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Acid and Bile Salt Tolerance of Presumptive Lactic Acid Bacteria Isolated from Tamarind (*Tamarindus indica* L.) Pulp

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Abstract

Probiotics are mostly dairy-derived, whereas acidic tropical fruits remain underexplored sources of lactic acid bacteria. Tamarind (*Tamarindus indica* L.) pulp is highly acidic, and, to our knowledge, its lactic acid bacteria have not been evaluated for acid and bile salt tolerance. This study aimed to isolate presumptive lactic acid bacteria from ripe tamarind pulp and screen their *in vitro* acid and bile salt tolerance. Bacteria were isolated on de Man, Rogosa and Sharpe agar with 0.5% calcium carbonate and characterized by colony morphology, Gram staining, and the catalase test. The isolates were exposed to broth at pH 3 or with 0.3% bile salt as basic screening conditions, and viable counts in three tubes per isolate were determined at 0, 2, and 4 hours and analyzed by two-way repeated-measures analysis of variance. Of fifteen colonies, five produced clear zones, and three were Gram-positive, catalase-negative presumptive lactic acid bacteria. At pH 3, relative viability was 86.10–90.15% after 2 hours, and counts exceeded the initial level after 4 hours (111.23–122.20%), possibly reflecting recovery or residual growth. In 0.3% bile salt, relative viability after 4 hours was 74.53–99.20%, and all reductions were below one logarithmic cycle. Isolate, time, and their interaction significantly affected counts in the acid assay, but only isolate did so in the bile salt assay. These isolates show preliminary probiotic-associated stress tolerance, and tamarind pulp may be a source of non-dairy probiotic candidates, although molecular identification and further functional and safety evaluations are required.

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Introduction

Gastrointestinal disorders remain a common health problem in Indonesia; the 2023 Indonesian Health Survey recorded a diarrhea prevalence of 7.4% among children under five years of age (Kementerian Kesehatan Republik Indonesia, 2023). An imbalance in the gut microbiota, or dysbiosis, weakens the intestinal barrier and facilitates colonization by pathogens, which has driven interest in food-based strategies that support gut health (Chandrasekaran et al., 2024). Probiotics, defined as live microorganisms that confer a health benefit on the host when administered in adequate amounts (Hill et al., 2014), are central to these strategies. Lactic acid bacteria (LAB) are the most widely used probiotic group because they ferment carbohydrates into lactic acid, produce antimicrobial metabolites such as organic acids and bacteriocins, and modulate host immunity (Wang et al., 2021; Latif et al., 2023).

A probiotic can only exert its effects if it reaches the intestine alive. After oral intake, the organism must first survive gastric juice, whose pH in healthy individuals ranges from about 1.5 to 3.5, and then bile salts in the small intestine, which occur at concentrations of roughly 0.03% to 0.3% (Karadaş et al., 2020; Farhangfar et al., 2021). For this reason, *in vitro* tolerance to low pH and bile salts is the first and most widely applied criterion in probiotic selection (De Melo Pereira et al., 2018; Wendel, 2022). Most candidate strains screened in this way have been isolated from dairy products and traditional fermented foods, such as yak milk cheese, fermented milk, and indigenous fermented dishes (Zhang et al., 2016; Mulaw et al., 2019; Vicky et al., 2022). Dairy-based probiotics, however, are less suitable for consumers with lactose intolerance or those who avoid animal products, which has increased the demand for non-dairy probiotic foods (Min et al., 2019). Fruits have emerged as a

promising alternative source, as LAB with probiotic traits have been recovered from pineapple juice, common table fruits, and various tropical fruits (Riani et al., 2020; Rodrigues et al., 2021; Vasundaradevi et al., 2025). Native LAB have also recently been isolated from Indonesian tropical fruit, such as matoa (*Pometia pinnata*), although those isolates were characterized only phenotypically and were not screened for gastrointestinal stress tolerance (Kamil et al., 2026).

