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Research Article

The Effectiveness of *Limnobium laevigatum* in Inhibiting the Growth of Microbes Found in Lake Water

Teryna Thu Pui Xing*, Teo Swee Sen

Department of Biotechnology, Faculty of Applied Science, UCSI University, No. 1, UCSI Heights, Jalan Puncak Menara Gading, Taman Connaught, 56000 Cheras, Malaysia
E-mail: teryna2my@gmail.com

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Abstract: *Limnobium laevigatum*, commonly called Amazon frogbit, belongs to the Hydrocharitaceae family and thrives in freshwater environments with abundant lighting. This perennial herb has a unique characteristic of floating above the water's surface but can also establish roots in shallow water. Several studies showed that the roots of *L. laevigatum* have high removal efficiencies of heavy metals in wasted water up to a concentration of 70%, particularly Zn. However, the investigation of *L. laevigatum* to treat microorganisms in contaminated water still needs to be explored. After serial dilution, a lake water sample was isolated to identify the types of microbes. Gram-staining was applied as a preliminary analysis for further identification of the types of microbes by observing under a compound microscope. Following identification, 6 gram-positive and 4 gram-negative bacteria were tested with different amounts of *L. laevigatum* powder, 0.1 g, 0.2 g, and 0.3 g using a mass spectrophotometer to determine the effectiveness of inhibiting the growth of selected microbes. Gram-staining revealed that 66% of isolates are categorized as gram-positive bacteria, while 34% are gram-negative. Further observation shows that 75.75% of isolates from gram-positive bacteria are bacillus-shaped, and 24.25% are cocci-shaped among all gram-positive. 0.1 g of powder shows the least effect on inhibiting Gram-positive bacteria growth, whereas 0.3 g of powder shows the most impact on bacteria. Graphs with standard deviation (SD) values were plotted to compare the effectiveness between different concentrations of *L. laevigatum* and the differences between Gram-positive and Gram-negative bacteria. The antimicrobial properties of *L. laevigatum* show minimal effect on Gram-negative than Gram-positive bacteria. This research aims to assess the efficacy of *L. laevigatum* for inhibiting microbes' growth, offering practical applications for sustainable water treatment, contributing to eco-friendly solutions, and promoting environmental sustainability.

***Keywords*:** lake water, gram-staining, gram-positive, gram-negative, *Limnobium laevigatum*, mass spectrophotometer, microorganism inhibition

1. Introduction

Limnobium laevigatum, commonly known as Amazon frogbit, is a member of the Hydrocharitaceae family [1]. This perennial herb displays a distinctive trait of floating on the water's surface while also being capable of rooting in shallow waters. The leaves of *L. laevigatum* are characterized by their subcircular shape, smooth texture, and glossy appearance on the upper surface. Beneath the surface, they contain a dense, air-filled, spongy tissue with a rounded or

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slightly cordate base. This unique feature allows the perennial herb to remain buoyant, consistently floating above the water's surface. However, the lower part of the plant and its roots extend downward, anchoring it in shallow waters, particularly in freshwater habitats with soft water and abundant lighting [1].

According to the research of the National Hydraulic Research Institute of Malaysia (NAHRIM) [2], 62% of lakes were eutrophic, and 38% were mesotrophic as their Trophic State Index (TSI) value exceeded 47.4, which is graded as bad. Subsequent investigations revealed that water quality in Malaysia is impacted by external sources, which can be classified into organic and inorganic pollutants [2]. In Malaysia, contaminated water has a wide range of effects on the environment and public health, as inadequate waste management, industrialization, urbanization, and agriculture have decreased the water quality by bringing pollutants, including pesticides, heavy metals, and pathogens, into lake habitats [2, 3]. In this case, lakes are no longer suitable for drinking or recreational purposes. Pollution, especially nutrients from sewage discharge and agricultural runoff, causes eutrophication, leading to algal blooms, oxygen deprivation, and disturbances in aquatic food chains. Fish population reductions, biodiversity loss, and deterioration of habitat quality have become major problems of this ecological imbalance [3, 4].

The first common way to treat wastewater is through physical water treatment. Sedimentation is a major physical wastewater treatment technique separating clean water and heavy or insoluble particles as these particles will sink to the bottom. Filtration is another method involving running the effluent through certain filters to remove impurities [5]. The other common way is chemical water treatment. An oxidising chemical called chlorine is often used to eliminate the microorganisms that contaminate water [5]. Neutralisation could also help adjust the pH of the water to its normal level of 7 by adding either an acid or a base. These chemicals can efficiently maintain the purity of the water by blocking the growth of microorganisms in water [5].

There are several researches and studies have shown that the roots of *L. laevigatum* have high removal efficiencies of lead (Pb) and zinc (Zn) in wasted water up to 70% of the concentration but are more effective towards Zn [6, 7]. Also, *L. laevigatum* is commonly bred in fish or shrimp tanks as it can efficiently remove extra nutrients in water [8]. According to Kokoszka et al. [9], sulfamethoxazole (SMX) and trimethoprim (TRI) from *L. laevigatum* can treat bacteria and protozoa in contaminated water. However, the investigation of *L. laevigatum* to treat microorganisms in contaminated water still needs to be explored.

In this research, *L. laevigatum* was tested in different concentrations against selected bacteria extracted from lake water to determine the effectiveness of *L. laevigatum* in preventing microbial development by observing the absorbance reading. In addition, this research aims to identify the availability of *L. laevigatum* in replacing chemicals such as chlorine to minimize microbe growth [10]. This plant has the potential to be a natural alternative to chemical agents such as chlorine for reducing microbe proliferation. This study attempts to support environmentally sustainable methods in water management by investigating the plant's potential as a natural solution.

