

Original Research Article

Probiotic potential, antioxidant, and antimicrobial properties of *Lactobacillus* spp. isolated from indigenous food plants in Ilocos Norte, Philippines

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Abstract

Purpose: To evaluate the *in vitro* probiotic, antioxidant, and antimicrobial properties of *Lactobacillus* spp. isolated from Philippine indigenous food plants (IFPs) as potential sources of non-dairy probiotic strains.

Methods: Four isolates were assessed for gastrointestinal tolerance across a pH range of 2.0 – 8.0 and at bile concentrations of 0.5 and 1 %. Cell-surface characteristics, such as auto-aggregation and cell surface hydrophobicity, were measured. Bile salt hydrolase (BSH) activity was qualitatively evaluated. Ethyl acetate fractions of cell-free supernatants (EAFs) were tested for antioxidant capacity using the DPPH assay and for antimicrobial effects against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

Results: *Lentilactobacillus hilgardii* DSB22 showed the highest survival at pH 2 (50.22 ± 3.32 %) and 1 % bile (80.50 ± 4.37 %) after 6 h. It also demonstrated superior auto-aggregation (79.35 ± 0.56 %) and cell surface hydrophobicity (63.36 ± 0.40 %). All isolates were BSH positive. The EAF of *L. hilgardii* DSB22 exhibited strong radical scavenging capacity (73.51 ± 0.58 %) and the greatest antimicrobial effect, especially against *S. aureus* (18.33 ± 2.89 mm).

Conclusion: *Lentilactobacillus hilgardii* DSB22 exhibits robust probiotic potential, and IFPs represent valuable sources of non-dairy probiotic strains.

Keywords: Antimicrobial, Auto-aggregation, Bile tolerance, Indigenous food plants, *In vitro*, *Lactobacillus*, Probiotics

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INTRODUCTION

Probiotics are live microorganisms that can provide health benefits to the host when consumed in sufficient amounts [1]. Among the

various probiotic groups, lactic acid bacteria (LAB), especially *Lactobacillus* spp., have been reported to support gut health, influence immune function, and contribute to the prevention and management of certain diseases [2-4]. The

demand for functional foods continues to rise as more consumers become conscious of the relationship between diet and health [5]. Interest in probiotic products has also grown, particularly among individuals seeking alternatives to dairy-based foods due to lactose intolerance, dairy allergies, or concerns related to fat content [4]. As a result, non-dairy and plant-based probiotic sources have attracted considerable attention.

Indigenous food plants (IFPs) offer a promising but still largely underexplored source of potentially beneficial probiotics [6]. Although the Philippines is rich in plant biodiversity, studies examining probiotic strains from IFPs remain limited [3]. In Ilocos Norte, many IFPs are commonly consumed and continue to be a part of the local diet. However, little information is available regarding their potential to harbor beneficial probiotic strains [7]. This represents a missed opportunity to utilize locally available plant resources in the development of functional foods and probiotic-based products.

Therefore, this study aimed to evaluate the *in vitro* probiotic potential and functional properties of *Lactobacillus* spp. isolated from bayog (*Bambusa spinosa*), nipa sap (*Nypa fruticans*), buga (*Dioscorea esculenta* var. *spinosa*), and kamangeg (*Dioscorea luzonensis*). This study identifies probiotic candidates from traditionally and locally consumed food plants to support the development of locally adapted non-dairy probiotic products.

METHODS

Microorganisms and growth conditions

Four *Lactobacillus* isolates were tested in this study, namely, *Lentilactobacillus hilgardii* DSB22, *Lactobacillus paracasei* 7060, *Lacticaseibacillus paracasei* B2, and *Limosilactobacillus fermentum* PJG11 (Table 1). These isolates were previously identified through 16S rRNA sequencing and maintained in glycerol stocks at -80°C until further use. *Lactobacillus plantarum* (ATCC 14917) and *Lactobacillus fermentum* (ATCC 9338) were used as reference strains. For experimental assays, each isolate was activated by inoculating it into de Man, Rogosa, and

Sharpe (MRS) broth and incubating it at 37°C for 18 – 24 hours under anaerobic conditions. Three bacterial pathogens, *Staphylococcus aureus* (ATCC 12600), *Escherichia coli* (ATCC 11775), and *Pseudomonas aeruginosa* (ATCC 25668), were used in the antimicrobial assay.

