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# Characterization of *Lactococcus garvieae* Isolated from *Wadi Papuyu* (*Anabas testudineus* Bloch) Fermentation of Indonesian Origin as a Probiotic Candidate

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**Abstract:** *Lactococcus garvieae* was isolated from the traditional fermented food *Wadi Papuyu* (*Anabas testudineus* Bloch) and characterized for its potential as a probiotic candidate. Growth assays demonstrated that *L. garvieae* could proliferate in MRS medium, reaching cell counts exceeding 107 CFU/mL after 72 h of anaerobic incubation. The isolate exhibited optimal growth at both 30 °C and 37 °C, as indicated by significant increases in absorbance at these temperatures. However, in the bile salt tolerance test using 0.5% (w/v) bile salts, *L. garvieae* showed a marked decline in growth, with absorbance values decreasing substantially after 24 and 48 h, indicating insufficient bile tolerance. The autoaggregation assay revealed values below 10%, suggesting limited autoaggregation capability and reduced potential for colonization in the gastrointestinal tract. In contrast, the co-aggregation assay showed that *L. garvieae* was able to coaggregate with pathogenic bacteria such as *Escherichia coli*, *Salmonella spp*., and *Shigella spp*., with co-aggregation percentages exceeding 40% after 5 h. Antimicrobial activity tests demonstrated that *L. garvieae* produced strong inhibition zones (diameters >10–20 mm and >20 mm) against Gram-positive and Gram-negative pathogenic bacteria. These findings indicate that while *L. garvieae* exhibits promising antimicrobial activity and co-aggregation ability, its limited bile salt tolerance and autoaggregation capacity are significant constraints in its development as a probiotic candidate.

## Introduction

*Wadi* is a popular fermented food in Central Kalimantan. *Wadi* is made from meat or fish, and the fish used for *Wadi* fermentation usually come from freshwater, swamp waters, or other stagnant waters, such as *Papuyu* fish (*A. testudineus* Bloch) (1). *Wadi* is traditionally processed by adding salt and other ingredients. Another ingredient added during *Wadi* fermentation was *samu/lamu. Samu/lamu* is made from roasted rice, pounded until smooth, and added to the fermented fish. During fermentation, *Samu/lamu* is a carbohydrate source for lactic acid bacteria (2). This is a spontaneous fermentation process without adding external bacterial starters (3).

Bacterial isolation from *Wadi Papuyu* has resulted in several bacteria, including *L. garvieae* (4). *L. garvieae* is one of the bacterial isolates that belong to the group of lactic acid bacteria that grow in various fermented fish or meat products. *L. garvieae* is a Gram-positive, cocci-shaped bacterium with morphological and biochemical similarities to enterococci. Although *L. garvieae* is recognized as an opportunistic pathogen, particularly in aquaculture and occasionally in humans, its pathogenicity is known to be strain-specific and largely dependent on certain virulence factors. The isolate used in this study was obtained from *Wadi Papuyu*, a traditionally fermented fish product consumed safely for generations, with no reported adverse health effects. Preliminary genetic screening of this isolate revealed the absence of key virulence genes such as hemolysin (HLY) and collagen-binding

protein (CBP), which are commonly associated with pathogenic strains. Additionally, this isolate demonstrated sensitivity to β-lactam and tetracycline antibiotics, in contrast to clinical strains that often exhibit resistance. Based on these observations, we hypothesize that this food-derived *L. garvieae* isolate possesses a safe genetic profile and holds probiotic potential, despite the general zoonotic reputation of the species. This study aims to characterize its probiotic properties further and assess its safety for potential application (5, 6).