2. Materials and methods

All research works for this project were conducted in the Microbiology Laboratory, UCSI University, Kuala Lumpur Campus, Malaysia. This research was carried out to determine the effectiveness of *L. laevigatum* in inhibiting the growth of selected microbes found in lake water.

2.1 *Procurement of instruments, apparatus, consumables, chemicals, reagents and materials*

2.1.1 *Instruments*

Table 1 shows the list of instruments and the manufacturer involved in this study, which mostly can be obtained in a microbiology lab at UCSI University, Kuala Lumpur Campus, Malaysia.

2.1.2 *Apparatus and consumables*

Table 2 shows the list of apparatuses and consumables used by the manufacturer in this research. All apparatuses are located in the laboratories of UCSI University, Kuala Lumpur Campus, Malaysia.

Table 1. List of instruments with the manufacturer

Instruments	Model	Manufacturer
Grinder	-	Kenwood, British
pH-meter	-	Fisher Scientific
Water bath	WNB 14	Memmert, Germany
Autoclave machine	HV-110	Hirayama
Chiller	PQS 201	Panasonic
Laminar air flow	AHC-4D1	ESCO, Singapore
Electromechanical agitator vortex	VTX-3000L	LMS
Shake incubator	IN30plus	Memmert, Germany
Mass spectrophotometer	UviLine 9400	AHS
Compound light microscope	-	Isodynamique Group of Companies, Malaysia
Analytical balance	EL-2000S	Setra

Table 2. List of apparatus and consumables with the manufacturer

Apparatus and consumables	Model	Manufacturer
Scott bottles	250, 1,000 mL	-
Measuring cylinder	10, 100 mL	Sigma Aldrich, USA
Timer	-	-
Inoculation loop	-	-
Petri dishes	90 mm	Biofil, Spain
Beaker	50 mL	Labmart
Micropipette	20-200, 100-1,000 µL	-
Micropipette tips	20-200, 100-1,000 µL	Gilsen Pipettes, Malaysia
Centrifuge tubes	15 mL	-
Tube rack	-	-
Kimwipes	-	Kimberly-Clark, USA
Disposable latex gloves	S size, powder-free	-
Glass spreader	-	-
Bunsen burner	-	-
Cuvette	-	-
Glass slide	Clear glass, ground edges	Sail Brand

2.1.3 *Chemicals and reagents*

Table 3 shows all chemicals and reagents with the supplier information used in this study.

Table 3. List of chemicals and reagents with the suppliers

Chemicals and reagents	Suppliers
Nutrient broth powder	-
Nutrient agar powder	-
Distilled water	Syarikat Copens Enterprise, Malaysia
Luria broth powder	-
MYP agar powder	-
MacConkey Agar powder	-
Chlorine pellet	Quanxingnan
Gram-stain kit	Chemiz, Germany

2.1.4 *Materials*

Table 4 shows the materials used in this research with the supplier's details.

Table 4. List of materials with the suppliers

Materials	Suppliers
L. laevigatum	Aquatic Laboratory, UCSI University

2.2 *Preparation of materials*

2.2.1 *L. laevigatum powder*

As *L. laevigatum* can be easily grown in freshwater during the tropical season and spread easily, 100 seedlings of *L. laevigatum* were obtained from the aquatic laboratory at UCSI University, Kuala Lumpur, and grown under the sun. The plants were grown until they matured, 10 times their previous weight. All the plants were washed clean with tap water and air-dried under hot sun for 3 days. After the plants were dried, they were ground into powder using a grinder.

2.2.2 *Nutrient broth*

The nutrient broth was prepared by adding 13.0 g of nutrient broth powder measured with analytical balance and 1,000 mL distilled water into a 1,000 mL Schott bottle. The powder was mixed to dissolve in water completely. The pH level of the mixture was fixed at 6.8 ± 0.2 and heated up to 50 °C using a water bath until the powder was completely dissolved. The solution was then autoclaved at 121 °C for 15 minutes for sterilization. Once cooled down after autoclaving, the sterilized solution was stored in the chiller at 4 °C.

2.2.3 *Luria broth (LB)*

The Luria broth was prepared by weighing 13.0 g of powder and suspending it into 1,000 mL distilled water in a 1,000 mL Schott bottle. The powder was correctly mixed before the pH level of the solution was fixed at 6.8 ± 0.2. The solution was then heated to 50 °C using a water bath until the powder was completely dissolved. The solution was sent to the autoclave for sterilization at 121 °C for 15 minutes. The sterile solution was stored in the chiller at 4 °C for further usage.

2.2.4 *Nutrient agar*

The nutrient agar was prepared by adding 28.0 g of nutrient agar powder weighed using an analytical balance with 1,000 mL distilled water into a 1,000 mL Schott bottle. After the powder was dissolved completely, the pH level was adjusted to 6.8 ± 0.2. The solution was then heated up to 50 °C using a water bath until the powder was completely dissolved, and it was sent to an autoclave at 121 °C for 15 minutes. After leaving it cool at room temperature of 25 °C, the solution was poured into empty Petri dishes, and one petri dish was approximately 20 mL of agar solution. This process was done under laminar airflow to prevent contamination during nutrient agar preparation. Once the nutrient agar was solidified, the plates were stored in the chiller at 4 °C for further use.

2.2.5 *Mannitol-egg yolk-polymyxin (MYP) agar*

MYP agar was prepared by suspending 46.0 g of the agar powder in 900 mL of distilled water and gently boiling it until it completely dissolved. The pH level was then adjusted at 7.2 ± 0.2 before heated up to 50 °C with a water bath till the powder was dissolved thoroughly. The solution was sterilized using an autoclave at 121 °C for 15 minutes. The solution was then left cool at room temperature, approximately 25 °C, and poured into empty petri dishes. One petri dish was approximately 20 mL of agar solution. This process was done under laminar airflow to prevent contamination during nutrient agar preparation. After the agar was solidified, the plates were stored in a chiller at 4 °C for further use.