Evaluation of pH tolerance

Tolerance to different pH conditions was tested using a method previously reported [11], with minor modifications. Cultures were harvested by centrifugation at 6,500 rpm for 10 min at 4°C after incubation. The cell pellets obtained were washed three times with sterile 0.85 % (w/v) NaCl solution and resuspended to obtain a uniform suspension. One milliliter of the suspension was transferred to 9 mL of NGYC (Nutrient, Glucose, Yeast, and Cysteine) medium. The pH of the medium was adjusted to 2.0, 4.0, 6.5, or 8.0 with HCl or NaOH. The inoculated media were incubated at 37°C , and samples were collected at 0, 3, and 6 h for analysis. Viable counts were determined by the spread plate method using MRS agar, followed by incubation at 37°C for 48 h. Percentage survival was calculated using Eq 1.

$$\text{Survival (\%)} = \{(\text{CFU/mL at } x \text{ Hour})/(\text{CFU/mL at } 0 \text{ Hour})\} 100 \dots\dots\dots (1)$$

Where time (x) is 3 and 6 hours.

Evaluation of bile tolerance

Tolerance to different bile concentrations was evaluated following a previously described method [11], with minor modifications. Cell suspensions were prepared as described for the pH tolerance assay. One milliliter of the suspension was transferred into 9 mL of NGYC medium, supplemented with 0.5 or 1 % (w/v) bile. The pH of the medium was adjusted to 6.9. The inoculated media were incubated at 37°C , and samples were collected at 0, 3, and 6 h for analysis. Viable cell counts were enumerated using the spread plate method on MRS agar following incubation at 37°C for 48 h. Percentage survival was calculated using Eq 1.

Table 1: IFP source and NCBI accession number of *Lactobacillus* isolates

Isolate	IFP Source	NCBI accession number	Reference
<i>Lentilactobacillus hilgardii</i> DSB22	<i>Bambusa spinosa</i>	PX363295	[8]
<i>Lactobacillus paracasei</i> 7060	<i>Nypa fruticans</i>	-	
<i>Lacticaseibacillus paracasei</i> B2	<i>Dioscorea esculenta</i> var. <i>Spinosa</i>	PV992281	[9]
<i>Limosilactobacillus fermentum</i> PJG11	<i>Dioscorea luzonensis</i>	PV992279	[10]

Auto-aggregation evaluation

Auto-aggregation was evaluated following previously reported methods [12], with minor modifications. After centrifugation at 8,000 rpm for 10 min, the bacterial cells were washed twice with phosphate-buffered saline (PBS; pH 7.4). The cell suspension was diluted to an OD₆₀₀ of 0.25 ± 0.05 , which corresponds to a bacterial concentration of $\approx 10^7$ – 10^8 CFU/mL before analysis. The cell suspensions were vortexed for 10 s and incubated under stationary conditions at room temperature for 2, 4, and 6 h. At each time point, the upper suspension was carefully collected without disturbing settled aggregates, and absorbance was measured at 600 nm. All assays were done in triplicate. *Lactobacillus plantarum* (ATCC 14917) was used as the reference strain due to its established safety and documented probiotic properties. Percentage auto-aggregation was calculated using Eq 2.

$$\text{Auto-aggregation (\%)} = \{(A_0 - A_t)/A_0\}100 \dots (2)$$

where A_t is the absorbance of the upper suspension at time (t) = 2, 4, and 6 h, and A_0 is the initial absorbance (baseline).

Evaluation of cell surface hydrophobicity

Cell surface hydrophobicity was evaluated using previously described methods [13], with minor modifications. Cell suspensions were obtained using the method employed in the auto-aggregation assay. Each cell suspension was mixed thoroughly with hexane at a 1:1 ratio using a vortex mixer for 2 min, and incubated at room temperature for 30 min without disturbing to allow phase separation. The aqueous phase was carefully collected, and its absorbance was measured at 600 nm. All assays were done in triplicate, with *Lactobacillus plantarum* (ATCC 14917) used as the reference strain. Percentage cell surface hydrophobicity was calculated using Eq 3.

$$\text{Cell surface hydrophobicity (\%)} = \{(A_0 - A_1)/A_0\}100 \dots (3)$$

where A_1 is the absorbance of the aqueous phase after two-phase system separation, and A_0 is the initial absorbance (baseline).

Evaluation of bile salt hydrolase activity

Bile salt hydrolase (BSH) activity was qualitatively evaluated as previously described [14]. Bile salt agar was prepared by supplementing MRS agar with 0.5 % (w/v) mixed bile salts from bovine origin (ox gall) and 0.37 g/L

CaCl₂. Sterile filter discs were saturated with overnight cultures of the *Lactobacillus* isolates and placed onto the bile salt agar plates. Plates were incubated at 37 °C for 72 h, with unsupplemented MRS agar serving as the control. The presence of opaque, granular white colonies or precipitate halos surrounding the discs was interpreted as a positive indication of BSH activity.

