Isolation and Probiotic Characterization of Lactic Acid Bacteria from the Phylloplanes of Edible and Culinary Leaves

Authors: Shashank S[1], Uday Kumar B V[2,3], Suvarna V C[1], Uddalak Das[4,5,3*]

[1] *Department of Agricultural Microbiology, University of Agricultural Sciences, Bangalore (UAS-B), Bengaluru, Karnataka, India 560 065*

[2] *School of Agricultural Biotechnology, College of Agriculture, Punjab Agricultural University (PAU), Ludhiana, Punjab, 141 044;*

[3] *Department of Plant Biotechnology, University of Agricultural Sciences, Bangalore (UAS-B), Bengaluru, Karnataka, India 560 065.*

[4] *Indian Council of Agricultural Research – National Institute of Plant Biotechnology (ICAR – NIPB), Indian Agricultural Research Institute (IARI), New Delhi, India 110 012*

[5] *School of Biotechnology (SBT), Jawaharlal Nehru University (JNU), New Delhi, India, 110 067*

Abstract

The phylloplane of edible and culinary plants harbours diverse microbial communities, including lactic acid bacteria (LAB), which remain underexplored for their probiotic potential. This study aimed to isolate LAB from the leaf surfaces of eight plant species: *Sauropus androgynus*, *Piper betle*, *Centella asiatica*, *Artocarpus heterophyllus*, *Pandanus amaryllifolius*, *Musa paradisiaca*, *Curcuma longa*, and *Ricinus communis*. The isolates were characterized for essential probiotic traits, including tolerance to acid, bile salts, NaCl, and phenol, temperature resistance, haemolytic activity, antibiotic susceptibility, auto-aggregation, and cell surface hydrophobicity. All LAB isolates exhibited γ-haemolytic activity, confirming their non-pathogenic nature. Notably, *Lactobacillus acidophilus* and LAB-10 displayed superior gastrointestinal stress tolerance, high hydrophobicity (72% and 60%, respectively), and strong auto-aggregation (42% and 36%). These strains tolerated NaCl concentrations up to 6%, phenol (0.4%), and grew optimally at 37–40 °C and pH 2.0–3.5. Antibiotic susceptibility varied, with some isolates resistant to multiple antibiotics. This study highlights the phylloplane as a valuable source of probiotic LAB strains with functional attributes suitable for potential applications in functional foods and therapeutic formulations.

Keywords: Phylloplane, Lactic Acid Bacteria, Probiotics, Acid and Bile Tolerance, Antibiotic Resistance

*Corresponding author; **Email**: uddalakdas1998@gmail.com

1. Introduction

The phylloplane, or aerial surface of plant leaves, provides a diverse and dynamic habitat for microbial colonization, including bacteria, fungi, and viruses. This niche supports a complex microbial community influenced by leaf morphology, age, physicochemical properties, and environmental factors such as humidity and temperature. These factors collectively shape the microbial diversity and functionality on the leaf surface, making it a critical interface between plants and the surrounding environment (1–4). A variety of leafy plants such as *Sauropus androgynus* (Chekkurmanis), *Piper betle* (Betel vine), *Centella asiatica* (Brahmi), *Artocarpus heterophyllus* (Jackfruit), *Pandanus amaryllifolius* (Pandan), *Musa* sp. (Banana), *Curcuma longa* (Turmeric), and *Ricinus communis* (Castor) are commonly consumed raw or used in culinary practices. These leaves are valued not only for their nutritional content, including proteins, essential minerals, and dietary fibre, but also for their bioactive properties such as antioxidant, antimicrobial, anti-inflammatory, and neuroprotective effects (5,6). Their traditional medicinal use includes lactation enhancement, cognitive support, blood pressure regulation, and improved skin health.

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating, facultative anaerobes that primarily ferment carbohydrates to produce lactic acid. They are ubiquitous in nature and have been isolated from diverse sources including fermented foods, plant surfaces, animal mucosa, and soil (7,8). LAB play a central role in the fermentation of a wide array of food products, enhancing preservation, flavor, and nutritional value (9). Major LAB genera include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Weissella*, many of which have undergone recent taxonomic reclassification (7). LAB associated with raw fruits and vegetables are of particular interest due to their potential as natural probiotics. Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (10). Many strains of *Lactobacillus* and *Bifidobacterium* have demonstrated probiotic properties, including antimicrobial activity, immunomodulation, and restoration of gut microbial balance (11,12). Their inhibitory effect against intestinal pathogens is largely attributed to the production of lactic acid, hydrogen peroxide, bacteriocins, and other bioactive compounds, as well as competitive exclusion mechanisms (13).