2.2.6 *MacConkey agar*

MacConkey agar was done by suspending 52.0 g of MacConkey agar powder in 1,000 mL of distilled water, and the pH level was fixed at 7.1 ± 0.2 before being heated up to 50 °C for the powder to dissolve thoroughly. The agar solution was autoclaved at 121 °C for 15 minutes and left to cool at room temperature, which is 25 °C. The solution was then poured into empty Petri dishes, and one petri dish was approximately 20 mL under a laminar air hood to avoid contamination. After the agar was solidified, the plates were stored in the chiller at 4 °C for further use.

2.3 *Serial dilution of lake water sample*

In this research, natural wastewater was used instead of synthetic wastewater, as natural wastewater reflects the actual composition of wastewater found in real-world scenarios. This is important in studies aiming to understand the behavior of contaminants and microorganisms under authentic conditions. The location and the details of the lake water sample collected are shown in Table 5.

Table 5. The location and the details of the lake water sample

Location	Address	Magnitude of location	Date collected	Time collected
Taman Tasik Permaisuri	Jalan Tasik Permaisuri 2, Bandar Tun Razak, 56000 Kuala Lumpur, Wilayah Persekutuan Kuala Lumpur	3.0980° N, 101.7190° E	19th August 2023	11.00 am

After the lake water samples were collected, serial dilution was made by labeling a few sterile test tubes with the dilution factor from 10^{-1} to 10^{-6}. 1 mL of lake water samples and 9 mL of distilled water was added into the test tube labeled 10^{-1} and mixed thoroughly. After mixing, 1 mL of 10^{-1} sample was transferred to a test tube labeled 10^{-2}. The serial dilution steps were then repeated till 10^{-6}.

2.4 *Microorganism isolation, cultivation and identification*

2.4.1 *Preliminary screening for microorganisms*

The spread plate method isolated the unidentified microbes from lake water samples by transferring 100 µL of samples onto the center of nutrient agar using a micropipette. The bent end of the glass spreader was dipped in alcohol and was sterilized by flaming it with a Bunsen burner. After cooling, the water sample was spread with the sterilized glass spreader over the surface of nutrient agar and then incubated inside the incubator for 48 hours at 37 °C. The nutrient agar was observed daily till approximately 30 to 300 colonies were formed.

2.4.2 *Isolation of microorganisms*

After incubation, multiple single colonies have grown on the nutrient agar, which can be visually seen. Fifty colonies were randomly selected from the nutrient agar. They were added to the nutrient broth to produce stock culture by inoculating with a sterile inoculation loop and were put into a labeled centrifuge tube containing 3 mL nutrient broth. The inoculated nutrient broth was incubated with a cell shaker incubator at 200 rpm, 37 °C for 24 hours.

2.4.3 *Gram-staining*

After the stock cultures were grown and shaken, a loopful of culture broth was placed on a labeled glass slide using a sterilization inoculation loop and spread in a circular motion to confirm the shape and types of bacteria. The smear was heat-fixed by passing the glass slide through the Bunsen burner with the smear facing up a few times. The smear was covered by a drop of crystal violet and left for 60 seconds. The crystal violet was then poured off and rinsed gently with tap water. The step was repeated using Gram's Iodine and decolourized with ethanol before it was counter-stained by Safranin. After air-drying the stained smear, the results were observed under the microscope, whereby shapes with either Gram-positive or Gram-negative bacteria could be identified. Purple colour should appear for Gram-positive whereas pink colour should be present for Gram-negative bacteria.

2.4.4 *Bacillus sp. colony screening and identification*

The identification of *Bacillus sp*. colonies was grown on specific agar, which is MYP agar. After being observed under a microscope, 10 Gram-positive bacteria were selected among the 50 stock cultures again and were grown on MYP agar prepared using the spread plate method. After incubating at 37 °C for 24 hours, visible colonies were seen on the MYP agar. 1 colony of selected microbes was picked and transferred into a new centrifuge tube containing 6 mL of Luria broth to produce a stock culture of the selected microbes by incubating using a cell shaker incubator at 200 rpm, 37 °C for 24 hours.

2.4.5 *Vibrio sp. colony screening and identification*

Like *Bacillus sp*., *Vibrio sp*. colonies were identified by growing on a specific MacConkey agar. However, after being observed under a microscope, only 8 Gram-negative with rod-shaped bacteria were found among the 50 stock cultures and were grown on MacConkey agar that was prepared previously using the spread plate method. After incubating at 37 °C for 24 hours, visible colonies were seen on the MacConkey agar. 1 colony of selected microbes was picked and transferred into a new centrifuge tube containing 6 mL of Luria broth to produce a stock culture of the selected microbes by incubating using a cell shaker incubator at 200 rpm, 37 °C for 24 hours.

2.5 *Determination of the efficiency in inhibiting growth of selected microbes*

To test the efficacy of *L. laevigatum* in inhibiting the growth of selected microbes, the shake culture undergoes serial dilution again to fix the bacterial concentration at OD0.3 in a new centrifuge tube. The number of bacteria per millimeter was calculated using the formula: colony-forming units (CFU)/mL = Average colony count/amount plated × dilution factor. The shake culture was incubated at 37 °C with shaking and read OD600 absorbance. 0.1 g of *L. laevigatum* powder was then added into the tube and was mixed evenly. Table 6 shows the amount of solution prepared for measurement of absorbance.

Table 6. The amount required for a diluted shake culture with *L. laevigatum* powder

Materials	Amount
Shake culture	0.5 mL
Luria Broth	4.5 mL
L. laevigatum powder	0.1 g

The absorbance was then read and collected at OD600 every 1 hour, and 6 data were collected. After taking all the data, the processes were repeated using 0.2 g and 0.3 g of *L. laevigatum* powder. These processes were also repeated with 0.5 mL of Gram-negative bacteria.