Tamarind (*Tamarindus indica* L.), locally known as "asam jawa", is a tropical legume widely used in Indonesia as a culinary acidulant and traditional remedy (Girmay et al., 2020). Its pulp is rich in phenolic compounds with antioxidant and antibacterial activities (Fagbemi et al., 2022) and contains fermentable sugars together with organic acids, giving it a pH of about 3 or lower (Bakayoko et al., 2024). Such a substrate-rich but strongly acidic niche could act as a natural selective filter, favoring microorganisms that can maintain intracellular pH homeostasis, a trait typical of LAB (Zapaśnik et al., 2022; Khushboo et al., 2023). LAB isolated from tamarind pulp could therefore already tolerate the low pH encountered in the stomach. Nevertheless, research on tamarind has focused mainly on its phytochemistry and bioactivity, and the probiotic potential of its indigenous LAB has not been systematically investigated. Bakayoko et al. (2024) detected LAB in tamarind pulp sold in Ivorian markets, but only within a food quality and safety framework, without assessing the isolates as probiotic candidates. The present study differs from these earlier reports in that it targets a fruit whose pulp is itself strongly acidic and evaluates the isolates directly against the acid and bile salt stresses used in probiotic screening. To the best of our knowledge, the isolation of LAB from tamarind pulp combined with an evaluation of their acid and bile salt tolerance has not been reported.

The present study therefore aimed to isolate presumptive LAB from ripe tamarind pulp, to characterize the isolates phenotypically, and to evaluate their viability during a four-hour exposure to pH 3 and to 0.3% bile salt as basic *in vitro* screening criteria for potential probiotics. We hypothesized that LAB naturally inhabiting tamarind pulp would tolerate acid and bile salt stresses, possibly because of adaptation to the acidic fruit environment. Because no isolates from less acidic sources were included, the study tests the tolerance of the tamarind isolates rather than this ecological explanation itself. Identification of probiotic candidates from tamarind may contribute to the development of locally sourced, plant-based functional foods and broaden the diversity of LAB available for further development.

Materials and Methods

Study Design, Time, and Location

This experimental laboratory study (*in vitro*) comprised an exploratory isolation stage followed by tolerance assays. The laboratory work was carried out over two weeks starting on 6 April 2026 at the Microbiology Laboratory, Faculty of Medicine, Universitas Pembangunan Nasional "Veteran" Jakarta, Indonesia. The study protocol was reviewed by the Research Ethics Committee of Universitas Pembangunan Nasional "Veteran" Jakarta (KEP-UPNVJ) and was granted an exemption from ethical approval (No. 6/II/2026/KEP, 20 February 2026). The tolerance assays followed a repeated-measures (split-plot) design: three presumptive LAB isolates (between-tube factor) were each followed over three exposure times (0, 2, and 4 h; within-tube factor) in separate replicate tubes, and every tube was sampled at all three times. The number of replicates was determined according to Federer's formula, $(t - 1)(r - 1) \geq 15$, where t is the number of treatment combinations (nine) and r is the number of replicates (Halim et al., 2023), giving three replicate tubes per isolate, that is, nine tubes and 27 observations per assay.

Materials

The main materials were de Man, Rogosa and Sharpe (MRS) agar (Merck), MRS broth (Merck), calcium carbonate ($CaCO_3$; Merck), bile salts for microbiology, a mixture of sodium cholate and sodium deoxycholate (Cat. No. B8756; Sigma-Aldrich, Merck), hydrochloric acid (HCl; Merck), sterile 0.9% sodium chloride (NaCl) solution (OneMed), 3% hydrogen peroxide (H_2O_2; OneMed), and Gram staining reagents, namely crystal violet, Lugol's iodine, 95% ethanol, and safranin (Indo Reagen). The main instruments were an autoclave (All American), a laminar air flow cabinet (ABL BSC100), an incubator (Memmert), an analytical balance (Livotech), micropipettes (Dlab), a mini blender (HanRiver), a pH meter (Merck), a colony counter (Stuart Scientific), and a light microscope (Olympus CH20).

Sample Collection and Preparation

Ripe tamarind fruits were collected on 7 February 2026 from a single tree in Tridaya Sakti Village, Tambun Selatan District, Bekasi Regency, West Java, Indonesia (6.2442° S, 107.0678° E); the number of fruits was not recorded. The botanical identity of a specimen from the same tree was confirmed as *T. indica* L. at Herbarium Bandungense, School of Life Sciences and Technology, Institut Teknologi Bandung. The pulp was separated aseptically from the seeds, placed together in a tightly closed, airtight glass jar sterilized in boiling water, and stored continuously at 4 °C (El-Rhouttais et al., 2025) for 58 days, until isolation on 6 April 2026. Laboratory work could begin only after the ethical exemption had been granted (20 February 2026), and isolation was further delayed until the required materials were available and laboratory time could be scheduled. Glassware and media were sterilized by autoclaving at 121 °C for 15–20 min, transfer tools were flame-sterilized, and all aseptic work was performed in the laminar air flow cabinet pre-exposed to ultraviolet light for 15–30 min (Dwyana et al., 2024).