Preparation of *Lactobacillus* spp. postbiotics

Freshly grown cultures were harvested through centrifugation at 6,500 rpm for 10 min at 4 °C. The resulting cell-free supernatant (CFS) containing postbiotic metabolites was collected and sterilized by filtration through a 0.22 µm membrane. The filtered CFS was lyophilized using a freeze dryer, reconstituted in ultrapure water and placed in a separatory funnel. An equal volume of ethyl acetate was added and the mixture was shaken and allowed to stand until the phase separation was complete. The ethyl acetate layer was collected and the extraction repeated three times with fresh solvent. The combined ethyl acetate fractions were concentrated under reduced pressure using a rotary evaporator and further dried using a speed vacuum concentrator. The dried ethyl acetate fraction of the cell-free supernatant (EAFP) was kept at 4 °C for subsequent antioxidant and antimicrobial analyses.

Evaluation of the antioxidant capacity

The radical scavenging capacity of the EAFPs was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [2], with slight modifications. A 0.01 mM (0.037 mg/mL) DPPH solution was prepared in methanol and incubated in a dark environment for 1 h. Each EAFP was dissolved in methanol to make serial concentrations of 1000, 100, 10, 1, and 0.01 µg/mL. L-Ascorbic acid served as the positive control.

In a 96-well microplate, 100 µL of each sample dilution was added to 100 µL of DPPH solution. The plate was incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a multimode microplate reader. The percentage of radical scavenging capacity was calculated using Eq 4.

$$\text{Radical Scavenging Capacity (\%)} = \{(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}\}100 \dots (4)$$

where A_{control} is the absorbance of the negative control, and A_{sample} is the absorbance of the samples at 517 nm.

Evaluation of the antimicrobial effect

The antibacterial effect of the EAFPs and two reference strains against three key pathogenic microorganisms: *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* was assessed using diffusion assays. Stock solutions of each EAFP were prepared by transferring the EAFPs into microcentrifuge tubes and diluting them with 2.5 % dimethyl sulfoxide in sterile ultrapure water to achieve the desired working concentration. Dimethyl sulfoxide was used as the diluent because of its ability to effectively dissolve bioactive compounds while having no reported activity at the concentration employed in a previous study [15].

Mueller-Hinton Agar plates were prepared by pouring 25 mL of molten medium into sterile Petri dishes and allowed to solidify. Then, 100 μ L of the standardized bacterial suspension was spread evenly over the agar surface after solidification. Wells of 5 mm diameter were aseptically made in the agar and loaded with 50 μ L of the corresponding stock solutions. Each treatment was carried out in triplicate. The inoculated plates were spotted with antibiotic discs containing clindamycin (2 μ g) and ceftriaxone (30 μ g), which served as positive controls [16].

All plates were incubated at 37 °C for 24 h in aerobic conditions. After incubation, antibacterial efficacy was determined by measuring the diameter of the inhibition zones (mm) around the wells and antibiotic discs. Values are expressed as mean $\pm$ SD ($n = 3$). Measurements were made in triplicate using a caliper. The measured inhibition zones were assessed by the qualitative classification described by [17], which classifies

inhibition zones as: inactive (< 10 mm), partially active (10 – 13 mm), active (14 – 19 mm), and very active (> 19 mm).

Statistical analysis

Statistical analysis was conducted in GraphPad Prism v10. Results are presented as mean $\pm$ SD based on triplicate measurements. One-way ANOVA was employed to determine significant differences among *Lactobacillus* isolates. Dunnett's multiple comparisons test was used to compare each isolate with the reference strains. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Tolerance to varying pH conditions

All isolates exhibited higher viability under neutral to alkaline conditions (pH 6.5–8) than at a strongly acidic pH of 2 (Figure 1). *Lentilactobacillus hilgardii* DSB22 exhibited stable survival at pH 6.5 (97.58 ± 2.77 % at 3 h; 103.99 ± 2.78 % at 6 h) but declined under acidity (50.22 ± 3.32 % at pH 2 after 6 h). *Lactobacillus paracasei* 7060 maintained high viability at pH 8 (195.63 ± 55.31 % after 6 h) and dropped sharply at pH 2 (35.58 ± 48.88 %). Similarly, *Lacticaseibacillus paracasei* B2 showed increased survival under optimal conditions (126.63 ± 11.96 % at pH 6.5 after 6 h) but decreased under acid stress (36.12 ± 5.39 % at pH 2). *Limosilactobacillus fermentum* PJG11 showed the widest variability, with remarkable survival at pH 6.5 (244.09 ± 155.95 %) and pH 8 (240.45 ± 38.77 %) after 6 h, but almost complete loss of viability at pH 2 (0.05 ± 0.01 %).