One of the food products that can provide positive effects is probiotic bacteria. Probiotics are defined as live microorganisms in food that are present in sufficient quantities and offer health benefits to the digestive system. The most commonly used microorganisms as probiotics in commercial products are *Lactobacillus* spp. and *Bifidobacterium* spp (7). It is also stated that most probiotic bacteria in food products have immunomodulatory effects and can survive in the digestive tract (8). Another study reported that the fermentation of *Bambangan* (a traditional food product from Malaysia) resulted in lactic acid bacteria strains, including *Lactobacillus brevis*, and *Lactobacillus plantarum*, which were tolerant to NaCl. Fermented *Mandai*, derived from the peel of chempedak fruit, successfully isolated nine bacterial strains, among which *L. plantarum* and *Pediococcus pentosaceus* were identified (9). All these lactic acid bacteria strains are considered promising probiotic candidates for functional food products.

The characterization of *L. garvieae* BCC43578 from fermented pork sausages showed that it produces the bacteriocin compound garvieacin Q (Gar Q), which can inhibit the growth of *Listeria monocytogenes* (10). *L. garvieae* obtained from fish intestinal fermentation is tolerant to sodium chloride, bile salts, and a wide pH range (pH 2-9) (11). *L. garvieae* obtained from traditional milk-based fermented products has been characterized as a starter culture in the dairy industry. This bacterium can hydrolyze milk proteins and casein and quickly exhibit high acidification activity during cheese (curd) production (12). The novelty of this study lies in the isolation and comprehensive characterization of indigenous *L. garvieae* strains from *Wadi Papuyu*, a traditional Indonesian fermented fish product, as potential probiotics. To date, a notable lack of research focuses on the characterization and safety assessment of *L. garvieae* strains derived from Indonesian fermented foods. Therefore, this research provides new insights into the probiotic potential and safety profile of local *L. garvieae* isolates, contributing valuable data to functional foods and probiotic development in Indonesia.

# Experimental Section

## Tools and Materials

LAF (Laminar Air Flow) brand ESCO®, analytical balance Sartorius® Type bp 221s, Incubator brand Faithful® GP-65BE, round ose, petri dishes, test tubes, autoclave brand GEA® YX-18LDJ, oven Memmert® Type UNB 30p, eppendorf, colony counter brand Bel-art® type Mini light box and UV-Vis Spectrophotometer (TECAN® Infinite 200 PRO Nano Quant). MRS agar (de Man Rogosa Sharpe Agar), MRS broth (de Man Rogosa Sharpe Broth), MHA (Mueller Hinton Agar), distilled water, and pathogenic bacteria such as *Staphylococcus aureus* ATCC 6537, *Salmonella sp*. ATCC 14028, and *Escherichia coli* ATCC 25922.

## Fish Fermentation

The fermentation process of *Wadi Papuyu* followed traditional methods adapted from Central Kalimantan practices. Fresh *Papuyu* fish were cleaned, cut into pieces, and mixed with 20% (w/w) salt and 10% (w/w) cooked rice as a carbohydrate source to promote microbial activity. The mixture was tightly packed into airtight ceramic jars to create an anaerobic environment and stored at room temperature (25–30 °C). No external yeast or starter cultures were added, as fermentation relied on indigenous microbiota, including *L. garvieae*, naturally present in the fish and rice. The process lasted 7–10 days, during which lactic acid bacteria dominated, lowering the pH and preserving the fish. Post-fermentation, samples were stored at -20 °C in 20% glycerol for long-term preservation.

## *In Vitro* Characterization of *Lactococcus Garvieae* Isolates

### Growth Test of *Lactococcus garvieae* Isolates

*L. garvieae* isolates were inoculated in MRSB, then incubated for 18-24 h at 37 °C. 1 mL suspension derived from the *L. garvieae* isolate was diluted gradually with distilled water from dilution levels $10^{-1}$ to $10^{-8}$. 1 mL of *L. garvieae* isolate suspension from $10^{-4}$ and $10^{-8}$ was put into each Petri dish, and 20 mL of MRSA was added. Petri dishes were shaken until the bacteria were distributed and allowed to solidify. All the petri dishes were placed in an anaerobic incubator for 72 h. The number of bacteria that grew at each dilution was counted at 24, 36, 48, 60, and 72 h (13).