Isolation of Presumptive Lactic Acid Bacteria

Isolation followed the methods of Putri and Kusdiyantini (2018) and Kurnia et al. (2020) with modifications. Ten grams of pulp were homogenized with 90 mL of sterile 0.9% NaCl in the sterilized mini blender for 2–3 min, and the suspension was pre-incubated at 37 °C for 24 h. The suspension was then serially diluted (1:10) in sterile NaCl to 10^{-5}, and 0.1 mL of each dilution was spread in triplicate on MRS agar supplemented with 0.5% $CaCO_3$. Plates were incubated at 37 °C for

24–144 h in a candle jar to provide microaerophilic conditions and were inspected every 24 h. Colonies with a typical LAB appearance and a clear zone around them, indicating acid production that dissolves $CaCO_3$ (Amaliah et al., 2018), were selected and purified by repeated streaking on MRS agar at 37 °C for 24–72 h (Goa et al., 2022). Pure cultures were maintained on MRS agar slants.

Morphological and Biochemical Characterization

Colony morphology of the purified isolates was described in terms of shape, margin, elevation, size, and color (Petersen and McLaughlin, 2016). Gram staining was performed according to Vicky et al. (2022) with modifications, using crystal violet (1 min), Lugol's iodine (1 min), decolorization with 95% ethanol, and safranin counterstain (1 min); smears were examined at 1000× magnification under oil immersion with the light microscope. Catalase activity was tested by mixing a colony with one or two drops of 3% H_2O_2 on a glass slide, where immediate bubbling indicated a positive reaction (Goa et al., 2022). Isolates that were Gram-positive and catalase-negative were regarded as presumptive LAB (Wasis et al., 2019) and were activated in MRS broth at 37 °C for 24–48 h before the tolerance assays (Mulaw et al., 2019).

Acid Tolerance Assay

The acid tolerance assay was adapted from Zhang et al. (2016) and Ko et al. (2022). MRS broth was adjusted to pH 3.0 by stepwise addition of 0.1 mol/L HCl, and the pH was confirmed with the calibrated pH meter before inoculation. For each isolate, a single preculture grown in MRS broth at 37 °C for 24–48 h was used to inoculate three separate assay tubes (replicates A–C), which were processed in parallel on the same day. In each tube, 1 mL of the culture was added to 9 mL of acidified broth, mixed, and incubated at 37 °C. Each tube was sampled immediately after mixing (0 h) and after 2 and 4 h. At each sampling point, 1 mL of the suspension was serially diluted (1:10) in sterile NaCl to 10^{-5}, and 0.1 mL of this dilution was spread on one MRS agar plate. Plates were incubated at 37 °C for 72 h, and colonies were counted with the colony counter. Viable counts were calculated as colony-forming units (CFU)/mL = number of colonies × dilution factor / volume plated, that is, colonies × 10^5 / 0.1 mL. All plates contained 30–300 colonies except one plate in the bile salt assay (isolate 1, 2 h, replicate B; 27 colonies), which was retained so that each mean was based on three tubes. The initial cell density of the inocula was not standardized between isolates; instead, the initial (0 h) count was measured in every tube, and the mean 0 h count of each isolate served as its baseline.

Bile Salt Tolerance Assay

The bile salt tolerance assay was adapted from Vicky et al. (2022). MRS broth was supplemented with bile salts to a final concentration of 0.3% (w/v; 0.21 g in 70 mL, weighed with the analytical balance) and homogenized until completely dissolved. A separate preculture of each isolate was prepared for this assay in the same way, and inoculation of three tubes per isolate, incubation, sampling at 0, 2, and 4 h, serial dilution, plating, colony counting, and calculation of CFU/mL were performed exactly as described for the acid tolerance assay.