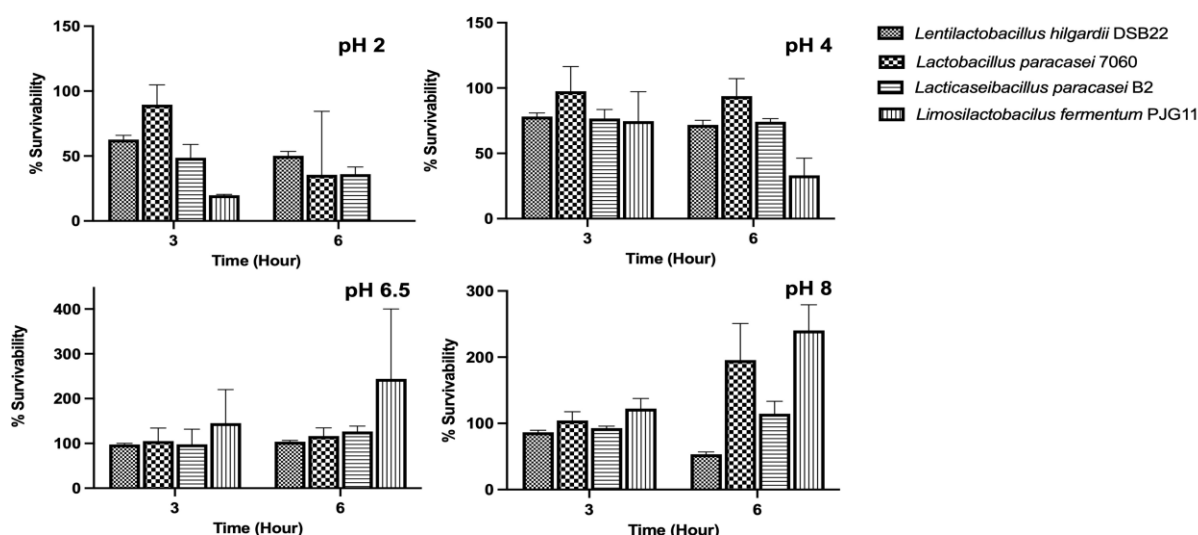


Figure 1: Percent survivability of *Lactobacillus* isolates in different pH conditions

Tolerance to varying bile concentrations

Exposure to 0.5 % bile for 3 hours resulted in high survival across all *Lactobacillus* isolates (Figure 2), with *L. paracasei* 7060 (94.94 ± 12.45 %) and *L. fermentum* PJG11 (94.92 ± 19.08 %) showing the highest tolerance. *Lentilactobacillus hilgardii* DSB22 (89.30 ± 4.23 %) and *L. paracasei* B2 (89.82 ± 3.59 %) also exhibited strong tolerance to 0.5% bile. After 6 hours, survival declined slightly, with *L. hilgardii* DSB22 (85.70 ± 2.41 %) and *L. fermentum* PJG11 (84.75 ± 11.46 %) maintaining the highest viability.

The inhibitory effect of bile was significantly amplified when the concentration was increased to 1 %. After 3 hours, *L. hilgardii* DSB22 showed the highest tolerance (88.50 ± 5.33 %), while *L. paracasei* 7060 and *L. fermentum* PJG11 declined to 50.67 ± 13.83 % and 56.10 ± 18.35 %, respectively. Prolonged exposure (6 h) further reduced survival, but *L. hilgardii* DSB22 remained the most resilient (80.50 ± 4.37 %), followed by *L. paracasei* B2 (68.62 ± 11.42 %).

Auto-aggregation

Lentilactobacillus hilgardii DSB22 consistently demonstrated the highest auto-aggregation property at every time point among the tested

strains, including the reference strain, *Lactobacillus plantarum* (ATCC 14917; Table 2). After 2 hours, *L. hilgardii* DSB22 exhibited an aggregation rate of 69.36 ± 0.21 %, which was significantly higher than that of the other isolates. The aggregation property of *L. hilgardii* DSB22 peaked at the 4-hour mark with 79.35 ± 0.56 % and remained highly stable at 78.92 ± 0.42 % after 6 hours. In contrast, the auto-aggregation percentages for the remaining strains were significantly lower, generally ranging between 10.02 % and 13.59 % at the 2-hour interval. Among the lower aggregating isolates, *L. paracasei* 7060 showed the greatest increase over time, reaching 46.38 ± 0.94 % at 6 hours, which was significantly higher than the auto-aggregation displayed by *L. paracasei* B2 (29.78 ± 0.73 %), *L. fermentum* PJG11 (26.13 ± 0.54 %), and the reference strain (23.17 ± 0.49 %).

