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Lactococcus Garvieae : CHARACTERIZATION AND ABILITY TO INHIBIT THE GROWTH OF FRESHWATER AQUACULTURE PATHOGENIC BACTERIA

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ABSTRACT

Lactococcus garvieae is a gram-positive ovoid cocci bacterium formerly classified as a member of the Lactococcus genus. This study aims to isolate *L. garvieae* from catfish rearing pond and characterize it as a potential probiotic candidate. *L. garvieae* was identified and characterized through phenotypic and genotypic observation, genomic % G~C content analysis, cell surface hydrophobicity assays, acidification test, in vitro antagonism, and a profile of antimicrobial activities. The MT597595.1 accession number corresponds to *L. garvieae*, as determined by a molecular identification test. Biochemical characterization was performed using API 50 CH kit. The genomics %G~C content of *L. garvieae* was 51.8. Findings from acidification ability tests, in vitro antagonism tests, and the ability of bacteria to grow in broth medium at pH 4 reveal that *L. garvieae* can inhibit the growth of *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Streptococcus iniae*, and *Edwardsiella ictaluri*. However, it does not suppress the growth of *L. garvieae Edwardsiella tarda*. Remarkably, *L. garvieae* has the ability to reduce the pH of neutral broth medium turning it acidic. Furthermore, *L. garvieae*'s hydrophobic cell surface exhibited an adhesive, hydrophobic, and protein surface cell content with a compact growth pattern consistent with postive SAT and MATH assay. Antimicrobial activity tests, encompassing 11 antibiotics, disclosed resistance to Nalidixic acid while displaying intermediate sensitivity to Streptomycin and Trimethoprim. In conclusion, *L. garvieae* demonstrates an inhibitory effect on the growth of pathogenic bacteria, underlining its potential as a probiotic candidate.

KEYWORDS: antimicrobial agent; *L. garvieae*; probiotic; pathogenic bacteria

INTRODUCTION

Aquaculture is a growing sector and a significant economic driver in numerous countries (FAO, 2020). However, the industry grapples with persistent challenges, notably the prevalence of diseases induced by pathogens, particularly bacteria. Common fish-infecting bacteria include *A. hydrophila*, *S. agalactiae*, *S. iniae*, and *E. ictalurid.* Bacterial infections have been a recurrent issue leading to significant mortality among tilapia populations, with detrimental economic consequences documented globally (Raja & Jithendran, 2015; Rodger, 2016). In Saudi Arabia, tilapia mortality attributed to *S. iniae* infections was first reported in 1992 and continues to occur in several countries (Al-Harbi, 2011). Brazil has also witnessed substantial losses in tilapia due to *S. agalactiae* infections (Delphino *et al*., 2019; Basri *et al*., 2020). It is worth noting, as outlined by Jin *et al*. (2023), that while *A. hydrophila* may constitute normal flora, and it possesses the potential to cause infections in fish. Additionally, *E. ictaluri* was initially isolated from channel catfish (*Ictalurus punctatus*) characterized by a high mortality rate (Abbas *et al*., 2022).

Vaccines, probiotics, and antibiotics have been developed to control bacterial diseases in fish culture (Munang'andu, 2018; Flores-Kossack, 2020). Unfortunately, the inappropriate use of antibiotics has given rise to a significant concern within the aquaculture industry. The emergence of antimicrobial resistance presents itself as a pressing and potentially catastrophic issue, akin to a time bomb with an uncertain detonation date. In this context, the use of bacteriocin in aquaculture emerges as a prom-

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ising avenue for future exploration. Bacteriocins are proteins synthesized by microorganisms, notably bacteria, and serve as innate antimicrobial agents (Woraprayote *et al*., 2016; O'Connor *et al*., 2020).

Lactococcus, recognized as a genus of lactic acid bacteria (LAB), is notable for its prolific production of bacteriocins. Bacteriocins, being antimicrobial peptides synthesized by ribosomes, are typically heat-inactivated and possess the ability to inhibit the growth of a wide spectrum of microorganisms (Nayak *et al*., 2021; Verma *et al*., 2022). Within the purview of bacteriocin production, *Lactococcus garvieae* has been, and continues to be, a subject of extensive research. Notable bacteriocins produced by L. garvieae include garviecin L1-5 (Villani *et al*., 2001), garvicin ML (Borrero *et al*., 2011), garvieacin Q (Tosukhowong *et al*., 2012), Qarvicin A (Maldonado-Barragán *et al*., 2013), and garvicin KS (Ovchinnikov *et al*., 2016). Furthermore, *L. garvieae* strains isolated from raw cow milk have been demonstrated their capacity to inhibit the growth of *Staphylococcus aureus*, enhance survival rates, and deter pathogenic bacterial infections in Tilapia (Abdelfatah & Mahboub, 2018). The present study focuses on the examination of *L. garvieae* isolated from a water pond and in vitro experiment, aiming to unveil its potential for inhibiting the growth of pathogenic bacteria in fish. This investigation also entails the characterization of hydrophobic surface cell properties and the assessment of antimicrobial activities.