Data and Statistical Analysis

Viable counts are reported as the mean ± standard deviation (SD) of the three replicate tubes (n = 3), in CFU/mL and as $\log_{10}$ CFU/mL. A self-control design was applied, in which the 0 h count of each isolate served as its baseline (De Melo Pereira et al., 2018; Wendel, 2022). Relative viability was calculated as RV (%) = (N_t/N_0) × 100, and the logarithmic change as $\Delta\log = \log_{10}N_t - \log_{10}N_0$, where N_0 and N_t are the mean counts (CFU/mL) at 0 h and at time t (2 or 4 h). An isolate was considered tolerant when it retained high relative viability with a reduction of less than one logarithmic cycle (Zhang et al., 2016; Vicky et al., 2022); this criterion was used for preliminary screening only. Because each tube was sampled repeatedly, the effects of isolate, exposure time, and their interaction on viable counts (CFU/mL) were tested by two-way mixed (repeated-measures) analysis of variance (ANOVA), with isolate as the between-subjects factor, exposure time as the within-subjects factor, and the replicate tube as the subject. The normality of residuals (Shapiro–Wilk test), the homogeneity of variances at each time point (Levene's test), and sphericity (Mauchly's test) were checked, and the Greenhouse–Geisser correction was planned in case sphericity was violated. Partial eta squared (η_p^2) was reported as the effect size. A significant isolate effect was followed by Tukey's honestly significant difference (HSD)

Table 1. Colony Morphology, Gram Reaction, and Catalase Activity of the Five Clear-Zone-Forming Isolates from Tamarind Pulp

Isolate	Colony morphology (shape; margin; elevation; size; color)	Cell shape (arrangement)	Gram reaction	Catalase	Selected
1	Circular; entire; convex; medium; opaque cream	Coccus (single/clustered)	+	−	Yes
2	Circular; entire; convex; medium; opaque cream	Coccus (single)	+	−	Yes
3	Circular; entire; convex; medium; opaque cream	Rod (single)	+	−	Yes
6	Circular; lobate; raised; small; cream	Coccus (clustered)	+	+	No
8	Circular; undulate; raised; small; opaque cream	Coccus (single)	+	+	No

All five isolates produced a clear zone on de Man, Rogosa and Sharpe agar supplemented with 0.5% $CaCO_3$. + = positive; − = negative. Qualitative observations; standard deviation is not applicable. Source: primary data of this

study.
test, a significant time effect by Bonferroni-adjusted pairwise comparisons, and a significant interaction by simple-effect comparisons between time points within each isolate with Bonferroni adjustment. As a sensitivity analysis, the ANOVA was repeated on $\log_{10}$-transformed counts. Analyses were performed with IBM SPSS Statistics version 29.0.2 (IBM Corp., Armonk, NY, USA), and $p < 0.05$ was considered significant.

Results and Discussion

Isolation and Phenotypic Characterization

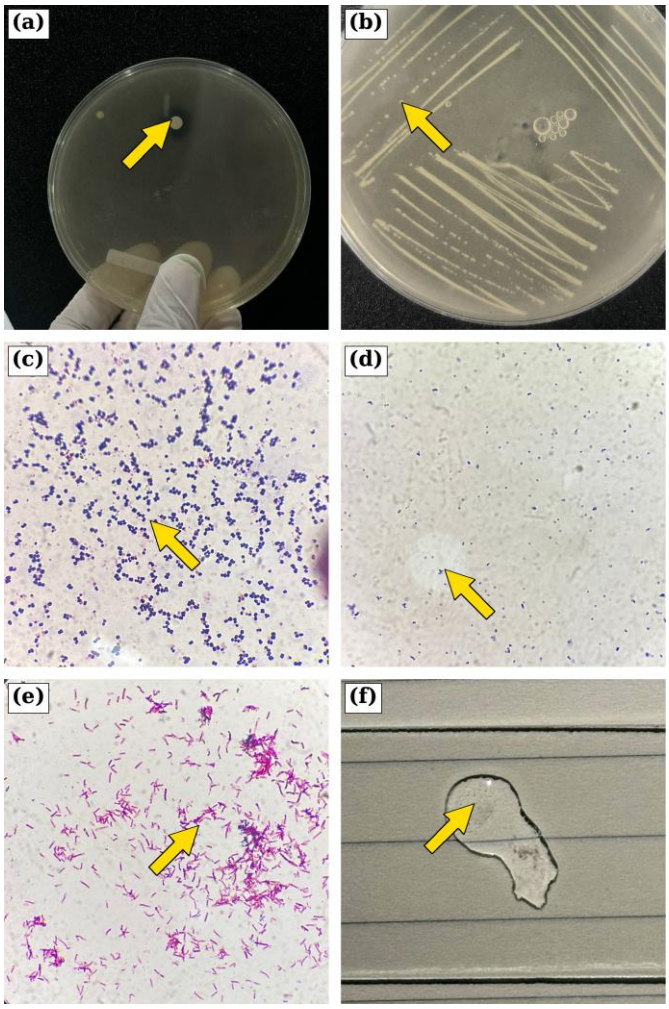


Figure 1. Isolation and Phenotypic Characterization of Presumptive Lactic Acid Bacteria from Tamarind Pulp. (a) Primary isolation plate (de Man, Rogosa and Sharpe agar + 0.5% $CaCO_3$) showing a colony of isolate 1 surrounded by a clear zone (arrow); (b) purified culture of isolate 1 obtained by streaking on de Man, Rogosa and Sharpe agar (arrow); (c–e) Gram-stained cells of isolates 1, 2, and 3, respectively (1000×, oil immersion; arrows indicate representative cells); (f) catalase-positive reaction of isolate 6 (gas bubbles, arrow), shown for comparison with the catalase-negative isolates 1–3 (Table 1). Photographs: primary documentation of this study.