Cell surface hydrophobicity (CSH)

Lentilactobacillus hilgardii DSB22 exhibited a statistically equivalent CSH (63.36 ± 0.40 %) to that of the reference strain, *L. plantarum* (ATCC 14917; 64.60 ± 1.42 %; Figure 3). Conversely, *L. fermentum* PJG11 demonstrated the lowest CSH capacity at 10.08 ± 0.21 %. *L. paracasei* 7060 showed a CSH of 30.07 ± 0.28 %, while *L. paracasei* B2 showed 14.42 ± 0.11 %.

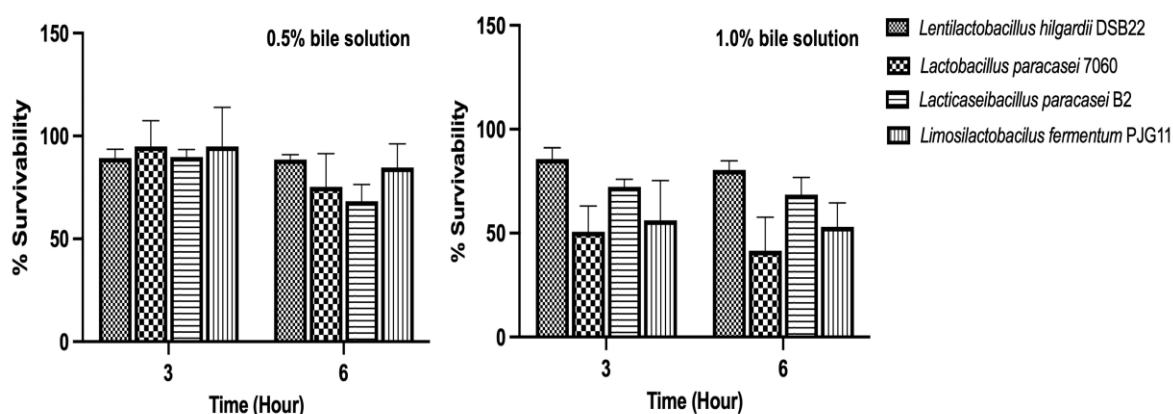


Figure 2: Percent survivability of *Lactobacillus* isolates in different bile concentrations

Table 2: Percentages of auto-aggregation (mean $\pm$ SD) of *Lactobacillus* isolates

Isolate	Auto-Aggregation (%)		
	2 h	4 h	6 h
<i>L. hilgardii</i> DSB22	69.36 ± 0.21^a	79.35 ± 0.56^a	78.92 ± 0.42^a
<i>L. paracasei</i> 7060	13.59 ± 1.27^b	22.46 ± 0.65^b	46.38 ± 0.94^b
<i>L. paracasei</i> B2	10.02 ± 3.64^b	15.89 ± 0.48^c	29.78 ± 0.73^c
<i>L. fermentum</i> PJG11	10.84 ± 0.34^b	11.49 ± 0.58^d	26.13 ± 0.54^d
<i>L. plantarum</i> (ATCC 14917) - Reference	10.10 ± 0.48^b	11.80 ± 0.79^d	23.17 ± 0.49^e

In a column, means with the same letter are not significantly different ($P < 0.05$)

Table 3: Percentages of cell surface hydrophobicity (mean $\pm$ SD) of *Lactobacillus* isolates

Isolate	Cell surface hydrophobicity (%)
<i>L. hilgardii</i> DSB22	63.36 $\pm$ 0.40 ^a
<i>L. paracasei</i> 7060	30.07 $\pm$ 0.28 ^b
<i>L. paracasei</i> B2	14.42 $\pm$ 0.11 ^c
<i>L. fermentum</i> PJG11	10.08 $\pm$ 0.21 ^d
<i>L. plantarum</i> (ATCC 14917) -Reference	64.60 $\pm$ 1.42 ^a

In a column, means with the same letter are not significantly different ($P < 0.05$)

Bile salt hydrolase (BSH) activity

All tested *Lactobacillus* strains and the reference strain *Lactobacillus plantarum* (ATCC 14917) demonstrated BSH activity, indicated by the presence of a precipitate halo (+; Table 4). The negative control (MRS Broth only) showed no precipitate halo (-), confirming assay specificity.