For a LAB strain to be considered probiotic, it must demonstrate acid and bile tolerance, adhesion to intestinal epithelial cells, and resistance to digestive enzymes (14,15). In addition, probiotic strains are known to synthesize B-group vitamins, short-chain fatty acids, and essential amino acids, and may produce enzymes such as esterases and lipases that aid host metabolism (16,17). Several LAB strains have also been reported to produce antimicrobial peptides with potential immunosuppressive or anticancer effects (18,19). The GRAS (Generally Recognized as Safe) status granted by the U.S. FDA and the QPS (Qualified Presumption of Safety) status by the EFSA further support their widespread use in food and therapeutic applications.

Given the growing consumption of raw leafy vegetables and their role as carriers of beneficial microbes, the phylloplane represents a promising yet underexplored niche for the isolation of LAB with probiotic potential. Therefore, the present study was undertaken to isolate LAB from the phylloplanes of selected edible and culinary leaves and to evaluate their probiotic characteristics through a range of functional and physiological assays. The findings aim to contribute to the identification of novel LAB strains that may be used in the development of functional foods, dietary supplements, or natural bio preservatives.

2. Materials and methods

2.1.Plant Source

The different leaves *viz, chekkurmanis* (*Sauropus androgynus*), *betel* vine *(Piper betle*), *brahmi* (*Centella asiatica*), jackfruit (*Artocarpus heterophyllus*), *pandan* (*Pandanus amaryllifolius*), banana (*Musa paradisiaca*), turmeric (*Curcuma longa*) and castor (*Ricinus communis*) were collected from horticulture medicinal garden and around UAS, GKVK campus for isolation and enumeration of microorganisms.

2.2.Isolation of Microorganisms from the Phylloplanes of Leaf Samples

The standard plate count method (for small phylloplane samples *i.e., chekkurmanis*, *betel* vine, *brahmi*) and cotton swab technique (for large phylloplane samples *i.e., pandan*, jackfruit, banana, turmeric and castor) were employed for isolation of microorganisms. The leaves samples (*chekkurmanis*, *betel* vine, *brahmi*) were weighed one gram and transferred to sterile water blank. A sterile cotton swab was employed to sample the microflora from

the surface of leaves within designated 10 cm^2 areas. This semi-quantitative approach enables enumeration of the microorganisms per cm^2 (20,21). The media *viz*, nutrient agar (NA) for bacteria, MRS agar for lactic acid bacteria and Martin's Rose Bengal Agar (MRBA) for fungi were used for isolation of microorganisms (22–24). The plates were incubated in an incubator at 25 °C for 48, 72 and 96 h for bacteria, LA bacteria and fungi respectively. The single discrete colonies were picked up and re-streaked on petri plates until pure cultures were obtained and were maintained on slants which were sub-cultured once in three months.

2.3.Probiotic parameters analysis

Bile Salt Tolerance: The tolerance of lactic acid bacterial (LAB) isolates to bile salts was assessed following the method of Ali et al. (2020) with minor modifications (25,26). Overnight cultures of LAB isolates grown in de Man, Rogosa and Sharpe (MRS) broth at 37 °C were inoculated (10^8 cfu/mL) into fresh MRS broth supplemented with varying concentrations of bile salts (0.5, 1.0, 1.5, and 2.0%, w/v). Cultures were incubated at 37 °C for 24 h and 48 h. Uninoculated tubes served as controls. Growth was assessed by measuring optical density (OD) at 660 nm.

Acid Tolerance: Acid tolerance was evaluated according to Mulaw et al. (2019) (27). LAB isolates were cultured overnight in MRS broth and harvested by centrifugation (5000 rpm, 10 min, 4 °C). Pellets were washed twice with phosphate-buffered saline (PBS, pH 7.2) and resuspended in MRS broth adjusted to pH values of 2.0, 3.5, 5.0, and 7.0 using 1N HCl. Uninoculated tubes were used as controls, cultures were incubated at 37 °C, and growth was measured as OD at 660 nm at 24-hour intervals for 48 hours.

Temperature Tolerance: Temperature tolerance was determined following Ali et al. (2020) (25). Overnight cultures of LAB isolates were inoculated (10^8 cfu/mL) into MRS broth and incubated at 25, 30, 37 and 40 °C for 24 and 48 hours. Uninoculated tubes were used as controls. Growth was quantified by OD measurement at 660 nm.