MATERIALS AND METHODS

Bacterial Isolation and Biochemical Profile

L. garvieae was successfully isolated from the water of a catfish (*Clarias gariepinus*) rearing pond, situated within the premises of the Main Center for Freshwater Aquaculture. This pond is geographically located at -6.906124° latitude, 106.934452° longitude, specifically within Sukabumi City, West Java 43114, Indonesia. The water samples were collected in bottles and promptly conveyed to a laboratory situated near the fish pond. In the laboratory, these water samples were inoculated onto *Lactobacillus* De Man, Rogosa, and Sharpe agar (MRS-agar), a specialized growth medium provided by Oxoid. Subsequently, the inoculated samples were placed within an incubator set at a temperature of 28°C and allowed to incubate for a period of 48 hours. A bacterial sub-culture procedure was meticulously performed upon the MRS-agar to isolate a single colony. The biochemical tests were conducted using the Analytic Profile Index (API) 50 CHL kit (BioMérieux) following the manufacturer's instructions.

Sequencing genome and Whole Genome Analysis

PT Genetika Science meticulously conducted the sequencing and comprehensive analysis of the 16S rRNA gene and the entire genome. These bacterial cultures were expertly cultured on MRS-agar, specifically labeled with the sample code P4. Subsequently, these cultures were precisely prepared and forwarded for molecular analysis to PT. Genetika Science Indonesia, www.ptgenetika.com, Rukan Great Wall Blok C 19-21, Green Lake City, Petir Village, Cipondoh Districts, Tangerang, Banten Province. Furthermore, to facilitate the analysis of the %C?G content, an advanced software tool is accessible at [https://www.sciencebuddies.org/science-fair-projects/references/genomics-g-c-content-calculator].

Acidification Ability Test

L. garvieae was initially inoculated on MRS-broth medium, after which it was incubated for 48 hours. This incubation was conducted at a constant temperature of 28°C. Following the incubation period, the culture was subjected to centrifugation for a duration of 10 minutes. This centrifugation was carried out at a controlled temperature of 4°C, with a centrifuge rotor speed set at 10,000 rpm. The bacterial pellet obtained from the centrifugation step was washed rigorously three times using a sterile 0.85% NaCl (Merck) solution. Resuspending bacterial cells in sterile 0.85% NaCl resulted in a turbidity level of 0.5 McFarland (Rewel). A total of 10 µl of the resulting bacterial suspension was accurately introduced into each 10 mL of three distinct broth media, namely Tryptic Soy Broth TSB (Oxoid), Brain Heart Infusion Broth (BHIB) (Oxoid), and Nutrient Broth (NB) (Oxoid). As an indispensable control measure, parallel incubations were carried out for the same broth media (TSB, NB, BHIB) but without *L. garvieae* inoculation. In these control tests, 0.85% NaCl solution was added to the broth medium. The sample was incubated at 26°C, 28°C, and 35°C. To thoroughly mix the broth medium, vortex agitation was diligently applied. *L. garvieae* isolation was effectively executed on MRS Agar medium. The pH levels of the respective media were systematically measured using pH paper (Merck). The identification of *L. garvieae* was perfomed through a carbohydrate fermentation test, utilizing the API 50 CH kit from BioMérieux, biochemical tests.

In vitro Antagonism Test

The handling of bacterial suspensions involving *L. garvieae, A. hydrophila, S. agalactiae, S. iniae*, *E. tarda,* and *E. ictalurid* was meticulously conducted according to the following detailed procedure: Sus-

pensions of each bacterium (*L. garvieae, A. hydrophila, S. agalactiae, S. iniae, E. tarda, and E. ictaluri*) were expertly prepared, each attaining a standardized concentration of 0.5 McFarland. For each specific test container, 10 mL of sterile broth medium was thoughtfully dispensed. In creating bacterial suspensions, 10µl of L. garvieae and 10µl of A. hydrophila suspension were accurately introduced into separate 10mL portions of TSB medium. The analogous methodology was meticulously applied to prepare suspensions *of S. agalactiae, S. iniae, E. tarda,* and *E. ictaluri*, all utilizing BHIB medium. To ensure comprehensive testing, control samples were thoughtfully prepared without the inoculation of *L. garvieae*. Each test was carried out with three replications. Subsequently, all test samples were incubated at 28°C for 48 h. The medium was mixed using a vortex. The turbidity of the medium was measured by a spectrophotometer (Thermo Scientific, Genesys 30). The bacteria culture was measured using pH paper (Merck). Subsequently, the bacteria were isolated on a selective agar media to corroborate bacterial growth and quantity of the Optical Density (OD_{600}). Selecting the appropriate selective agar medium for each bacterium was crucial for successful isolation. *A. hydrophila* was isolated on Rimler-Shootts (RS) medium (Himedia). *S. agalactiae* and *S. iniae* were cultured on KF Streptococcus Agar medium (Merck). *E. ictaluri* and *E. tarda* were isolated on TSA medium. *L. garvieae* was cultured on MRS-Agar (Oxoid). A comprehensive suite of biochemical tests was conducted to further validate the presence of bacteria in the samples, utilizing API 20E and API 50CH kits.