Table 4: Bile salt hydrolase activities of *Lactobacillus* isolates

Isolate	Presence (+) or Absence (-) of Precipitate Halo
<i>L. hilgardii</i> DSB22	+
<i>L. paracasei</i> 7060	+
<i>L. paracasei</i> B2	+
<i>L. fermentum</i> PJG11	+
<i>L. plantarum</i> (ATCC 14917) -Reference	+
MRS Broth only (Negative control)	-

Antioxidant capacity

Radical scavenging capacity was concentration-dependent for all tested EAFPs and the positive control, L-Ascorbic acid (Table 5). At 1000 μ g/mL, scavenging capacity varied among samples, and although none reached the level of L-ascorbic acid (97.09 $\pm$ 0.50 %), *L. fermentum* PJG11 (83.23 $\pm$ 2.11 %) exhibited the highest value. *L. hilgardii* DSB22 EAFP recorded

a high scavenging capacity of 73.51 $\pm$ 0.58 % at 1000.0 μ g/mL, statistically comparable to *L. paracasei* 7060 EAFP (74.35 $\pm$ 1.09 %) and *Lactobacillus plantarum* (ATCC 14917) EAFP (70.08 $\pm$ 6.24 %). At lower concentrations, 0.1 and 1.0 μ g/mL, the scavenging capacities across all *Lactobacillus* EAFPs were modest, ranging between 44.16 and 52.12 %. At the intermediate concentration of 100.0 μ g/mL, *L. hilgardii* DSB22 EAFP (54.70 $\pm$ 0.48 %) showed comparable activity to *L. fermentum* PJG11 EAFP (55.67 $\pm$ 1.38 %) and the *L. fermentum* reference strain EAFP (55.43 $\pm$ 0.93 %), which formed the highest scavenging group among EAFPs at this level.

Antimicrobial effect

Among the *Lactobacillus* EAFPs, *L. hilgardii* DSB22 exhibited the strongest overall antimicrobial profile (Table 6). *L. hilgardii* DSB22 EAFP produced the largest inhibition zone for *S. aureus* (18.33 $\pm$ 2.89 mm), comparable to clindamycin (21.67 $\pm$ 1.53 mm), and showed moderate inhibition for *E. coli* (14.33 $\pm$ 1.15 mm), exceeding other EAFPs. For *P. aeruginosa*, *L. paracasei* B2 EAFP exhibited the greatest inhibition (15.0 $\pm$ 3.0 mm), followed by *L. hilgardii* DSB22 EAFP (13.67 $\pm$ 1.15 mm). Notably, the reference strain *L. plantarum* (ATCC 14917) EAFP showed no measurable inhibition against *P. aeruginosa*.

Table 5: Percentage DPPH radical scavenging capacity (mean $\pm$ SD) of EAFPs derived from *Lactobacillus* isolates

Treatment	Radical Scavenging Capacity (%)				
	0.1 μ g/mL	1.0 μ g/mL	10.0 μ g/mL	100.0 μ g/mL	1000.0 μ g/mL
<i>L. hilgardii</i> DSB22 EAFP	52.12 $\pm$ 0.12 ^a	52.12 $\pm$ 0.47 ^a	52.28 $\pm$ 0.46 ^b	54.70 $\pm$ 0.48 ^{bc}	73.51 $\pm$ 0.58 ^c
<i>L. paracasei</i> 7060 EAFP	44.16 $\pm$ 0.54 ^b	45.35 $\pm$ 0.43 ^a	46.31 $\pm$ 0.52 ^b	50.33 $\pm$ 0.72 ^d	74.35 $\pm$ 1.09 ^c
<i>L. paracasei</i> B2 EAFP	46.69 $\pm$ 0.44 ^{ab}	46.39 $\pm$ 0.57 ^a	48.39 $\pm$ 1.16 ^b	50.96 $\pm$ 0.92 ^{cd}	76.37 $\pm$ 1.81 ^{bc}
<i>L. fermentum</i> PJG11 EAFP	51.17 $\pm$ 1.46 ^a	51.55 $\pm$ 1.68 ^a	52.15 $\pm$ 1.85 ^b	55.67 $\pm$ 1.38 ^b	83.23 $\pm$ 2.11 ^b
<i>L. fermentum</i> (ATCC 9338) EAFP -Reference	51.13 $\pm$ 0.55 ^a	51.35 $\pm$ 0.61 ^a	51.96 $\pm$ 0.27 ^b	55.43 $\pm$ 0.93 ^b	83.68 $\pm$ 3.21 ^b
<i>L. plantarum</i> (ATCC 14917) EAFP - Reference	48.61 $\pm$ 4.29 ^{ab}	47.99 $\pm$ 4.40 ^a	48.64 $\pm$ 3.83 ^b	52.11 $\pm$ 3.12 ^{bd}	70.08 $\pm$ 6.24 ^c
Ascorbic acid (Positive control)	49.57 $\pm$ 4.33 ^{ab}	52.20 $\pm$ 4.50 ^a	92.09 $\pm$ 4.75 ^a	97.11 $\pm$ 0.32 ^a	97.09 $\pm$ 0.50 ^a

