24 hrs	48 hrs
Bacillus velezensis	148.47	64.22	42.19
Lycinibacillus capsici	159.86	77.68	47.67

Discussion

The current study explored the isolation and characterization of antibacterial and larvicidal bacterial strains from the gut microbiome of the Nile tilapia, *Oreochromis niloticus*. The pore plate method yielded 69 distinct bacterial colonies, which were further purified and evaluated for their biological activities. Two bacterial isolates, designated as FG16 and FG61, demonstrated exceptional antibacterial and larvicidal properties compared to the other isolates. Phylogenetic analysis based on 16S rRNA gene sequencing identified FG16 as *Bacillus velezensis* and FG61 as *Lysinibacillus capsici*.

Both strains exhibited broad-spectrum antibacterial activity against a panel of human and fish pathogens, including *Escherichia coli*, *Klebsiella pneumonia*, *Aeromonas caviae*, *Staphylococcus aureus*, and *Aeromonas hydrophilia*. The inhibition zones produced by these strains ranged from 10 to 30 mm, suggesting their potential as sources of antibacterial compounds. Baharudin *et al*., (2021) [5] *Bacillus velezensis* strain PD9, isolated from stingless bee products, exhibited potent antimicrobial capabilities against methicillin-resistant *Staphylococcus aureus*, presenting a promising alternative therapeutic approach to conventional antibiotics. Research by Dahmana *et al*., (2020) [10] demonstrated the significant larvicidal potential of various bacterial species against mosquito larvae. Among these, *Bacillus nealsonii* emerged as the most effective, achieving a remarkable 70% larval mortality rate.

The larvicidal potential of *B. velezensis* and *L. capsici* was evaluated against fourth-instar *Culex quinquefasciatus* mosquito larvae. Both strains displayed significant larvicidal activity, with *B. velezensis* exhibiting the highest mortality rate of 70.2% at the highest tested concentration. The larvicidal effect was concentration-dependent, with higher supernatant concentrations resulting in greater larval mortality. (Falqueto *et al*., 2020) [13] demonstrated the remarkable larvicidal efficacy of *Bacillus velezensis* strains against *Aedes aegypti* larvae, with *B. velezensis* B64a and B15 generating crude lipopeptide extracts (CLEs) exhibiting LC50 values of 0.11 mg/mL and 0.12 mg/mL, respectively. In our study the LC50 values for *B. velezensis* and *L. capsici* were 42.19 mg/50mL and 47.67 mg/50mL, respectively, at 48 hours of exposure, indicating their potent mosquito-controlling properties. The antibacterial and larvicidal properties of *B. velezensis* and *L. capsici* can be attributed to their capacity to synthesize diverse bioactive metabolites, enzymes, and toxins. Drawing from previous research by (Arguelles-Arias *et al*., 2009) [4], *Bacillus* species are known for producing a comprehensive array of antimicrobial compounds, including lipopeptides, polyketides, and bacteriocins, which effectively suppress pathogenic bacterial growth. Similarly, *Lysinibacillus* species demonstrate notable insecticidal and larvicidal characteristics, with certain strains exhibiting antimicrobial potential through the production of bacteriocins, peptide antibiotics, and other therapeutic molecules, as highlighted by (Jamal and Ahmad 2022) [17].

The observed antibiotic resistance patterns of *B. velezensis* and *L. capsici* suggest that these strains may be promising candidates for the development of alternative antimicrobial and larvicidal agents. The high resistance to ampicillin and variable susceptibility to other antibiotics indicate that these bacterial isolates possess unique mechanisms to withstand certain classes of antibiotics, which could be further explored and exploited for therapeutic applications.

Conclusion

This study successfully isolated and characterized two potent bacterial strains, Bacillus velezensis (FG16) and *Lysinibacillus capsici* (FG61), from the gut microbiome of *Oreochromis niloticus*. Both isolates exhibited significant broad-spectrum antibacterial activity against various human and fish pathogens. B. *velezensis* showed the highest sensitivity to Tetracycline, while L. capsici was most susceptible to Streptomycin. Additionally, both strains demonstrated remarkable larvicidal efficacy against *Culex quinquefasciatus*, with B. velezensis achieving maximum mortality and LC50 values after 48 hours of exposure. The antibiotic resistance profiles and biological activities of these strains suggest their potential as sources for novel antibacterial and larvicidal agents. The findings emphasize the diversity of bioactive compound-producing bacteria within the fish gut microbiome and their potential applications in therapeutic and vector control strategies. Further research on isolating, characterizing, and optimizing these bioactive compounds could lead to new biological control agents and therapeutic solutions. This study provides valuable insights into the biotechnological potential of fish gut microbiota and opens new avenues for sustainable mosquito control and antibacterial drug development.

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