Fifteen morphologically distinct colonies were recovered from tamarind pulp on MRS agar containing $CaCO_3$. Eleven colonies were circular and four were irregular, and their color ranged from cream, opaque cream, and translucent cream to yellow and white. Only five colonies (isolates 1, 2, 3, 6, and 8) were surrounded by a clear zone (Figure 1a, isolate 1) and were therefore purified (Figure 1b, isolate 1); the remaining ten were excluded.

The purified isolates were circular, with entire margins, convex elevation, and medium size for isolates 1–3, and lobate or undulate margins, raised elevation, and small size for isolates 6 and 8 (Table 1). All five isolates stained purple and were classified as Gram-positive (Figure 1c–e). Isolates 1, 2, 6, and 8 were cocci, whereas isolate 3 was rod-shaped. Isolates 1, 2, and 3 were catalase-negative, while isolates 6 and 8 produced gas bubbles and were catalase-positive (Figure 1f, isolate 6). Consequently, only isolates 1, 2, and 3 met the combined criteria for presumptive LAB and were carried forward to the tolerance assays.

The clear zone forms when organic acids, mainly lactic acid, secreted by the colony dissolve the surrounding $CaCO_3$, which makes it a practical first indicator of the acid-producing capacity that defines LAB (Amaliah et al., 2018). The candle jar provided the reduced-oxygen atmosphere that favors LAB, which are aerotolerant anaerobes (Dwijastuti and Dewi, 2023). The morphological heterogeneity among isolates suggests that more than one type of bacterium was recovered from tamarind pulp, as also observed in other LAB-rich substrates (Parlindungan et al., 2021), whereas the four isolates recently obtained from matoa fruit were uniform Gram-positive rods with cream, circular colonies (Kamil et al., 2026). A Gram-positive reaction reflects the thick peptidoglycan layer that retains the crystal violet–iodine complex and is a basic trait of LAB (Rahmi et al., 2016; Putri and Kusdiyantini, 2018). The presence of both rods and cocci is consistent with the morphological diversity of LAB; however, cell shape and arrangement cannot be used to assign a genus or species (Zheng et al., 2020). The isolates are therefore referred to as presumptive LAB throughout, and their taxonomic identity remains to be determined by 16S rRNA gene sequencing.

The catalase-positive isolates, despite forming clear zones, are most likely non-LAB acid-tolerant organisms that grew on the selective medium, which is plausible given that tamarind pulp supports a mixed microbial community (Bakayoko et al., 2024). This finding underlines that LAB screening should combine the clear-zone reaction, Gram staining, and the catalase test rather than rely on a single criterion (Wasis et al., 2019).

Acid Tolerance

All three isolates survived exposure to pH 3 with a similar pattern: viable counts decreased at 2 h and then rose above the baseline at 4 h (Table 2; Figure 2a). After 2 h, relative viability ranged from 86.10% to 90.15%, and the logarithmic reductions were only 0.045–0.065, far below one log cycle; all isolates therefore met the screening criterion applied in this study (Zhang et al., 2016; Vicky et al., 2022). At 4 h, counts exceeded the baseline in all isolates (111.23–122.20%). The repeated-measures ANOVA showed significant effects of isolate ($F(2, 6) = 125.87$, $p < 0.001$), exposure time ($F(2, 12) = 434.75$, $p < 0.001$), and their interaction ($F(4, 12) =$

31.74, $p < 0.001$), all with large effect sizes (Table 3). Simple-effect comparisons showed that, in every isolate,

Table 2. Viable Counts, Relative Viability, and Logarithmic Change of Isolates 1–3 during Exposure to pH 3 and 0.3% Bile Salt