A. hydrophila and *S. agalactiae* isolates were obtained from the Research Institute for Freshwater Aquaculture and Fisheries Extension, Bogor. *S. iniae* was collected at Center for Freshwater Aquaculture Sei Gelam, Jambi. *E. ictaluri* (Mawardi *et al*., 2018) and *E. tarda* were obtained from Main Center for Freshwater Aquaculture, Sukabumi.

Growth Test of Bacteria in a Aroth Medium With a pH Value of 4

The preparation of BHIB and TSB media adhered to the manufacturer's instructions. Sterile 1N HCl solution was added to the medium until pH reached 4. Ten mL medium was put into a sterile test tube. Abacterial Inoculum, containing strains of *L. garvieae, A. hydrophila, S. agalactiae, S. iniae*, *E. tarda,* and *E. ictaluri* was prepared to a turbidity of 0.5 McFarland. Subsequently, ten ml of bacterial suspension was added to the medium and then incubated at a temperature of 28°C for 48 hours. The medium was then mixed using vortex. Following incubation, the bacteria were diligently isolated on an agar medium and with the incubation period set at 28°C for 48 hours. The grown colonies were prepared for identification by API 20E and API 50CH kit biochemical assay. To complete the process, the turbidity of the medium was measured using a spectrophotometer.

Cell Surface Hydrophobicity Assays

Soft-Agar technique: The Soft-Agar medium was prepared by adding 0.15% Bacto Agar (Himedia) to Brain Heart Infusion Broth (Oxoid). The medium was sterilized in an autoclave for 15 minutes at a temperature of 121°C. *L. garvieae* was inoculated onto the soft-agar medium by touching the medium with a loop needle. The samples were vigorously mixed using a vortex at a medium speed for a duration of 2 to 3 seconds. Subsequently, the samples were incubated at 35°C for 24 hours. In this assay, *Lactobacillus paracasei* spp. was utilized as the control. The growth pattern of bacteria was then carefully observed.

Salt aggregation test (SAT). Ammonium sulfate $(NH_4)_2SO_4$ (Merck) was carefully prepared in distilled water, generating varying concentrations of 0.3 M, 0.6 M, 1.2 M, 1.8 M, 2.0 M, 2.4 M, 2.8 M, and 3.0 M. The 10^{12} CFU mL^{-1} of *L. garvieae* suspension was prepared in distilled water. Next, 20 µl of ammonium sulphate solution and 20 µl of *L. garvieae* suspension were mixed slowly on an object glass. Observations were made regarding the agglutination of the samples within a time frame of less than 1 minute. Interpretation of the results involved categorizing SAT values: a value below 1 M indicated strong results, while values between 1.0 M and 2.0 M were considered moderate. SAT values between 2.0 and 4.0 indicated weak results, and SAT value of 4.0 M indicated that the sample was not hydrophobic (hydrophilic). Positive reactions were observed for samples exhibiting strong and moderate SAT results.

Microbial adherence to the n-hexadecane (MATH) assay. A suspension of *L. garvieae,* comprising 4 mL in sterile distilled water, was prepared, attaining a turbidity level of 0.5 McFarland. with a turbidity of 0.5 McFarland. Subsequently, 400 µl of n-hexadecane (Merck) was meticulously introduced into the suspension. The sample was then placed in a water bath at 37°C for a duration of 10 minutes. The samples were vortexed and then incubated for 15-20 minutes at room temperature. Using a spectrophotometer, the optical density of the sample was measured at $OD_{640nm,}$ both for the aqueous phase and original bacterial suspension. The optical density of the aqueous phase was determined using a bacterial suspension without hexadecane. The percentage of hydrocarbons was determined by calculating the ratio of the opti-

cal density at 640nm (OD_{640nm}): [OD_{640nm} (original bacterial suspension) - OD_{640nm} (aqueous phase)/ OD_{640nm} (original bacterial suspension) x 100]. Bacterial adherence characteristics were categorized as : (1) highly hydrophobic (strong) at 50%; (2) moderately hydrophobic within the range of >20 to <50%; and (3) a hydrophilic surface at 20%. Positive reactions were identified for samples exhibiting strong and moderate adherence characteristics. The Salt aggregation test (SAT) and Microbial adherence to the n-hexadecane test (MATH) were performed following the method established by Mattos-Guaraldi et al. (1999).