As for positive control, *L. laevigatum* powder was replaced by 1 mL of chlorine water by adding 2 chlorine pellets into 500 mL of distilled water to test the ability of *L. laevigatum* to replace chemicals for inhibiting the growth of microbes.

2.6 *Statistical analysis*

The absorbance reading obtained from conducted tests was subjected to standard analysis using Microsoft Excel for statistical analysis. The triplicate readings for all tests were calculated using the average reading using the Excel formula "= AVERAGE". Standard deviation values were also obtained by the Excel formula "= STDDEV". After that, the averaged data were plotted into a bar chart to study the pattern of the graph of the efficiency of *L. laevigatum* in inhibiting microbes' growth. One-way analysis of variance (one-way ANOVA) was done to compare the efficiency of *L. laevigatum* in inhibiting the growth of selected microorganisms over time. SPSS software was also used to identify the significant differences among all raw data. Differences were statistically significant for *p*-values smaller than 0.05 ($P < 0.05$).

3. Results

3.1 *Gram-staining of bacteria*

After isolating 50 selected colonies into the nutrient broth, Gram-staining was done to identify types of bacteria. The shapes and arrangements of 50 colonies were also observed under a compound microscope. However, sample S-24 has both purple and pink colours after undergoing Gram-staining. This is due to sample S-24 containing Gram-positive and Gram-negative as it was not appropriately isolated due to technical error. In this case, colony S-24 was then isolated once again. In this experiment, the bacteria are divided into rod and sphere shapes. The bacteria are categorized into a few types: single, diplo, staphylo, strepto, and palisade. Table 7 presents the final results of the characteristics of 50 colonies isolated from lake water samples.

Table 7. The characteristics of 50 selected colonies

Sample	Shape	Gram	Arrangement
S-01	Sphere	+	Single, Staphylo, Strepto
S-02	Rod	-	Single, Staphylo, Strepto
S-03	Sphere	+	Single, Strepto
S-04	Sphere	-	Single, Staphylo, Strepto
S-05	Rod	+	Single, Strepto
S-06	Sphere	-	Staphylo
S-07	Rod	-	Staphylo
S-08	Rod	+	Single, Staphylo, Strepto
S-09	Rod	+	Single, Strepto
S-10	Rod	-	Single, Strepto
S-11	Sphere	-	Single, Staphylo, Strepto
S-12	Rod	+	Single, Strepto
S-13	Sphere	-	Single
S-14	Rod	+	Single, Staphylo, Strepto
S-15	Rod	-	Staphylo
S-16	Sphere	-	Single, Diplo, Strepto
S-17	Rod	-	Single, Staphylo
S-18	Rod	+	Single, Strepto
S-19	Rod	+	Single, Diplo
S-20	Sphere	-	Single, Staphylo, Strepto
S-21	Sphere	+	Staphylo, Strepto, Diplo
S-22	Rod	+	Single, Strepto, Palisade
S-23	Rod	+	Single, Staphylo, Strepto
S-24	Rod	+	Staphylo, Strepto
S-25	Rod	+	Staphylo, Strepto
S-26	Rod	+	Single, Staphylo, Strepto
S-27	Rod	-	Staphylo
S-28	Rod	+	Single, Staphylo
S-29	Rod	+	Single, Strepto
S-30	Rod	+	Single
S-31	Rod	+	Single, Staphylo, Strepto
S-32	Rod	+	Single, Staphylo, Strepto
S-33	Rod	+	Single, Staphylo
S-34	Rod	+	Single, Staphylo, Strepto
S-35	Rod	+	Single, Staphylo, Strepto
S-36	Rod	+	Single, Strepto

Table 7. (cont.)

Sample	Shape	Gram	Arrangement
S-37	Rod	-	Single, Strepto
S-38	Rod	+	Single, Staphylo, Strepto
S-39	Rod	-	Single, Staphylo, Palisade
S-40	Rod	+	Single
S-41	Rod	+	Single, Strepto
S-42	Sphere	+	Single, Diplo, Staphylo
S-43	Sphere	+	Single, Diplo, Staphylo, Strepto
S-44	Rod	+	Single, Strepto
S-45	Sphere	+	Single, Strepto
S-46	Sphere	-	Single, Diplo, Staphylo
S-47	Sphere	+	Single, Staphylo, Strepto
S-48	Rod	-	Single, Staphylo
S-49	Rod	-	Single, Staphylo, Strepto
S-50	Sphere	+	Single, Diplo, Strepto

This study proves the biodiversity of bacteria in lake water. Fifty colonies were isolated and identified from lake water, and Gram-staining was applied as a preliminary analysis to identify the microbes derived from the water samples. The bacteria were then classified according to physical characteristics (Table 7). According to the results from Table 7, 66% of isolates are categorized as Gram-positive bacteria, while 34% are Gram-negative. Additional research reveals that out % of all gram-positive bacteria isolates, 75.75% are bacillus-shaped bacteria, and 24.25% are cocci-shaped bacteria. Mostly, all isolates have the arrangement of single, staphylo and strepto. Among all Gram-positive bacteria, 33% are under single, staphylo and strepto arrangement, whereas 29.4% of Gram-negative bacteria have the same arrangement. Other arrangements, such as diplo and palisade, were found during observation. Figure 1 shows Gram-positive (left) and Gram-negative (right) bacteria isolated from lake water samples under 10× magnification.