Haemolytic Activity: Haemolytic activity was tested using the method described by Bergey (1930) (28) . LAB isolates were inoculated into wells (5 mm diameter) of sheep blood agar plates prepared using 5% (v/v) defibrinated sheep blood in Tryptic Soy Agar (TSA). Plates were incubated at 30 °C for 72 hours. Haemolysis was assessed by

observing the diameter of the clear zone around the wells. Sterile distilled water served as a control.

Antibiotic Susceptibility: Antibiotic susceptibility was evaluated using the disc diffusion method described by Bauer et al. (1966) (29). LAB cultures were grown in MRS broth for four days at 37 °C and adjusted to a final population of 5 × 10 cfu/mL. MRS agar was seeded with 5mL of culture per 100mL medium. Antibiotic discs (ampicillin 10 µg, azithromycin 15 µg, chloramphenicol 30 µg, gentamycin 10 µg, nystatin 50µg, and amphotericin-B 20µg) were placed on agar surfaces and incubated at 37 °C for 48 hours. Zone of inhibition were measured in centimetres.

NaCl Tolerance NaCl tolerance was tested as described by Escamilla-Montes et al. (2015) (30). LAB isolates were inoculated (10^8 cfu/mL) into MRS broth supplemented with 5, 6, and 7% (w/v) NaCl and incubated at 37 °C for 24 and 48 hours. Growth was measured spectrophotometrically at 600 nm. Tubes without NaCl served as controls.

Phenol Resistance Phenol resistance was assessed following the protocol of Jena et al. (2013) (31). LAB isolates were grown in MRS broth containing 0.4% (v/v) phenol and incubated at 37 °C for 24 hours. Cell viability was determined by standard plate count method and expressed as log CFU/mL.

Auto-aggregation ability: Auto-aggregation was evaluated according to Zommiti et al. (2017) (32). Overnight cultures were centrifuged (8000 rpm, 10 min, 4 °C), washed twice in PBS, and resuspended in the same buffer. Absorbance of the upper suspension was recorded at 600 nm at 0, 1, 2, 3, 4, and 5 hours. Auto-aggregation (%) was calculated as:

$$\text{Auto} - \text{aggregation (in \%)} = \left(1 - \frac{A_0}{A_t}\right) X\ 100$$

Where A is the absorbance at 0 h and A at a specific time.

Cell Surface Hydrophobicity Cell surface hydrophobicity was determined using microbial adhesion to hydrocarbons (MATH) method (Rokana et al., 2018) (33). LAB cells were harvested, washed with PBS, and absorbance (A) recorded at 600 nm. A 3mL cell suspension was mixed with 1mL xylene and incubated at 37 °C for 1 hour without agitation. After phase separation, aqueous phase absorbance (A) was measured. Hydrophobicity (%) was calculated as:

$$\text{Hydrophobicity (in \%)} = \left(1 - \frac{A_0}{A_t}\right) X\ 100$$

2.4. Statistical Analysis

Data were analysed using analysis of variance (ANOVA) with OPSTAT 2.0 software. Differences among means were assessed using Duncan's Multiple Range Test at a significance level of $p \leq 0.05$ (34).

3. Results and discussion

LAB isolated from phylloplanes that exhibited significant antibacterial and antifungal activity were selected and labelled as LAB-1 to LAB-15 for further evaluation of their probiotic potential. To be considered probiotic, LAB must remain viable and functional under the harsh conditions of the gastrointestinal tract (GIT). Key attributes assessed included haemolytic activity, antibiotic sensitivity, surface hydrophobicity, auto-aggregation, NaCl and phenol tolerance, temperature and acid tolerance, and bile salt resistance.

3.1. Haemolytic Activity

LAB must be non-haemolytic to be considered safe for probiotic use. Haemolysis was assessed on trypticase soy agar supplemented with 5% sheep blood after four days of incubation. Haemolytic activity is categorized as α-haemolysis (partial, greenish zone), β-haemolysis (complete, clear zone), or γ-haemolysis (none). All tested LAB isolates showed γ-haemolysis, indicating no haemolytic activity (**Table 1**). This is consistent with findings by Tatsaporn and Kornkanok (2020), and Chandel (2019), who reported negative haemolytic activity for *Pediococcus pentosaceus*, *Enterococcus faecium*, and *Lactobacillus* spp., confirming their safety for probiotic application (35,36).

3.2. Antibiotic Sensitivity

Isolates *Lactobacillus acidophilus*, LAB-3, and LAB-10 were resistant to six antibiotics—gentamicin, chloramphenicol, azithromycin, ampicillin, nystatin, and amphotericin B (**Table 1**). All isolates were resistant to the two antifungal agents tested. Dowarah et al. (2018) reported similar findings in LAB isolated from piglet feces, with

resistance to ciprofloxacin, ofloxacin, gatifloxacin, vancomycin, and co-trimoxazole, but sensitivity to penicillin and other antibiotics. Likewise, Sirichoat et al. (2020) reported acquired resistance among vaginal LAB strains (37).