Antimicrobial activities

The assessment of antimicrobial activity was conducted in strict accordance with the well-established disk diffusion method prescribed by the Clinical and Laboratory Standards Institute (CLSI 2018). In this meticulous examination, an array of antibiotics was thoughtfully selected to ensure comprehensive evaluation: Chloramphenicol (30 mg), Ampicillin (10 mg), Ampicillin/ sulbactam (20 mg), Nalidixic acid (30 mg), Erythromycin (15 mg), Streptomycin (10 mg), Cephalothin (30 mg), Trimethoprim (5 mg), Gentamicin (10 mg), Enrofloxacin (5 mg), Tetracycline (30 mg), and Oxytetracycline (30 mg). The methodological rigor of this investigation was underscored by the meticulous execution of experiments, with each test being thoughtfully replicated twice to ensure the robustness and reliability of the results.

Data Analysis

The research data were subjected to descriptive analysis employing Microsoft Excel, wherein calculations were performed to determine the optical density's percentage, mean, standard deviation, and clear zone diameter.

RESULTS AND DISCUSSION

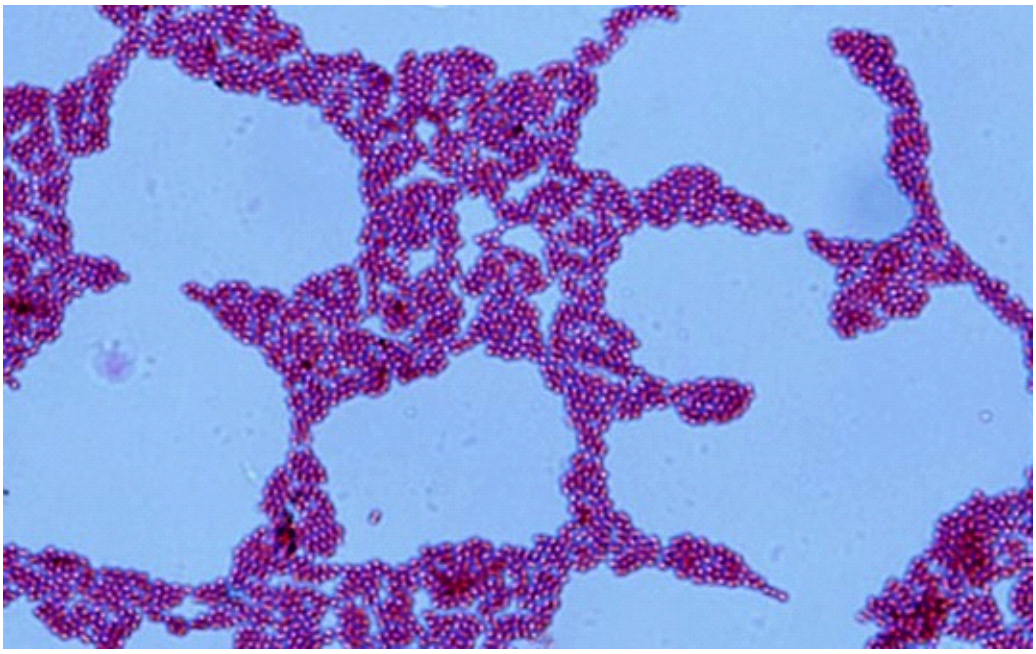

Figure 1. Morphology of *L. garvieae* cells using Gram-staining (1000x magnification) (Olympus BX53).

Isolation and Biochemical Profile

When cultivated on MRS-agar, the bacterial isolates exhibited growth characterized by the formation of smooth, circular, and white colonies. Figure 1 depicts the morphology of ovoid cocci-shaped cells and purple-colored Gram-positive bacteria.

Biochemical characterization by API 50CH system, the strain exibits positive reactions for D-Ribose, D-Galactose, D-Glucose, D-Fructose, D-Mannosem, N-Acetylglucosamine, Amygdalin, Arbutin, Esculin Ferric citrate, Salicin, D-Cellobiose, D-Maltose, D-Trehalose, and Gentiobiose (Table 1).

Genome sequencing and whole genome analysis

After extraction, the genomic DNA quantification was 23.6 (ng/il) $A_{260/280}$ 1.97 and $A_{260/230}$ 0.35.

Moreover, the sequence of the whole construct was analyzed by the genomics %G~C content calculator software. Adenine (A) 325, Thymine (T) 404, Guanine (G) 330, Cytosine (C) 452, and the %G~C content was 51.8. This section contains the complete sequence of *L. garvieae* (1511 base pairs).