Isolate	Time (h)	Acid (pH 3)			Bile salt (0.3%)		
		Count[a]	RV (%)[b]	Δlog[b]	Count[a]	RV (%)[b]	Δlog[b]
1	0	9.70 ± 0.46	–	–	5.37 ± 0.38	–	–
	2	8.73 ± 0.60*	90.03	−0.046	4.07 ± 1.19	75.78	−0.120
	4	11.23 ± 0.70*	115.81	+0.064	4.00 ± 0.20	74.53	−0.128
2	0	17.27 ± 0.80	–	–	8.37 ± 0.75	–	–
	2	15.57 ± 0.71*	90.15	−0.045	8.40 ± 0.79	100.40	+0.002
	4	21.10 ± 1.06*	122.20	+0.087	8.30 ± 1.04	99.20	−0.003
3	0	12.47 ± 0.60	–	–	7.10 ± 0.40	–	–
	2	10.73 ± 0.47*	86.10	−0.065	6.50 ± 0.95	91.55	−0.038
	4	13.87 ± 0.45*	111.23	+0.046	5.90 ± 0.61	83.10	−0.080

[a]Viable count (×10^7 CFU/mL), mean ± standard deviation of three replicate tubes (n = 3). [b]RV = relative viability, (N_t/N_0) × 100; Δlog = $\log_{10}N_t - \log_{10}N_0$; both calculated from mean counts, so no standard deviation is given. – = baseline (0 h). *Significantly different from 0 h within the same isolate (Bonferroni-adjusted simple effects, $p < 0.05$); time points were not compared in the bile salt assay (Table 3). Source: primary data of this study.

the 2 h count was significantly lower and the 4 h count significantly higher than the 0 h count (Table 2); the interaction reflected differences in the magnitude of these changes, with the largest rise in isolate 2. Because the inocula were not standardized, the absolute counts differed among isolates from the start (0 h: 0.97–1.73 × 10^8 CFU/mL, a difference of 0.25 log units), so the isolate effect largely reflects initial density; comparisons among isolates are therefore based on relative viability and Δlog rather than on absolute counts.

Tolerance to gastric acidity is the first barrier a probiotic must overcome, because low pH damages the cell wall and membrane and denatures proteins (Karadaş et al., 2020; Yang et al., 2025). The initial decline observed here is consistent with proton influx that disturbs intracellular pH and metabolic activity (Guan and Liu, 2020). The increase at 4 h, however, should not be interpreted as acid adaptation. Because MRS broth remained nutritionally supportive throughout the experiment and no unstressed growth control was included, the rise may reflect recovery of sublethally injured cells, residual growth of the surviving population, or variation in plating and counting, and the present design cannot distinguish among these explanations. The assay should therefore be interpreted primarily as a survival assessment rather than as a measure of acid resistance mechanisms. Acid stress responses such as proton extrusion by F_0F_1-ATPase, amino acid-dependent deacidification, and membrane remodeling have been described in LAB (Guan and Liu, 2020; Li et al., 2023), but they were not measured here. Ko et al. (2022) likewise showed that pH and exposure time interact in determining LAB viability, and a similar increase has been reported for a pineapple juice isolate whose count increased by 0.4 log CFU/mL after 2 h at pH 2 (Riani et al., 2020); the increase observed in the present study was more moderate (0.046–0.087 log).

For context, Zhang et al. (2016) found that 29 of 69 lactobacilli from yak milk cheese retained at least 90% viability after 2 h at pH 2 or 3, with a maximum of 97% at pH 3, while Vicky et al. (2022) reported 91.6% viability for isolate DNH-16 after 4 h at pH 3, and Nawaz et al. (2017) observed good tolerance at pH 3 in all isolates from fermented foods. Fruit-derived *Lactiplantibacillus* strains have also been shown to withstand pH 2 (Vasundaradevi et al., 2025). These comparisons are only indicative, because the studies differ in strains, media, initial cell density, pH adjustment, exposure time, and enumeration methods. The uniformly high acid survival of the three tamarind isolates is consistent with, but does not demonstrate, the hypothesis that the naturally acidic pulp (Bakayoko et al., 2024) favors acid-tolerant bacteria, because isolates from less acidic sources were not tested under identical conditions.