### Growth Test of *Lactococcus garvieae* Isolates at Specific Temperature

*L. garvieae* isolates were inoculated in MRSB, then incubated for 18-20 h at 37 °C. A total of 1 mL of the bacterial suspension was inoculated in 9 mL of MRSB and incubated at 5 °C, 30 °C, and 37 °C for 24 h. The total plate count method calculated the number of living cells (4).

### Growth Test of *Lactococcus garvieae* Isolates Under the Influence of Bile Salts

*L. garvieae* isolates were inoculated in MRSB, then incubated for 18 - 24 h at 37 °C. 1 mL of the bacterial suspension was *re-inoculated* in MRSB containing 0.5% b/v bile salts, and incubated at 37 °C for 24 h. The turbidity of the culture was determined at a wavelength of 600 nm (14).

### Auto-aggregation Assay of Lactococcus garvieae Isolate

*L. garvieae* was inoculated into MRSB and incubated for 24 h at 37 °C. The bacterial cells were centrifuged at 3,500 rpm for 20 min. The pellet was washed twice with phosphate-buffered saline (PBS) and then resuspended in PBS to a final concentration of $10^8$ CFU/mL. The auto-aggregation assay measured the initial absorbance (0 h) and the final absorbance (5 h) after incubating the suspension at 25–27 °C. A total of 0.1 mL of the upper part of the suspension was transferred into 3.9 mL of PBS, and the absorbance was measured at a wavelength of 600 nm. The percentage of auto-aggregation is expressed in **Equation 1** (15).

$$Auto-aggregation\,(\%) = 1 - \frac{A_t}{A_0} \times 100$$ **Equation 1**

where $A_t$ is absorbance at time t (5 h) and $A_0$ is absorbance at time 0 h.

### Co-aggregation Assay of *Lactococcus garvieae* Isolate from Wadi Papuyu Fish

*L. garvieae* and test bacteria (*S. aureus, E. coli, Salmonella* sp., *Shigella* sp., and *S. epidermidis*) were inoculated into MRSB and incubated for 24 h at 37 °C. The cultures were centrifuged at 3,500 rpm for 20 min. The pellets were washed twice and resuspended in PBS to reach a final concentration of $10^8$ CFU/mL. A total of 2 mL from each bacterial suspension was mixed in a single tube (a mixture of *L. garvieae* suspension with the selected pathogenic bacterial suspension). Controls were prepared in separate tubes containing 2 mL of *L. garvieae* and pathogenic bacterial suspensions. Co-aggregation was determined by measuring the initial absorbance (0 h) and the final absorbance (5 h) after incubation at 25–27 °C. A total of 0.1 mL of the upper part of the suspension was transferred into 3.9 mL of PBS, and the absorbance was measured at a wavelength of 600 nm. The percentage of co-aggregation is expressed in **Equation 2** (15).

$$Co-aggregation\,(\%) = \frac{(\frac{A_t + A_y}{2}) - A_{x+y}}{A_x + A_y} \times 100$$ **Equation 2**

where $A_x$ = absorbance of *Lactococcus garvieae* isolate suspension, $A_y$ = absorbance of the pathogenic isolate suspension, and $A_{x+y}$ = absorbance of the mixed suspension of Lactococcus garvieae and the pathogen.

### Antimicrobial Activity Test of *Lactococcus garvieae* Isolates

The antimicrobial activity of *L. garvieae* isolates was tested against three pathogenic bacteria: *S. aureus* ATCC 6537, *Salmonella sp*. ATCC 14028, and *E. coli* ATCC 25922 (all obtained from ATCC®, USA). *L. garvieae* isolates were inoculated in MRSB, then incubated for 24 h at 37 °C. The bacteria's culture suspension turbidity was adjusted to McFarland 0.5 turbidity (equivalent to 1.5 × $10^8$ CFU/mL). The culture of the *L. garvieae* isolate was centrifuged at 3,500 rpm for 20 min. A total of 15 µL of the supernatant was dripped onto a 6 mm diameter paper disc. A paper disk containing 15 µL MRSB was used as the control. A total of 40 µL of the test bacterial suspension was spread on the surface of MRSA and MHA, and a paper discwas placed on the surface of the medium. The Petri dishes were incubated at 37 °C for 18–24 h. The clear area around the disc paper represents the diameter of inhibition of the bacterial isolate against bacteria (4).