Table 1. The biochemical tests of bacteria isolates on API 50 CHL kit

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
(0) Control	-	(1) Glycerol	-	(2) Erythritol	-	(3) D-arabinose	-	(4) L-arabinose	-	5) D-Ribose	+
(6) D-Xylose	-	(7)L-Xylose	-	(8) D-Adonitol	-	(9) Methyl-ß-D-xylopyranoside	-	(10)D-Galactose	+	(11)D-Glucose	+
(12)D-Fructose	+	(13)D-Mannose	+	(14)L-Sorbose	-	(15) L-Rhamnose	-	(16) Dulcitol	-	(17) Inositol	-
(18)D-Mannitol	-	(19)D-Sorbitol	-	(20) Methyl-α-D-mannopyranoside	-	(21) Methyl-α-D-glucopyranoside	-	(22) N-Acetylglucosamine	+	(23) Amygdalin	+
(24) Arbutin	+	(25) Esculin Ferric citrate	+	(26) Salicin	+	(27)D-Cellobiose	+	(28) D-Maltose	+	(29) D-Lactose (bovine origin)	-
(30) D-Melibiose	-	(31)D-Saccharose (sucrose)	-	(32) D-Trehalose	+	(33) Inulin	-	(34)D-Melezitose	-	(35) D-Raffinose	-
(36) Starch (amidon)	-	(37) Glycogen	-	(38) Xylitol	-	(39) Gentiobiose	+	(40)D-Turanose	-	(41)D-Lyxose	-
(42)D-Tagatose	-	(43)D-Fucose	-	(44)L-Fucose	-	(45)D-Arabitol	-	(46)L-Arabitol	-	(47) Potassium gluconate	-
(48) Potassium 2-ketogluconate	-	(49) Potassium 5-ketogluconate	-								

(+) indicates a positive reaction, where a noticeable color change occurs.
(-) indicates a negative reaction, where there is no observable alteration in color.

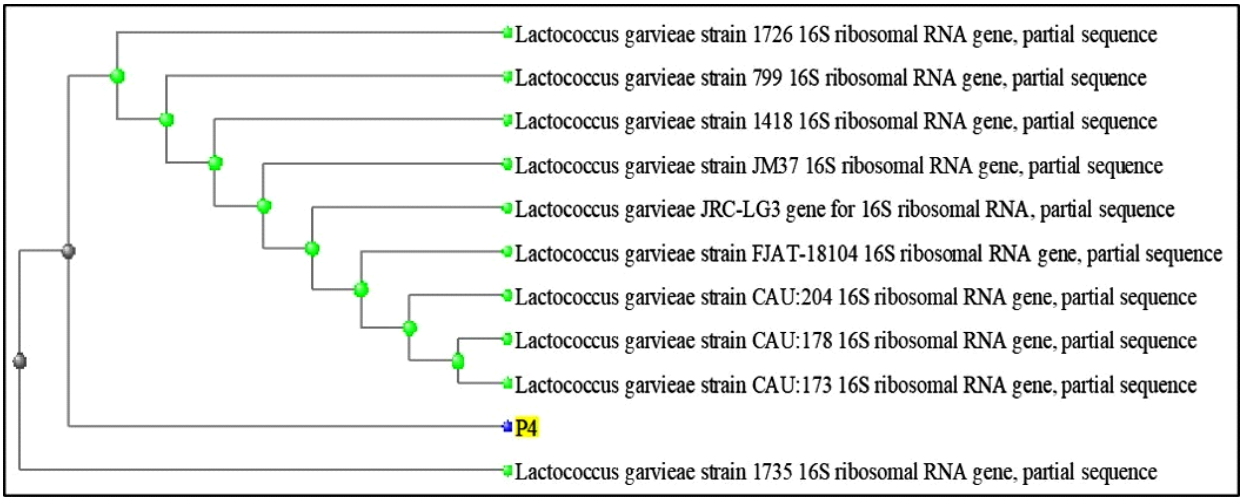

Figure 2. Phylogenetic tree of *L. garvieae*, MT597595.1 (the sample code is P4). The identification results are comparable to *L. garvieae* with accession number of MT597595.1 exhibiting 100% identity.

Acidification Ability Test

The evaluation of *L. garvieae* 's capacity to reduce the pH of liquid media involced the assessment of the growth of *L. garvieae* in each liquid media, specifically TSB, BHIB and NB and incubated at different temperature conditions. Following incubation, the

GGTTACCTTGTTACGACTTCACCCCAGTCATCGGTCTTACCTTAGGAAGCGCCCT
CCTTGCGGTTAGGCAACCTACTTTGGGTACTCCCAACTTCCGTGGTGTGACGGGCGGT
GTGTACAAGGCCCGGGAAC
GTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGCGA
GTTGCAGCCTGCAATCCGA
ACTGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGGCGACTCGTTGTACCA
TCCATTGTAGCACGTGTGT
AGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTAT
CACCGGCAGTCTCACTAGA
GTGCCCAACTTAATGATGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTAAC
CCAACATCTCACGACACGA
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GAAAATAGCAAGCTATCT
TCAATCATCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTC
CTGAGCCATGATCAAACTCTA

acidification ability of *L. garvieae* was reflected in the pH values of TSB, BHIB, and NB media (Table 2). In contrast, the pH value of the media without *L. garvieae* inoculum was 7 across all incubation temperatures. *L. garvieae* reduced the pH of TSB, BHIB, and NB media after incubation at various temperatures. Moreover, *L. garvieae* exhibited growth in media containing *L. garvieae* suspension.