In a column, means with the same letter are not significantly different ($P < 0.05$)

Table 6: Antimicrobial effect (mean $\pm$ SD) of EAFPs from *Lactobacillus* isolates against pathogenic microorganisms

Treatment	Zone of Inhibition (mm)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
<i>L. hilgardii</i> DSB22 EAFP	18.33 $\pm$ 2.89 ^a	14.33 $\pm$ 1.15 ^b	13.67 $\pm$ 1.15 ^{bc}
<i>L. paracasei</i> 7060 EAFP	7.67 $\pm$ 0.50 ^c	ND	12.00 $\pm$ 4.36 ^{bc}
<i>L. paracasei</i> B2 EAFP	8.67 $\pm$ 0.58 ^c	10.00 $\pm$ 1.00 ^c	15.00 $\pm$ 3.00 ^b
<i>L. fermentum</i> PJG11 EAFP	6.33 $\pm$ 0.58 ^c	6.33 $\pm$ 0.58 ^d	8.33 $\pm$ 0.58 ^c
<i>L. fermentum</i> (ATCC 9338) EAFP - Reference	7.33 $\pm$ 0.58 ^c	8.67 $\pm$ 0.58 ^c	12.00 $\pm$ 1.00 ^{bc}
<i>L. plantarum</i> (ATCC 14917) EAFP - Reference	12.33 $\pm$ 0.58 ^b	8.33 $\pm$ 1.15 ^{cd}	ND
Clindamycin	21.67 $\pm$ 1.53 ^a	-	-
Ofloxacin	-	26.00 $\pm$ 1.00 ^a	-
Ceftriaxone	-	-	21.33 $\pm$ 0.58 ^a
2.5% DMSO	ND	ND	ND

In a column, means with the same letter are not significantly different ($P < 0.05$); "ND" means no inhibition detected

DISCUSSION

Probiotic functionality depends on *Lactobacillus* spp. to survive gastrointestinal stress, as their ability to resist acid and bile is critical for survival in the intestine [14]. All isolates showed higher survival under neutral-to-alkaline pH. In contrast, the viability of cells significantly decreased upon exposure to pH 2, particularly with longer incubation times [18,19]. This trend has also been observed in other *Lactobacillus* spp., where prolonged exposure to highly acidic environments results in disturbance of the membrane and inhibition of metabolism [20].

In the present study, *L. hilgardii* DSB22 showed higher acid tolerance than the other isolates with > 50% survival after 6 h at pH 2. In contrast, *L. fermentum* PJG11 exhibited a rapid decline in viability in the same conditions, indicating a much weaker acid resistance profile. These variations within species are consistent with findings that *Lactobacillus* acid tolerance is very strain-dependent [21]. The survival of LAB under acidic stress strongly depends on the behavior of the H⁺-ATPase system under different pH conditions, the composition of its cytoplasmic membrane, as well as factors related to genetic background, nutritional environment, and incubation conditions [22]. Thus, the results suggest that *L. hilgardii* DSB22 has more efficient acid-adaptation systems than *L. fermentum* PJG11 under the tested conditions, which shows its potential to better tolerate the gastric transit.

The results of the bile tolerance obtained in this study are consistent with previous reports. All isolates survived well in the presence of 0.5 % bile, a concentration similar to that found in the small intestine. This degree of tolerance is relevant for *Lactobacillus* strains because the ability to withstand the membrane-disrupting effects of bile determines whether these bacteria

colonize the gut and carry out probiotic functions such as bile salt hydrolysis and pathogen inhibition [23]. Moreover, testing higher bile concentrations is also important since bile levels in the duodenum may temporarily increase to around 1% (>10.34 mM) after food intake [24]. At this more challenging concentration, only *L. hilgardii* DSB22 and *L. paracasei* B2 survived above 80%, suggesting that these isolates have strong adaptive mechanisms to withstand bile stress. This result is consistent with the report [25] of > 80 % survival for *L. fermentum* strains at 1 % bile. *Lactobacillus* strains with high bile tolerance generally have effective bile salt hydrolase activity and membrane adaptation mechanisms, which allow them to withstand bile toxicity [26]. In contrast, the lower survival of *L. paracasei* 7060 at high bile levels is consistent with previous report that showed pronounced intra-species variability in response to bile [26]. Such variability may be explained by the loss of bile-resistance genes in some strains as they evolve in bile-free environments, leading to decreased tolerance over time [27]. Overall, these results suggest that *L. hilgardii* DSB22 is the most physiologically resilient isolate under normal and high bile conditions, displaying great potential as a probiotic candidate that can survive gastrointestinal transit.