## Results

The growth testing of *L. garvieae* (see **Table 1**) demonstrated its ability to survive and proliferate under various environmental conditions. This isolate consistently grew on the culture medium throughout the incubation periods, both at high and low dilutions.

Moreover, growth was still detectable at the latest incubation time tested. When tested at different temperatures (see **Table 2**), the *L. garvieae* isolate showed changes in absorbance values after 24 h of incubation, indicating growth activity at all tested temperatures. These results suggest that the bacteria can adapt to a range of environmental temperature conditions.

In the bile salt test (see **Table 3**), the isolate exhibited an increase in absorbance values over time. This indicates that *L. garvieae* has resistance to the presence of bile salts, which are commonly found in the digestive tract environment.

However, in the auto-aggregation test (see **Table 4**), the isolate did not show a significant ability to form aggregates with itself. Conversely, in the co-aggregation test (see **Table 5**), the isolate demonstrated the ability to interact and aggregate with several pathogenic bacteria, including *Shigella* sp., *E. coli*, and *Salmonella* sp.

Furthermore, the antimicrobial activity test (see **Table 6**) showed that the *L. garvieae* isolate was able to inhibit the growth of several pathogenic bacteria, as reflected by the formation of inhibition zones on the test media.

**Table 1.** Growth test results of bacterial isolates on media.

| Bacterial Isolates | Dilution | Number of Colonies (CFU/mL) at Time (t, Hours) | | | | |
|---|---|---|---|---|---|---|
| | | $t_{24}$ | $t_{36}$ | $t_{48}$ | $t_{60}$ | $t_{72}$ |
| *L. garvieae* | $10^{-4}$ | >3x10$^6$ | >3x10$^6$ | >3x10$^6$ | >3x10$^6$ | >3x10$^6$ |
| | $10^{-8}$ | 2x10$^6$ | 4x10$^6$ | 8x10$^6$ | 8x10$^6$ | 8x10$^6$ |

**Note:** $t_{42}$ = time at 24 h, $t_{36}$ = time at 36 h, $t_{48}$ = time at 48 h, $t_{60}$ = time at 60 h, and $t_{72}$ = time at 72 h.

**Table 2.** Growth test results of bacterial isolates at specific temperatures.

| Bacterial Isolates | Temperature (°C) | $A_0$ ± SD | $A_{24}$ ± SD | $A_{24}$ - $A_0$ |
|---|---|---|---|---|
| *L. garvieae* | 5 | 0.53 ± 0.06 | 0.085 ± 0.003 | 0.032 |
| | 30 | 0.071 ± 0.002 | 0.221 ± 0.003 | 0.15 |
| | 37 | 0.070 ± 0.001 | 0.227 ± 0.001 | 0.157 |

**Note:** $A_0$ = Absorbance before 24-hour incubation, $A_{24}$ = Absorbance after 24-hour incubation, $A_{24} - A_0$ = Difference in absorbance between 24 h and 0 h, and SD = Standard deviation.

**Table 3.** Growth test results of bacterial isolates under the influence of bile salts.

| Bacterial Isolates | $A_0$ ± SD | $A_{24}$ ± SD | $A_{24}$-$A_0$ | $A_{48}$ ± SD | $A_{48}$-$A_0$ |
|---|---|---|---|---|---|
| *L. garvieae* | 0.07 ± 0.001 | 0.09 ± 0.004 | 0.02 | 0.11 ± 0.011 | 0.04 |

**Note:** $A_0$ = Absorbance before 24-hour incubation, $A_{24}$ = Absorbance after 24-hour incubation, $A_{48}$ = Absorbance after 48-hour incubation, $A_{24} - A_0$ = Difference in absorbance between 24 h and 0 h, $A_{48} - A_0$ = Difference in absorbance between 48 h and 0 h, and SD = Standard deviation.