Table 2. The pH values of the media inoculated with *L. garvieae*

Inoculum	pH value		
	26°C	28°C	35°C
TSB medium	7	7	7
NB medium	7	7	7
BHIB medium	7	7	7
TSB + *Lactococcus garvieae*	4	4	4
NB + *Lactococcus garvieae*	4	4	4
BHIB + *Lactococcus garvieae*	4	4	4

In vitro antagonism test

Experiments were conducted to substantiate the inhibitory capabilities of *Lactococcus garvieae* on the proliferation of pathogenic bacteria. The approach employed involved co-culturing *L. garvieae* bacteria with individual pathogenic bacterial strains within a liquid growth medium. The findings obtained through these experiments demonstrated that *L. garvieae* possesses the capacity to impede the growth of numerous pathogenic bacteria in contrast to a control group, which consisted of pathogenic bacteria devoid of any introduced *L. garvieae.*

Table 3 provides a comprehensive representation of the growth patterns observed in the context of various bacterial strains. Specifically, *A. hydrophila, S. agalactiae, S. iniae, E. tarda,* and *E. ictaluri* demonstrated growth in the absence of *L. garvieae.* Conversely, when co-cultured with *L. garvieae* in the respective broth media, negative identifications were ascertained for *A. hydrophila, S. agalactiae, S. iniae, and E. ictaluri.* Notably, a positive identification result was obtained for *E. tarda* following its co-cultivation with *L. garvieae*. Subsequent to an incubation period, the optical density was measured to assess bacterial growth. It is noteworthy that the pH value of the media containing the *L. garvieae* suspension exhibited a tendency to decrease, transitioning from an initial pH of 7 to a final pH of 4.

Table 3. Growth test results of *L. garvieae* inoculated co-cultured with pathogenic bacteria

Inoculum	Optical Density (OD_{600})	pH value	Identification
A. hydrophila	0,625±0,008	7	Positive
S. agalactiae	0,607±0,002	7	Positive
S. iniae	0,347±0,003	7	Positive
E. ictaluri	0,586±0,003	7	Positive
E. tarda	0,583±0,002	5	Positive
L. garvieae	0,835±0,002	4	Positive
L. garvieae + *A. hydrophila*	0,850±0,001	4	*A. hydrophila;* negative *L. garvieae:* positive
L. garvieae + *S. agalactiae*	0,853±0,002	4	*S. agalactiae*; negative *L. garvieae:* positive
L. garvieae + *S. iniae*	0,870±0,004	4	*S. iniae;* negative *L. garvieae:* positive
L. garvieae + *E. ictalurid*	0,847±0,004	4	*E. ictaluri*; negative *L. garvieae:* positive
L. garvieae + *E. tarda*	0,981±0,004	4	*E. tarda*; positive *L. garvieae:* positive

The results indicate disparate outcomes in the behavior of *E. tarda bacteria*, specifically, that *E. tarda* bacteria exhibit continued growth when co-cultured alongside *L. garvieae* bacteria in broth media. In contrast to the control group, where *E. tarda* was cultured in the absence of *L. garvieae*, a notable decrease in the pH value of the media was observed, transitioning from an initial pH of 7 to a final pH of 5. These findings collectively suggest that *E. tarda* bacteria possess the capacity to effectively lower the pH of the surrounding medium and demonstrate resilience in adapting to an environment characterized by a reduced pH level.

Growth test in the broth media with a pH of 4

Bacterial growth testing in broth media with a pH of 4 was performed to determine bacteria toleration of low pH. This test also supports the results of the in vitro antagonism test that *L. garvieae* has the ability to inhibit the growth of *A. hydrophila*, *S. agalactiae*, *S. iniae,* and *E. ictaluri bacteria*. The growth test for *A. hydrophila, S. agalactiae, S. iniae,* and *E. ictaluri* in broth media with a pH of 4 yielded negative results (Table 4). However, *E. tarda* and *L. garvieae* showed positive results. On a medium with a pH of 4, *A. hydrophila, S. agalactiae, S. iniae, and E. ictaluri* did not grow. Meanwhile, it was discovered that *E. tarda* and *L. garvieae* could thrive in a medium with a pH of 4.