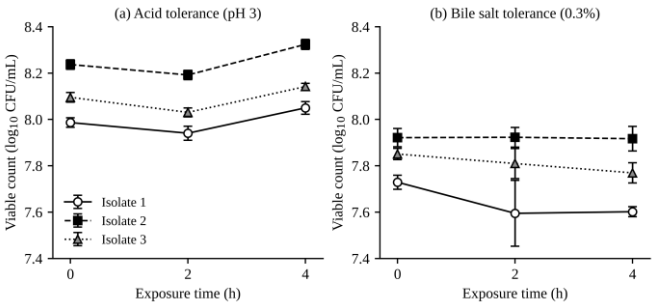


Figure 2. Viable Counts of Isolates 1–3 during Exposure to Acid and Bile Salt. Viable counts ($\log_{10}$ CFU/mL) during exposure to (a) de Man, Rogosa and Sharpe broth at pH 3 and (b) the same broth containing 0.3% bile salt. Points are means of three replicate tubes (n = 3), and error bars indicate the standard deviation of the $\log_{10}$-transformed counts. Source: primary data of this study.

Bile Salt Tolerance

In 0.3% bile salt, the viable counts of each isolate remained relatively stable over the 4 h exposure, and the three isolates were clearly separated from one another (Table 2; Figure 2b). Relative viability at 2 and 4 h was 100.40% and 99.20% for isolate 2, 91.55% and 83.10% for isolate 3, and 75.78% and 74.53% for isolate 1. The largest logarithmic reduction was 0.128 (isolate 1 at 4 h); thus, all isolates retained more than 70% of their initial count, none lost one log cycle, and all met the screening criterion applied in this study (Zhang et al., 2016; Vicky et al., 2022). The repeated-measures ANOVA showed a significant isolate effect ($F(2, 6) = 74.19$, $p < 0.001$), whereas the effects of time ($F(2, 12) = 2.78$, $p = 0.102$) and of the isolate × time interaction ($F(4, 12) = 0.78$, $p = 0.557$) were not significant (Table 3). The absolute

counts followed the order isolate 2 > isolate 3 > isolate 1

Table 3. Two-Way Repeated-Measures Analysis of Variance of Viable Counts in the Acid and Bile Salt Tolerance Assays

Source of variation	df	Acid (pH 3)			Bile salt (0.3%)		
		F	*p*	η_p^2	*F*	*p*	η_p^2
Isolate	2, 6	125.87	< 0.001*	0.977	74.19	< 0.001*	0.961
Time	2, 12	434.75	< 0.001*	0.986	2.78	0.102	0.317
Isolate × Time	4, 12	31.74	< 0.001*	0.914	0.78	0.557	0.207
Shapiro–Wilk, *p*			0.226			0.985	
Levene's test, *p* (range)			0.284–0.803			0.046–0.645	
Mauchly's test, *W* (*p*)		0.734	0.461		0.984	0.961	

Isolate was the between-subjects factor and exposure time the within-subjects factor; the subject was the replicate tube (n = 3 per isolate). df = degrees of freedom (effect, error); η_p^2 = partial eta squared; W = Mauchly's statistic. The Shapiro–Wilk test was applied to the residuals. Sphericity was not violated; with equal group sizes, the ANOVA is robust to the slight variance heterogeneity at 4 h in the bile salt assay (Levene, $p = 0.046$). *$p < 0.05$. Tukey's test: all isolate pairs differed in both assays ($p \leq 0.008$); in the acid assay, all time points differed (Bonferroni, $p < 0.001$). Log_{10}-transformed counts gave the same pattern of significance. Source: primary data of this study.

already at 0 h (0.54–0.84 × 10^8 CFU/mL, a difference of 0.19 log units), so the isolate effect partly reflects initial density; the relative viability values, which are normalized to the initial density of each isolate, followed the same order.

Bile salts act as biological detergents that disrupt membrane lipids, increase permeability, chelate calcium and iron, and cause oxidative DNA damage (Urdaneta and Casadesús, 2017). The concentration of 0.3% used here corresponds to the upper level reported for the small intestine and is the most common screening threshold (Farhangfar et al., 2021). Viable counts did not change significantly over the 4 h exposure (Table 3). The early decrease in isolate 1 (75.78% at 2 h, a value lowered by the single plate with 27 colonies) may reflect the loss of cells lacking adequate protection, but it should be interpreted with caution because neither time nor the isolate × time interaction was significant. Bile tolerance can involve several mechanisms, including bile salt hydrolase (BSH) activity (Foley et al., 2019; Bourgin et al., 2021) and changes in membrane composition, but none of these was measured, and survival in 0.3% bile salt alone does not indicate which mechanism operates in the tamarind isolates. Likewise, whether bile tolerance reflects previous exposure to the stressful, acidic fruit environment cannot be determined from the present design.