Table 1: Antibiotic sensitivity and haemolytic activity of LA bacterial isolates

Isolates	Antibiotic sensitivity						Haemolytic activity
	Antibacterial				Antifungal		
	Gentamycin (EC 10µg/disc)	Chloramphenicol (EC 30µg/disc)	Azithromycin (EC 15µg/disc)	Ampicillin (EC 10 µg/disc)	Nystatin (EC 50µg/disc)	Amphotericin - B (EC 20µg/disc)	
Control	NA	NA	NA	NA	NA	NA	-
LA	R	R	R	R	R	R	-
LAB-3	R	R	R	R	R	R	-
LAB-5	R	S	S	S	R	R	-
LAB-10	R	R	R	R	R	R	-
LAB-15	R	S	S	S	R	R	-

Note: '+'- positive, '-' – negative, 'R'- resistance, 'S'- susceptible, LAB - Lactic acid bacteria, LA- *Lactobacillus acidophilus*.

3.3. Cell Surface Hydrophobicity

Hydrophobicity is an important factor in bacterial adhesion to intestinal epithelial cells. The isolates showed hydrophobicity ranging from 19% to 72% (**Table 2**). *Lactobacillus acidophilus* showed the highest hydrophobicity (72%), followed by LAB-10 (60%). LAB-3 and LAB-5 had less than 40%. Dowarah et al. (2018) reported 64% hydrophobicity in *Lacp28* (38), while Meena et al. (2022) observed 75.3 ± 2.76% in *Lactobacillus delbrueckii subsp. bulgaricus* KMUDR1 (39).

Table 2: Percent auto-aggregation and cell hydrophobicity of LA bacterial cultures at intervals

Isolates	Auto aggregation (%)					Cell hydrophobicity (%)
	1h	**2h**	**3h**	**4h**	**5h**	**24h**
Control	00	00	00	00	00	00
LA	19	24	30	31	42	72
LAB-3	2	4	4	7	11	38
LAB-5	10	12	19	24	28	19
LAB-10	10	15	25	29	36	60
LAB-15	19	21	22	24	32	57

Note: LAB- Lactic acid bacteria, numerical values are mean of three replicates, LA- *Lactobacillus acidophilus*

3.4. **Auto-Aggregation**

Auto-aggregation correlates with bacterial adherence capability. After 5 hours, auto-aggregation ranged from 11–42% (**Table 2**). *Lactobacillus acidophilus* exhibited the highest (42%), followed by LAB-10 (36%). Xu et al. (2009) observed 51.8% auto-aggregation in *Bifidobacterium longum* B6. Topcu et al. (2020) found 17.62% auto-aggregation in *Pediococcus pentosaceus* K41 (40).

3.5. NaCl Tolerance

NaCl tolerance is essential for LAB survival in osmotic stress conditions. All isolates grew at 4–6% NaCl, with growth declining at higher concentrations due to salt's inhibitory effects (**Table 3**). *Lactobacillus acidophilus* showed the highest growth, followed closely by LAB-10. Pundir et al. (2013) similarly reported LAB growth up to 6–6.5% NaCl and reduced growth at 7%, with no survival at 8–10% (41).

Table 3: Absorbance of LA bacterial cultures at different NaCl and phenol concentrations

Isolates			Control	LA	LAB- 3	LAB- 5	LAB- 10	LAB- 15
NaCl concentrations (%)	**4**	**3 h**	0.00^{d}	0.73^{a}	0.18^{c}	0.15^{c}	0.58^{b}	0.53^{b}
		6 h	0.00^{d}	0.87^{a}	0.21^{c}	0.17^{c}	0.79^{b}	0.77^{b}
		24 h	0.00^{d}	0.89^{a}	0.21^{c}	0.17^{c}	0.81^{b}	0.79^{b}
	5	**3 h**	0.00^{e}	0.7^{a}	0.15^{d}	0.14^{d}	0.55^{b}	0.48^{c}
		6 h	0.00^{d}	0.86^{a}	0.20^{c}	0.15^{c}	0.76^{b}	0.72^{b}
		24 h	0.00^{d}	0.88^{a}	0.20^{c}	0.15^{c}	0.77^{b}	0.74^{b}
	6	**3 h**	0.00^{e}	0.68^{a}	0.15^{d}	0.13^{d}	0.53^{b}	0.47^{c}
		6 h	0.00d	0.83^{a}	0.16^{c}	0.15^{c}	0.73^{b}	0.71^{b}
		24 h	0.00^{d}	0.85^{a}	0.16^{c}	0.15^{c}	0.75^{b}	0.72^{b}
Phenol (0.4 %)		**24 h**	0.00^{b}	0.10^{a}	0.07^{a}	0.07^{a}	0.08^{a}	0.08^{a}
		0 h	0.00^{e}	0.31^{c}	0.27^{c}	0.14^{d}	0.50^{a}	0.44^{b}