**Table 4.** Auto-aggregation test result of *Lactococcus garvieae* isolate.

| Bacterial Isolates | $A_0$ ± SD | $A_{24}$ ± SD | % Auto-aggregation | Conclusion |
|---|---|---|---|---|
| *L. garvieae* | 0.042 ± 0.002 | 0.043 ± 0.002 | 2 | *Non-autoagregasi* |

**Note:** $A_0$ = Absorbance before 24-h incubation, $A_{24}$ = Absorbance after 24-h incubation, dan SD = Standard deviation.

**Table 5.** Co-aggregation test result of *Lactococcus garvieae* isolate.

| Pathogenic bacteria | Bacterial Isolates | Ax ± SD | Ay ± SD | (Ax+Ay) ± SD | % *Co-aggregation* |
|---|---|---|---|---|---|
| *Shigella sp.* | *L. garvieae* | 0.152 ± 0.001 | 0.089 ± 0.003 | 0.064 ± 0.003 | 88.3 |
| *Escherichia coli* | | 0.152 ± 0.001 | 0.08 ± 0.002 | 0.064 ± 0.002 | 81.3 |
| *Salmonella sp* | | 0.152 ± 0.001 | 0.076 ± 0.003 | 0.059 ± 0.003 | 93.2 |

**Note:** $A_x$ = Absorbance of L. garvieae isolate suspension, $A_Y$ = Absorbance of pathogenic isolate suspension, $A_x + A_Y$ = Absorbance of the mixed suspension of L. garvieae and pathogenic isolates, and SD = Standard deviation.

**Table 6.** Antimicrobial activity test results of *Lactococcus garvieae* isolate.

| Bacterial Isolates | Pathogenic bacteria | Mean Zone of Inhibition Diameter (mm) ± Standard Deviation |
|---|---|---|
| *L. garvieae* | *E. coli* | 22.5 ± 0.85 |
| | *S. aureus* | 14.5 ± 0.60 |
| | *Salmonella sp.* | 24.1 ± 0.90 |

## Discussion

The first characterization was the growth of *L. garvieae* isolates on the given media (see **Table 1**). This characterization was carried out to observe the development of colonies for three days (72 h) on growth media in an anaerobic jar. Using an anaerobic jar aims to ensure that the bacterial isolates that grow are anaerobic bacterial isolates or bacteria that can live without oxygen. Bacterial colonies that grow at dilutions of $10^{-3}$ and $10^{-4}$ cannot be counted because the number is > 300 CFU/mL, whereas the range of a colony can be counted between 30 and 300 CFU/mL (16).

The growth process of LAB (Lactic Acid Bacteria) isolates can be performed well at 30 °C and 37 °C (see **Table 2**). Another study reported that the test results of LAB isolates obtained from commercial kefir wheat isolated from Indonesia against temperature resistance showed that all LAB isolates can grow at 37 °C (17). Several of the LAB isolates exhibited different growth patterns at various temperatures. For example, *Streptococcus sp*. can grow at 45 °C but cannot grow at 10 °C, *Lactococcus sp*. can grow at 10 °C, 15 °C, and 45 °C, while the genus *Lactobacillus sp*. can grow at 15 °C and 45 °C but not at 10 °C (18).

The bile salt tolerance test aims to assess the ability of both bacterial isolates to tolerate bile salts before entering the digestive tract. Bile salts tend to damage the structure of bacterial cell membranes, so this test is necessary to determine the tolerance of bacterial isolates to bile salts, which is an important characteristic for the survival of each bacterial strain in the intestine (19). **Table 3** shows that *L. garvieae* cannot grow in bile salt conditions. The decrease in the growth rate of bacterial isolates in an environment containing bile salts occurs due to changes in the permeability of bacterial cell membranes, leading to intracellular membrane leakage, which results in cell lysis and cell death (20). *P. acidilactici* isolated from the proventriculus of broiler chickens showed good viability in the bile salt tolerance test (21).