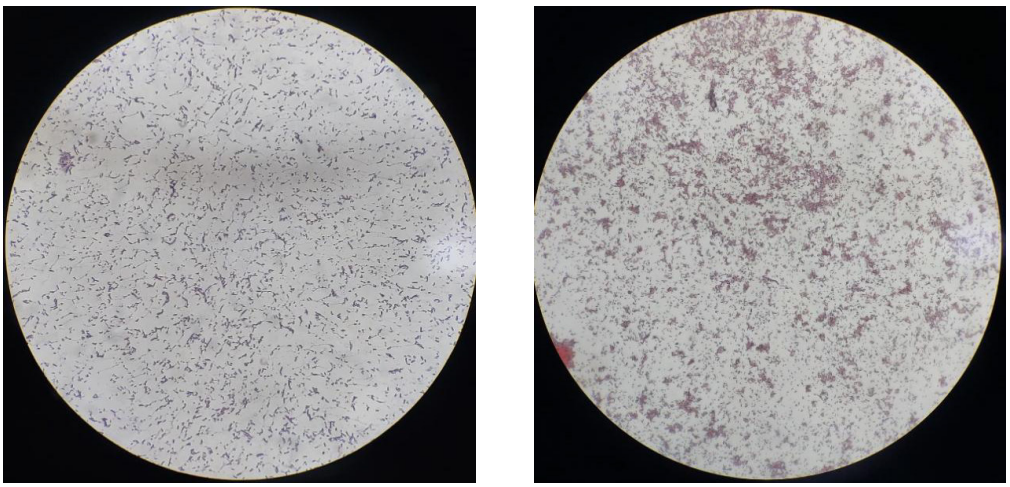

Figure 1. Gram-positive (left) and Gram-negative (right) bacteria isolated from lake water samples under 10× magnification

3.1.1 *Gram-positive, rod-shaped bacteria*

For further identification, colony screening was done by isolating 10 random Gram-positive and 10 random Gram-negative bacteria with specific agar, MYP agar for Gram-positive, and MacConkey agar for Gram-negative bacteria. Table 8 shows the characteristics of 10 selected Gram-positive, rod-shaped bacteria grown on MYP agar after incubation.

Table 8. The characteristics of 10 selected Gram-positive, rod-shaped bacteria grown on MYP agar

Gram-positive, rod shaped samples	Colour of colonies	Presence of white precipitate around colonies	Colour changes of MYP agar	
			Before	After
S-09	White	Yes	Red	Orange-pink
S-11	White	Yes	Red	Yellow-orange
S-14	White	No, too little	Red	Yellow-orange
S-18	White	Yes	Red	Orange-pink
S-19	White	Yes	Red	Orange-pink
S-23	White	Yes	Red	Orange-pink
S-25	White	Yes	Red	Yellow
S-36	White	Yes	Red	Pink
S-41	White	Yes	Red	Orange-pink
S-44	White	Yes	Red	Yellow

According to Table 8, all colonies produced are white, but 6 of MYP agar changed its colour to orange-pink while the remaining 4 did not alter their orange-yellow colour. MYP agar is commonly used to isolate *B. cereus* in unprocessed food, soil, or wastewater. Gram-negative bacteria cannot grow on MYP agar. In contrast, some bacteria change the pH of the agar by fermenting mannitol, which can cause colour changes in the agar to orange-yellow. As *Bacillus sp*. are mannitol-negative and lecithinase-positive on MYP agar, only S-09, S-18, S-19, S-23, S-36 and S-41 are selected for the next step as they did not show any colour changes on MYP agar.

3.1.2 *Gram-negative, rod-shaped bacteria*

Despite this, among the 50 stock cultures that were grown on MacConkey, only 8 Gram-negative, rod-shaped bacteria were discovered upon microscopic analysis. The characteristics of selected Gram-negative, rod-shaped bacteria on MacConkey agar are shown in Table 9.

However, 4 Gram-negative isolates could not grow on MacConkey agar as no growth was seen after 24 hours of incubation. These four isolates also did not show any colour changes of the MacConkey agar and showed white opaque colonies. The breakdown of lactose by the fermentation process produces organic acids, especially lactic acid, leading to a decrease in the pH of the agar. The MacConkey agar contains a pH indicator exhibiting pink colouration under acidic conditions. Gram-negative bacteria ferment lactose, known as lactose fermenters, form pink colonies, while non-lactose fermenters produce off-white and opaque colonies [11]. Throughout these colony screenings, it was proven that these four isolates were non-lactose fermenters and had the possibility of being bacteria such as *Salmonella*, *Pseudomonas*, *Shigella*, and others. Therefore, isolates S-02, S-10, S-48 and S-49 were continued for the next step.

Table 9. The characteristics of selected Gram-negative, rod-shaped bacteria on MacConkey agar

Gram-negative, rod shaped samples	Colonies		
	Presence	Lactose-fermenter	Colour
S-02	Yes	No	White, opaque
S-07	No	-	-
S-10	Yes	No	White, opaque
S-15	No	-	-
S-17	No	-	-
S-37	No	-	-
S-48	Yes	No	White, opaque
S-49	Yes	No	White, opaque

3.2 *Determination of the efficiency of L. laevigatum in inhibiting selected microbes' growth*

The absorbance of selected Gram-positive and Gram-negative bacteria with different amounts of *L. laevigatum* powder and chlorine was recorded. Each absorbance reading was taken down after every 1 hour of incubation at room temperature. All absorbance readings were taken triplicate for a more accurate result. To compare all data, 10 graphs in total, 6 graphs of Gram-positive bacteria and 4 graphs of Gram-negative bacteria, were plotted between different amounts of *L. laevigatum* powder.