Table 4. Bacterial Growth in the Broth medium with a pH of 4

Inoculum	OD_{600} of pH 4 medium	pH value	Identification
A. hydrophila	0,005±0,000	4	Negative
S. agalactiae	0,008±0,000	4	Negative
S. iniae	0,008±0,000	4	Negative
E. ictaluri	0,006±0,000	4	Negative
E. tarda	0,587±0,003	4	Positive
L. garvieae	0,850±0,007	4	Positive

Cell surface hydrophobicity assays

The growth pattern of *L. garvieae* colonies on Soft-Agar media is described in this section. The growth pattern colonies exhibited notable differences between *Lactobacillus paracasei* sp. and *L. garvieae*, as depicted in Figure 3. Specifically, the collonies of *L. garvieae* demonstrated a compact growth pattern (Figure 3A). The SAT results showed that *L. garvieae* was agglutinated at a 0.6 M ammonium sulfate concentration, indicating a positive test. Furthermore, the MATH (Microbial Adhesion to Hydrocarbon) result for the *L. garvieae* isolate recorded a value of 32.02%, indicating further a positive test result.

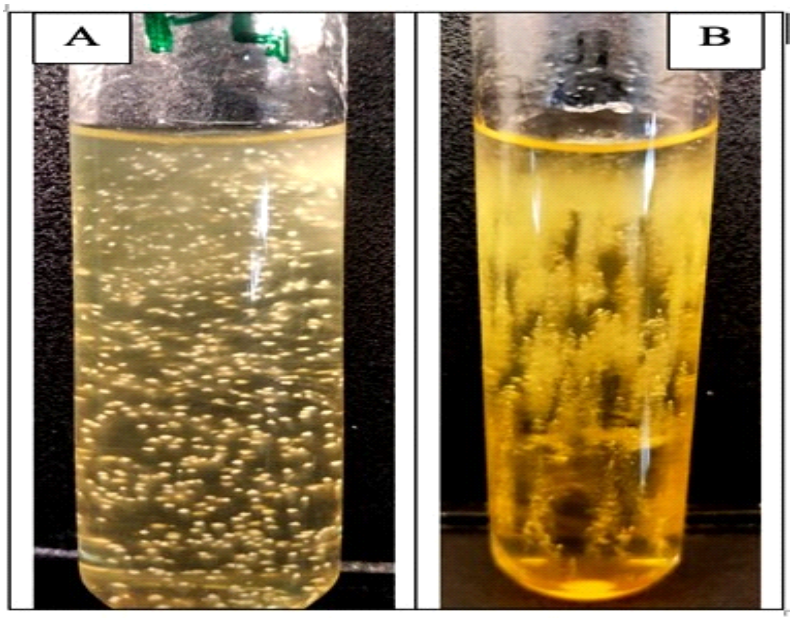

Figure 3. Formation of compact colonies in Soft Agar medium. Compact colonies are characteristic of *L. garvieae* (A) and diffuse colonies are characteristic of *L. paracasei* sp. (B).

Antimicrobial activities

The antibiotic resistance test revealed that *L. garvieae* was resistant to Nalidixic acid, an intermediate to Streptomycin and Trimethoprim (Table 5).

L. garvieae isolated from the rearing pond water of catfish (Clarias gariepinus), exhibited distinctive characteristics. The isolated bacteria were visually characterized by their hue and absence of odor. The observed state of the rearing pond was notably free from deceased fish, with the fish population displaying robust health and growth. Water was taken in a sampling bottle and transported to the laboratory for biological analysis. Fig. 1 depicts gram-positive bacteria with cocci-shaped cells. *L. garvieae* is a gram-positive bacterium with cocci-shaped cells (Varsha & Nampoothiri., 2016), is catalase-negative, facultative, anaerobic, and nonhemolytic (Russo *et al*., 2012), is also referred to as *Enterococcus seriolicida* (Ferrario *et al*., 2013). The biochemical analysis was performed using API 50 CHL kit; the outcomes are outlined in Table 1. Molecular identification demonstrated 100% identity with *L. garvieae* (accession number MT597595.1). The detailed sequence of the entire construct was determined using genomics %G~C content calculator software, revealing content of 51.8. Garg & Sharma., (2020) stated that sequencers C phosphodiester G Island (CpGI) are DNA sequence regions with high-frequency CG nucleotides compared to non-CGI regions, with a minimum concentration of 50% C+G. According to Xue *et al*., (2019), CpG DNA has demonstrated the ability as an effective immune adjuvant. CpG dinucleotides flanked by certain bases may serve as non-self-pattern recognition motifs for receptors in the innate immune system to detect infecting pathogens (Schuüller *et al*., 2011). In this study, *L. garvieae* had the potential a GpC island.