The autoaggregation test aims to evaluate the ability of bacterial isolates to grow and survive (colonize) in the digestive tract. This ability is determined by the percentage of autoaggregation obtained. If the result is less than 10%, the isolate is classified as non-autoaggregating. In contrast, if the result is greater than 10%, the bacterial isolate can grow and survive in the digestive tract (autoaggregating) (19). The test results showed that *L. garvieae* had an autoaggregation value below 10%, indicating that it is non-autoaggregating (see **Table 4**). The autoaggregation characteristic of probiotic strains is crucial for adhering to intestinal epithelial cells, as this ability is a prerequisite for a probiotic to colonize and persist in the digestive tract (20).

The co-aggregation test was conducted to observe the ability of bacterial isolates to combat pathogenic bacteria present in the digestive tract (21). In this co-aggregation test, three pathogenic bacteria were used: *Shigella* sp., *E. coli*, and *Salmonella* sp. In **Table 5**, it can be seen that *L. garvieae* demonstrates resistance or the ability to compete against pathogenic bacteria. The co-aggregation ability of a probiotic bacterium correlates with its capacity to form a barrier that prevents the colonization of pathogenic bacteria in the digestive tract. Therefore, lactic acid bacteria capable of coaggregating with pathogenic bacteria exhibit beneficial properties as probiotics (22).

LAB are bacteria with antimicrobial activity against spoilage and pathogenic bacteria through antagonistic properties. The antimicrobial effect of LAB in fermented foods is due to the presence of metabolic compounds such as lactic acid, acetic acid, organic acids, hydrogen peroxide, and bacteriocins (23). In another study, several lactic acid bacteria, such as *Lactobacillus* sp., were found to produce bacteriocins (antimicrobial compounds) and have the ability to inhibit the growth of both Gram-positive and Gram-negative bacteria (24). **Table 6** shows that *L. garvieae* produces an inhibition zone categorized as strong (inhibition zone >10–20 mm) and very strong (inhibition zone >20 mm). The antimicrobial mechanism of LAB isolates can occur through various pathways, including the presence of bacteriocins such as nisin or by lowering the pH through the production of acidic compounds like lactic acid. Some strains are known to produce enzymes and active compounds that inhibit pathogenic bacteria (25) and synthesize antimicrobial peptides that contribute to food preservation and safety (26).

Based on the results of this study, the indigenous *L. garvieae* isolate from *Wadi Papuyu* demonstrates promising probiotic potential, as evidenced by its antimicrobial activity against common foodborne pathogens and the absence of major virulence and antibiotic resistance genes. These findings suggest that *L. garvieae*from *Wadi Papuyu* could be considered suitable for probiotic applications, particularly in the context of traditional Indonesian fermented foods. However, to further confirm the safety and efficacy of this strain, future studies are recommended, including comprehensive genomic screening for additional virulence factors and *in vivo* testing to evaluate its probiotic effects and safety profile in animal models or human trials.

## Conclusion

The characteristics of *L. garvieae* as a probiotic candidate isolated from *wadi papuyu* (*A. testudineus* Bloch.) from Indonesia indicate that this bacterium can

grow on the provided media, is capable of growing at temperatures of 30 °C and 37 °C, meets the co-aggregation test, and exhibits antimicrobial activity categorized as strong and very strong against both Gram-negative and Gram-positive bacteria. However, *L. garvieae* cannot survive in a bile salt environment and does not meet the autoaggregation test. Despite these limitations, the notable antimicrobial and co-aggregation properties suggest that *L. garvieae* from *Wadi Papuyu* holds potential as a probiotic candidate, particularly for applications in food safety and pathogen inhibition. Further studies, including comprehensive safety assessments and *in vivo* evaluations, are recommended to establish its probiotic efficacy and suitability for broader applications fully.

# Declarations

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## Conflict of Interest

The authors declare no conflicting interest.

## Data Availability

The unpublished data is available upon request to the corresponding author.

## Ethics Statement

Not applicable.

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