Auto-aggregation and cell-surface hydrophobicity are important characteristics for a probiotic to attach to mucosal surfaces and remain within the gastrointestinal tract [28]. Auto-aggregation also contributes to biofilm formation, which protects bacterial cells against environmental stresses and favors survival in the gut [29]. The auto-aggregation of *L. hilgardii* DSB22 was consistently high (> 69 % from 2 hours onward), similar to the auto-aggregation of the *Lactobacillus* strains with strong auto-aggregation [30](> 50 %). The rapid and stable aggregation suggests the involvement of surface-associated components such as

exopolysaccharides or aggregation-promoting proteins that facilitate efficient cell-to-cell interaction and microcolony formation [31].

The cell surface hydrophobicity (CSH) of *L. hilgardii* DSB22 ($63.36 \pm 0.40\%$) further supports its strong adhesion potential. Its hydrophobicity was comparable to that of the well-established probiotic reference strain *L. plantarum* (ATCC 14917; $64.60 \pm 1.42\%$), which is remarkable, as strains with higher hydrophobicity usually show better adherence to epithelial tissues [5,32]. In contrast, isolates such as *L. fermentum* PJG11 had significantly lower hydrophobicity and it has been previously reported that hydrophobicity varies widely among *Lactobacillus* spp. [33]. Such strains may be more reliant on specific receptor-mediated adhesins rather than non-specific hydrophobic interactions for successful host attachment [32]. These results indicate that *L. hilgardii* DSB22 is a promising probiotic candidate. Its strong aggregation ability and high hydrophobicity may favor adhesion to intestinal surfaces, which is an important step for successful colonization and persistence in the gut.

All isolates showed bile salt hydrolase activity, which is consistent with previous reports that BSH is widely expressed among *Lactobacillus* species [29, 34]. Bile salt hydrolase participates in bile salt deconjugation, and thus enhances bacterial tolerance to bile and host cholesterol metabolism [34]. The detection of bile salt hydrolase activity across all isolates indicates their ability to survive in the small intestine and to be involved in bile-related physiological processes.

All isolates also exhibited concentration-dependent radical scavenging capacity with EAFPs. This trend follows antioxidant patterns previously seen in the postbiotics of *Lactobacillus* spp. This may be due to the presence of non-enzymatic substances and intracellular antioxidant enzymes [2]. Specifically, *L. hilgardii* DSB22 showed high antioxidant capacity (73.51 ± 0.58 at $1000 \mu\text{g/mL}$), similar to those reported in other *Lactobacillus* strains isolated from pineapple puree [2] and fermented foods [35]. Although ascorbic acid naturally showed greater potency, the high scavenging capacity of the EAFPs suggests that they are important for the mitigation of oxidative stress.

The EAFPs also showed antimicrobial effects, which further enhance the functional properties of the isolates. The inhibition shown by *L. hilgardii* DSB22 against both Gram-positive and Gram-negative pathogens suggests the

presence of secreted bioactive compounds with broad-spectrum effects, consistent with previous studies reporting antimicrobial effects in *Lactobacillus* spp. due to organic acids, bacteriocins, and other metabolites [36]. The strong inhibition of *L. hilgardii* DSB22 against *S. aureus* indicates the production of antimicrobial metabolites and potential importance for competitive exclusion and pathogen suppression in vivo.

CONCLUSION

This study shows the potential of Philippine indigenous plants as sources of functionally diverse *Lactobacillus* strains with probiotic potential. Among the tested isolates, *Lentilactobacillus hilgardii* DSB22 showed promising in vitro properties such as tolerance to gastrointestinal stress conditions, high auto-aggregation and hydrophobicity properties, bile salt hydrolase activity, and production of bioactive metabolites with antioxidant and antimicrobial effects. The results indicated that the strain could be used as a non-dairy probiotic. While the results provide a strong preliminary foundation, further investigations in animals and human patients are required to confirm the probiotic properties and safety of these isolates. This study highlights the importance of the local microbial biodiversity for the development of functional foods and probiotic products.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

Ethical approval

Not required.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Use of artificial intelligence

Artificial intelligence tools were used solely for editing grammar and phrasing. The authors remain fully responsible for the content and interpretation of the work.

Use of research reporting tool

No research reporting tool was used in this study.

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