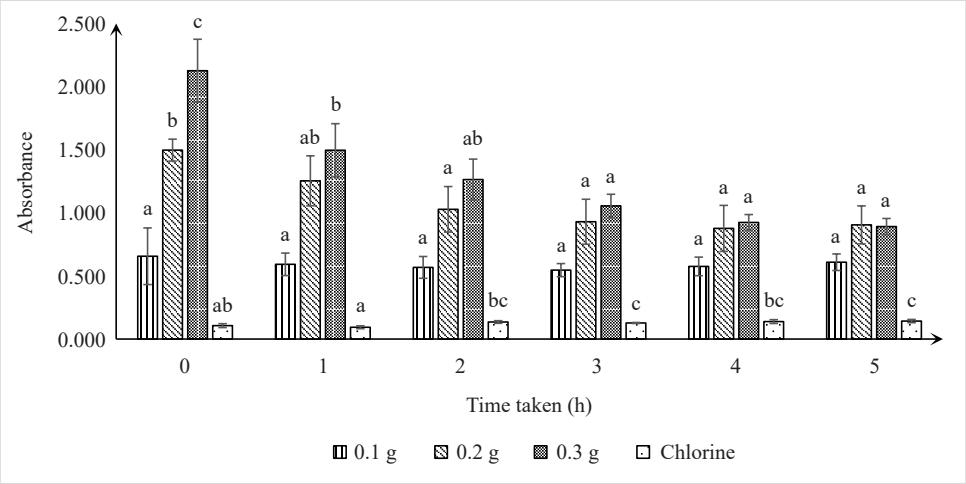

Figure 2. Graph of absorbance S-41 affected by different amounts of *L. laevigatum* and chlorine over time (h)

3.2.1 *Gram-positive, rod-shaped bacteria*

In total of 6 graphs were plotted for each Gram-positive rod-shaped bacterium inhibited by different amounts of *L. laevigatum* powder and chlorine over 5 hours. The graphs show a distinct pattern of bacterial inhibition over time

for each concentration of *L. laevigatum* powder and chlorine. All plots show relatively high absorbance values at the beginning of the experiment, which shows minimal bacterial inhibition. However, the absorbance reading gradually decreases, indicating a decreasing bacterial growth rate. A higher concentration, 0.3 g powder, generally exhibits more significant bacterial inhibition. However, chlorine showed a huge effect on bacterial inhibition at the beginning of the experiment, but the microbial growth increased in the following hour. Figure 2 shows a graph of absorbance affected by different amounts of *L. laevigatum* and chlorine over time (h).

Overall, the results highlight the interaction between bacteria growth and different concentrations of *L. laevigatum* over time. Additionally, the positive control, chlorine, shows critical inhibition and acts as a standard to measure the antimicrobial activity of *L. laevigatum*. Table 10 indicate the average absorbance of each sample with different amounts of *L. laevigatum* and chlorine over time.

Table 10. The average absorbance of S-41 with different amounts of *L. laevigatum* and chlorine over time

S-41	Time taken (h)					
	0	1	2	3	4	5
0.1 g	0.656 ± 0.22	0.592 ± 0.09	0.568 ± 0.09	0.546 ± 0.05	0.575 ± 0.07	0.609 ± 0.07
0.2 g	1.497 ± 0.09	1.253 ± 0.20	1.028 ± 0.18	0.929 ± 0.18	0.877 ± 0.18	0.904 ± 0.15
0.3 g	2.126 ± 0.25	2.496 ± 0.21	1.263 ± 0.16	1.054 ± 0.09	0.924 ± 0.06	0.891 ± 0.06
1 mL Chlorine	0.106 ± 0.02	0.093 ± 0.01	0.135 ± 0.01	0.127 ± 0.00	0.138 ± 0.02	0.142 ± 0.01

3.2.2 *Gram-negative rod-shaped bacteria*

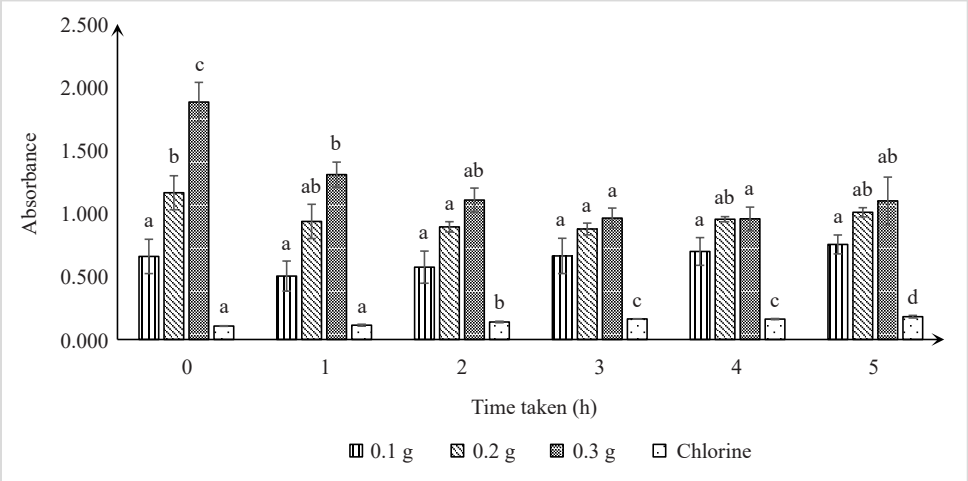

Figure 3. Graph of absorbance S-02 affected by different amounts of *L. laevigatum* and chlorine over time (h)

Similar to Gram-positive rod-shaped bacteria, the experiment was also conducted with Gram-negative rod-shaped bacteria. 4 bar charts for each sample were plotted to compare the absorbance affected by different amounts of *L. laevigatum* powder and the positive control, chlorine. Notably, there are various changes in the trend line for some samples. For S-02 and S-10, a high absorbance value was shown at the beginning of the experiment. However,

the absorbance value decreases at the first hour, indicating the inhibition of microbial growth, but gradually increases after a few hours of the experiment, which shows no inhibition effect on bacterial development. For S-48 and S-49, the absorbance value started high initially and gradually decreased over time. As for positive control, chlorine shows the elimination of bacterial growth, as a low absorbance value was observed at the start of the experiment. However, the absorbance value increases after 1st hour, indicating no antimicrobial efficacy for Gram-negative bacterial growth. Like Gram-positive bacteria, the highest concentration of *L. laevigatum* powder, 0.3 g, shows the greatest inhibition effect of bacterial growth for Gram-negative bacteria. Figures 3 and 4 show the graph of absorbance of each Gram-negative rod-shaped bacteria sample affected by different concentrations of *L. laevigatum* and chlorine over time (h).