For context, Vicky et al. (2022) reported 66.5% viability for isolate DNH-16 after 4 h in 0.3% bile, compared with the 74.53–99.20% retained by our isolates over the same period. Nawaz et al. (2017) found that fermented-food isolates survived well at 0.3% and 0.4% bile but that only three of 25 tolerated 0.75–1.0%, while 72% of the yak cheese lactobacilli studied by Zhang et al. (2016) were classified as bile-tolerant at 0.3%. As with the acid assay, these values are not directly comparable, because of differences in strains, culture media, initial cell density, bile composition, exposure time, and enumeration methods. The near-complete survival of isolate 2, whose viable population remained virtually unchanged, justifies testing it at higher bile concentrations in future work.

Probiotic Potential of the Tamarind Isolates and Study Limitations

Survival of both gastric acidity and intestinal bile is a prerequisite for a strain to reach the intestine in sufficient numbers to be beneficial (De Melo Pereira et al., 2018; Sarita et al., 2025). The three tamarind isolates satisfied the preliminary *in vitro* acid and bile salt tolerance criteria used here, retaining more than 85% viability after 2 h at pH 3 and more than 74% after 4 h in 0.3% bile salt, with all reductions well below one log cycle, and their mean viable counts remained between 4.0 × 10^7 and 2.1 × 10^8 CFU/mL throughout both assays. These results identify them as presumptive LAB with preliminary probiotic-associated stress tolerance, not as probiotics. Because no composite selection criterion was defined in advance, no single isolate is designated as the lead candidate; nevertheless, isolate 2, a Gram-positive, catalase-negative coccus, showed the highest relative viability in bile salt (99.20% at 4 h) and an acid survival comparable to or higher than that of the other isolates, which makes it a logical first candidate for identification. Acid and bile tolerance are only the first step of probiotic characterization: adhesion to intestinal epithelial cells, autoaggregation and coaggregation, antimicrobial activity, exopolysaccharide production, antibiotic susceptibility, and safety must be evaluated before probiotic status can be considered (De Melo Pereira et al., 2018). Safety assessment should include hemolytic activity, antimicrobial susceptibility and transferable resistance genes, virulence and toxin genes, biogenic amine production, and whole-genome analysis if the isolates progress toward application (Roe et al., 2022). Because tamarind is an inexpensive, widely consumed local commodity of plant origin, isolates derived from it could support the development of non-dairy functional foods (Min et al., 2019).

This study has several limitations. Identification was based only on colony morphology, Gram staining, and the catalase test, so the isolates remain presumptive LAB whose genus and species must be confirmed by 16S rRNA gene sequencing. The pH 3 and 0.3% bile salt assays are basic *in vitro* screening models and do not reproduce gastrointestinal transit, which involves dynamic pH, pepsin and other digestive enzymes, food matrix effects, variable residence times, mixed bile acids, intestinal osmolarity and nutrient availability, and interactions with the gut microbiota. No unstressed growth control or reference probiotic strain (e.g., *Lacticaseibacillus rhamnosus* GG) was included, the

initial inoculum density was not standardized (e.g., by a McFarland standard or optical density with plate-count confirmation), and the pH of the medium was verified before inoculation but not monitored at each sampling time. The three replicate tubes of each isolate originated from one preculture and one experimental run, so they are not fully independent biological replicates. The pulp came from a single tree at one site and was stored at 4 °C for 58 days before isolation, which may have altered the culturable community; the results therefore show that presumptive LAB can be recovered from the sampled material rather than that tamarind pulp in general is a reliable source of probiotics. Finally, mechanisms such as BSH activity and acid stress responses were discussed on the basis of the literature and were not tested directly. Other functional properties and safety traits of the isolates were not evaluated. Future studies should therefore standardize the inoculum, include an unstressed growth control and a reference probiotic strain, use independent biological replicates prepared on different days, and sample fruits from several trees and locations.

Conclusion

Presumptive lactic acid bacteria were isolated from ripe tamarind pulp, and all three Gram-positive, catalase-negative isolates retained their viability at pH 3 and in 0.3% bile salt *in vitro*, with losses of less than one logarithmic cycle. In the acid assay, viable counts were significantly affected by the isolate, exposure time, and their interaction, whereas in the bile salt assay only the isolate effect was significant. The isolates satisfied preliminary *in vitro* criteria associated with probiotic potential, particularly acid and bile salt tolerance, but they cannot yet be regarded as probiotics: molecular identification, further functional characterization, and safety assessment remain necessary. Tamarind pulp may therefore be a source of non-dairy probiotic candidates for functional food development.

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