Table 5. Antimicrobial activities of *L. garvieae*

Antibiotic	Disk Content	Diameter zone (mm)	Interpretive	CLSI 2018 (Enterobacteriaceae) diameter zone standard (mm)		
				S	I	R
Chloramphenicol	30 µg	23,4 ±0,08	S	≥ 18	13-17	≤ 12
Ampicillin	10 µg	25,2 ±0,02	S	≥ 17	14-16	≤ 13
Ampicillin/ sulbactam	20 µg	24,8 ±0,98	S	≥ 15	12-14	≤ 11
Nalidixic acid	30 µg	7,1 ±0,15	R	≥ 19	16-18	≤ 13
Erythromycin*	15 µg	22,3 ±0,07	S	≥ 13		≤ 12
Streptomycin	10 µg	12,6 ±0,59	I	≥ 15	12-14	≤ 11
Trimethoprim	5 µg	11,4 ±0,31	I	≥ 16	11-15	≤ 10
Gentamicin	10 µg	16,7 ±0,55	S	≥ 15	13-14	≤ 12
Enrofloxacin**	5 µg	19,2 ±0,14	S	≥ 21	16-20	≤ 15
Tetracycline	30 µg	26,5 ±0,38	S	≥ 15	12-14	≤ 11
Oxytetracycline	30 µg	25,2 ±0,05	S	≥ 14	11-13	≤ 10

Refers to *Azithromycin (15 mg) **Ciprofloxacin (5 mg), S= sensitive, I=intermediate, R= resistance

L. garvieae was inoculated on NB, BHIB, and TSB media for an acidification capacity test (Table 2). Based on the result, the pH of the medium inoculated with *L. garvieae* changed to 4 at different temperatures. The pH of the control medium without adding *L. garvieae* was 7. The test is corroborated by the results of an in vitro antagonism test (Table 3). The pH of the combined culture of pathogenic bacteria and *L. garvieae* was 4. The confirmation test identified that *L. garvieae* inhibits the growth of pathogenic bacteria by altering the pH of media, except *E. tarda*. The result confirmed that *E. tarda* could thrive in a broth medium with a pH of 4 (Table 4). *E. tarda* was found to grow in broth medium with a pH of 4 without adding *L garvieae*, and confirming that *E. tarda* and *L. garvieae* were able to grow in the broth media with a pH of 4. In this study the bacteriocin product produced by *L. garvieae* represents its greatest potential. According to Dubey *et al*., (2022), bacteriocins produced by *L. garvieae* KS1546 were able to inhibit the growth of several fish pathogens, including *S. agalactiae, A. hydrophila, Salmonicida* strain 6421, but did not able to inhibit the growth of *E. tarda, Salmonicida* strain 6422 and *Yersinia ruckeri*. Maldonado-Barragán., (2022) reports that *L. garvieae* produces an immune protein, bacteriocin, which is chemically synthesized and inhibits the growth of *Listeria monocytogenes*, *Listeria ivanovii,* and *Enterococcus faecalis.* Moreover, Chi & Holo., (2018) state that gervicin KS bacteriocin isolated from *L. garvieae* inhibits 19 species of Gram-positive bacteria, has the ability to produce bacteriocin, and exhibits synergy with antimicrobials.

The result of *L. garvieae's* surface hydrophobicity assays on soft agar media revealed compact colonies (Fig. 3). The colonial growth pattern showed that *L. garvieae* possessed robust adhesion and hydrophobicity. Positive results were observed for bacterial growth on soft agar media, including SAT and MATH. It is consistent with the surface protein of cells (Wibawan & Lammler., 1991). Surface proteins or glycolipids complexed with cell surface protein structures mediate adhesion to n-hexadecane. Vendrell *et al*., (2009) state that *L. garvieae* adheres well to liver cells and polystyrene material.

L. garvieae isolates were tested for antimicrobial activity using the disk diffusion method on class eight antibiotics (Table 5). The classes and antimicrobial agents used were penicillins (ampicillin), b-lactams (ampicillin-sulbactam), aminoglycosides (gentamicin, streptomycin), macrolides (erythromycin), tetracyclines (tetracycline, oxytetracycline), quinolones and fluoroquinolones (enrofloxacin, nalidixic acid), folate pathway antagonists (trimethoprim), and phenicol (chloramphenicol). Eleven antimicrobial agents have been used, of which one is resistant (nalidixic acid) and two are intermediates (streptomycin and trimethoprim). The environment and contaminants that penetrate the aquatic environment can influence the antimicrobial resistance of microorganisms (Lee *et al*., 2020; Menciìa-Ares *et al*., 2020). In this study, it is also possible that *L. garvieae* is resistant to the antibiotic nalidixic acid, a contaminant found in aquatic environments.

CONCLUSIONS

In this study, it was observerd that *L. garvieae* exhibited the capacity to impede the growth of pathogenic bacteria encompassing both Gram-positive and Gram-negative types. Furthermore, the surface hydrophobicity characteristic of *L. garvieae* cells demonstrated an affinity for adherence to salt, soft-agar test, and hydrocarbon. Moreover, *L. garvieae* could produce novel antimicrobial agents in the form of bacteriocins and a strong ability to adhere to properties.

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