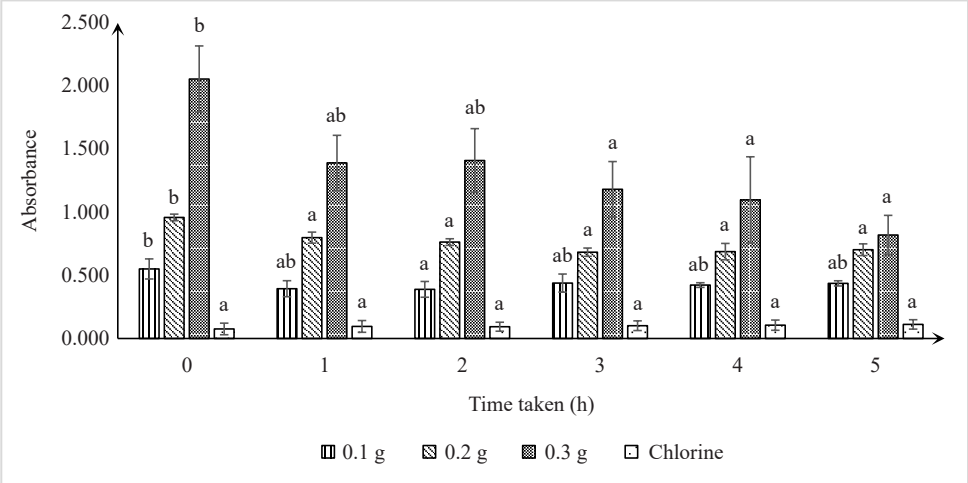

Figure 4. Graph of absorbance S-49 affected by different amounts of *L. laevigatum* and chlorine over time (h)

Overall, the results show the microbial growth interacting with varying *L. laevigatum* concentrations. Additionally, the positive control, chlorine, shows critical inhibition and acts as a standard to measure the antimicrobial efficacy of *L. laevigatum*.

4. Discussion

The study confirms that aquatic plants possess a unique bioactive compound with antimicrobial properties, effectively reducing bacterial growth, particularly in Gram-positive and Gram-negative bacteria. Many research studies focus on the capability of aquatic plants to phytoaccumulation, absorb, and decompose high levels of heavy metals through their roots and green parts. These investigations generally aim for the application in phytoremediation, including phytoextraction, rhizo-filtration, phytodegradation, phyto-stabilization and phytovolatilization [12].

Table 7 shows that 66% of isolates are Gram-positive bacteria, while 34% are Gram-negative. Further investigation reveals that of % of all gram-positive bacteria isolates, 75.75% are bacillus-shaped bacteria, and 24.25% are cocci-shaped bacteria. Benson et al.'s studies indicate that mostly general bacterial and *Pseudomonas* populations are dispersed randomly throughout the surfaces of lake waters or that there are still some factors influencing this distribution, which was not examined in his studies [13]. *Pseudomonas* species are Gram-negative, aerobic bacilli with a single polar flagellum [14]. It does not ferment lactose and produces a colourless colony when isolated on MacConkey agar. As no further tests were performed in this study, there remains a possibility that the Gram-negative bacteria could be *Pseudomonas* species. Therefore, other biochemical tests and further identification should be done to confirm the bacterial species accurately. As for Gram-positive bacteria, the research found that Actinobacteria was highly known in the freshwater drainage channels connected to the sea [15]. *Actinobacteria* are filamentous bacteria that are Gram-

positive and are characterized by a high guanine-plus-cytosine (G + C) content in their genomes [16]. These bacteria are commonly found freely in aquatic, soil and freshwater ecosystems. As both *Actinobacteria* and *Bacillus* genes are typically Gram-negative bacillus, further steps should be taken to identify the bacteria in lake water in this experiment.

According to the research of Dhir [17], aquatic plants have few ways to remove pathogens, including chemical components and antimicrobial compounds produced by the roots of aquatic plants. In Barznji's studies [18], *Saliviniacucullata* is one of the examples of a free-floating submerged plant. Like *L. laevigatum*, *Saliviniacucullata* has short roots, which effectively remove metals in water, including lead and cadmium. The short roots accumulate pathogens and then filter the pathogens from wastewater. Biofilms around the plant rhizosphere zone support the growth of bacterial communities. This leads to the removal of pathogens in contaminated water. Additionally, research from Yamasaki et al. [19] also supported that natural compounds extracted from plants such as apples, hop, green tea, 'neem', 'guazump', 'daio' and elephant garlic can inhibit microbes' growth or cholera toxin (CT), which can be found in *V. cholerae*. Research from Hasegawa et al. [20] reported that natural compounds from wasabi have antimicrobial effects on *E. coli*, *S. typhi*, *P. aeruginosa*, and other types of bacteria.

When comparing the effectiveness of different concentrations of *L. laevigatum* powder, 0.1 g of powder shows the least effect on inhibiting Gram-positive bacteria growth, whereas 0.3 g of powder shows the most impact on bacteria. The concentrations were insufficient to have the intended effect, which made the bacteria insensitive to them [21]. This might result in a decreased concentration of the bioactive compounds in *L. laevigatum*, as it has been discovered that bacterial sensitivity rises with the concentration of *L. laevigatum* powder. This result was also supported by Chen et al. [22], who discovered that the growth control ratio would negatively correlate with the immediate mortality rate. Not only that, as most microorganisms may develop resistance to antimicrobial agents at lower concentrations, exposing longer durations and necessary concentrations of disinfectants is important to achieve the highest inhibition of microbial growth [23].

However, the antimicrobial properties of *L. laevigatum* show minimal effect on Gram-negative rod-shaped bacteria compared to Gram-positive rod-shaped bacteria. The resistance of Gram-negative bacteria could be explained by the structure of the unique cell wall surrounding them. As Gram-negative bacteria have a strong permeability barrier made from an outer membrane composed primarily of lipopolysaccharides, the plant extract was effectively restricted from penetrating the barrier, unable to perform antibacterial activities against Gram-negative bacteria [24].

One of the most common ways to inhibit the growth of microorganisms in wastewater is chlorination. Chlorination eliminates most pathogens, including bacteria and viruses, responsible for waterborne diseases such as cholera, salmonellosis, and typhoid fever by adding chemical chlorine to water sources. As a positive control of this experiment, chlorine shows the highest antimicrobial effect at first but decreases over time. Chlorination will promote antibiotic resistance genes (ARGs) in bacteria multiple times, which makes disinfection of pathogens less effective. This is because the disinfection of drinking water might contribute to the release of DNA, including different types of ARGs and mobile genetic elements (MGEs) from deceased donors, such as integrons, insertion sequences and plasmids to the environment, resulting in triggers bacteria with stronger resistance to chlorination and have higher adaptation towards environmental DNA, which is ARGs and MGEs [25]. However, chlorine must be used to disinfect wastewater from time to time and maintain a volume above 0.2 mg/L as the concentration of chlorine will decrease due to consumption. Chemical reactions involving chlorine and other water constituents, as well as the interaction with biofilm and tubercles, developed on the pipe wall materials, are responsible for the consumption of chlorine, which leads to a decrease in the chlorine content in water [26]. This statement is also supported by Sheikhi et al. [27], who mentioned that longer duration will affect the loss of the concentration of free chlorine, which evaluated that the highest inhibitory effect of microorganisms shows at the initial hour but gradually decreases, enhancing the growth of selected microorganisms.

Overall, *L. laevigatum* plays a valuable role in aquatic ecosystems, which can contribute to a healthier ecosystem and environment by using it in excessive nutrient uptake and water filtration. *L. laevigatum* has also become one of the main research pathways in these recent years. For example, Martino et al. [28] have proved the potential phytoremediation system using *L. laevigatum* to remove excess chromium (Cr) from contaminated sediments. There are also studies of the biosorption of uranium (U) from aqueous solutions using *L. laevigatum* [29]. Even though it can help to improve water quality by absorbing heavy metals and promoting overall ecosystem health, studies for its antimicrobial effects are still very limited. However, further research could explore the potential antimicrobial properties of *L. laevigatum* to understand more about this plant and its vital role in the environment.

5. Conclusion

The effectiveness of *L. laevigatum* in inhibiting the growth of microorganisms has become a subject of research in recent years. This study investigated the antimicrobial properties of *L. laevigatum* against selected microbes found in lake water and its ecosystem restoration efforts. It is essential to reflect on this topic's findings, implications, and further research directions.

At the beginning of the study, fifty random colonies were isolated after the serial dilution of lake water samples to identify the types and characteristics of freshwater microorganisms. 66% of isolates are found to be Gram-positive bacteria, and 34% are Gram-negative bacteria. It is also observed that 75.75% are bacillus-shaped bacteria and 24.25% are cocci-shaped bacteria out of all gram-positive bacteria isolates. Additional identification tests, including catalase or glucose tests, can be focused on further studies of this topic to provide a more comprehensive understanding of the presence of microorganisms in lake water.

Furthermore, the investigation of the antimicrobial activity of *L. laevigatum* was continued using a mass spectrophotometer by observing the absorbance value of different concentrations of *L. laevigatum* powder with 6 Gram-positive and 4 Gram-negative bacteria. All graphs for Gram-positive bacteria show a high value initially and gradually decrease over time. The highest concentration, 0.3 g, exhibits the greatest bacterial inhibition. In contrast, among all 4 samples, 2 Gram-negative bacteria indicate no inhibition, but another 2 samples show inhibition of microbial growth. However, 0.3 g of *L. laevigatum* powder still exhibits the greatest inhibition level among 0.1 g and 0.2 g. The positive control, chlorine, shows a huge effect of elimination of bacterial growth at first but gradually decreases in the following hours.

Even though this study proves that *L. laevigatum* can inhibit selected microorganisms from lake water, further studies must be done. They could focus more on identifying lake water samples and the differences in antimicrobial properties between other aquatic plants. Additionally, *L. laevigatum* could be an alternative and a more sustainable way to replace chemical agents such as chlorine for water purification by inhibiting microorganisms in water sources, as excessive use of chemicals will have a harmful impact on consumers and the environment. In addition, the highest concentration of *L. laevigatum* powder exhibits the most efficacy in inhibiting microbial growth. Throughout this research, the determination of phytoremediation of *L. laevigatum* could be identified.

In conclusion, the findings of this study highlight the necessity of investigating and utilizing nature-based solutions, such as *L. laevigatum*, for addressing microbial contamination in freshwater ecosystems. A more sustainable future for future generations can be created by introducing natural components to practical applications for sustainable water treatment, contributing eco-friendly solutions, and promoting environmental sustainability.

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Conflict of interest

The authors declare no competing financial interests.

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