Note: LAB- Lactic acid bacteria, LA- *Lactobacillus acidophilus*, numerical values are mean of three replicates. The treatment means with the different letters as superscript in the same columns as determined by DMRT ($p \leq 0.05$).

3.6. Phenol Tolerance

LAB must resist toxic phenolic compounds produced during digestion. Phenol tolerance was evaluated at 0.4% phenol after 24 hours at 600 nm. LAB-10 exhibited the highest tolerance (0.50), followed by LAB-15 (0.44) (**Table 3**). Vizoso-Pinto et al. (2006)

and Reuben et al. (2019) also reported phenol resistance among LAB strains, with varying OD values indicating survivability.

3.7. Temperature Tolerance

Survivability at body temperature is a key probiotic trait. *Lactobacillus acidophilus*, LAB-10, and LAB-15 showed optimal growth at 37–40 °C, while LAB-3 and LAB-5 had suboptimal growth at all tested temperatures (**Figure 1**). Reuben et al. (2019) and Divyashree et al. (2021) reported similar findings, with optimal LAB growth at 37 °C after 24 hours (42,43).

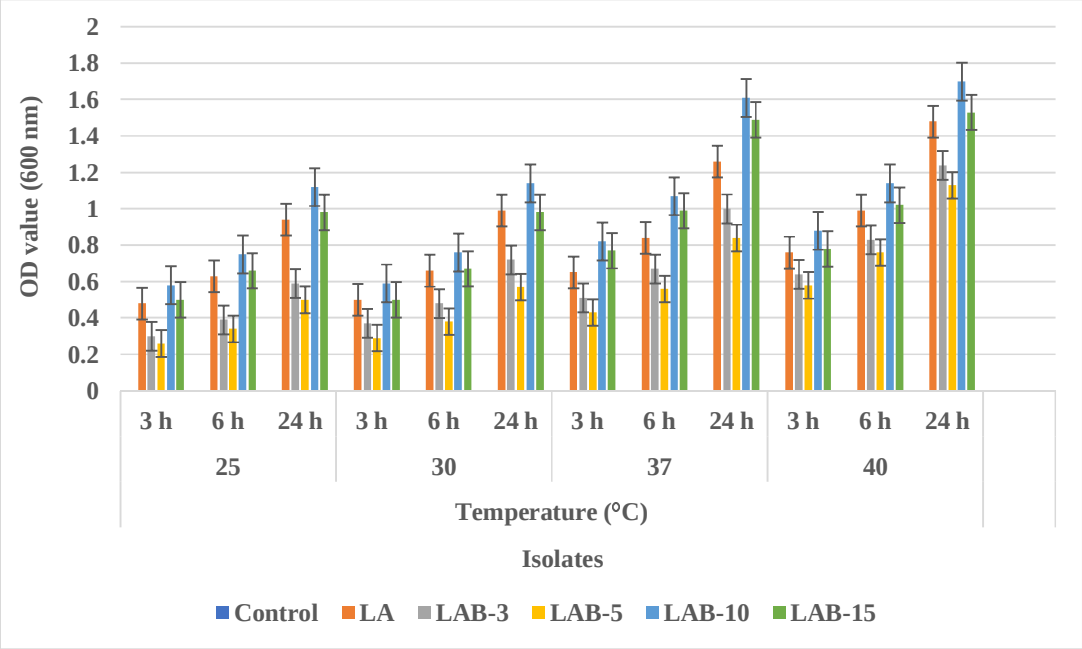

Figure 1: Effect of temperature on growth of LA bacterial isolates in MRS broth at intervals

3.8. Acid Tolerance

Acid tolerance enables LAB to survive gastric conditions. All isolates survived at pH values ranging from 2.0 to 8.0, with better survival at pH 2.0–3.5. LAB-10 showed the highest survival rate, followed by *Lactobacillus acidophilus*. LAB-5 had the lowest viability across all pH levels (**Figure 2**). Comparable results were reported by Chen et al. (2020) and Vanniyasingam et al. (2019), confirming the acid tolerance of *Lactobacillus plantarum* and other LAB strains at pH as low as 2.0 (44,45).

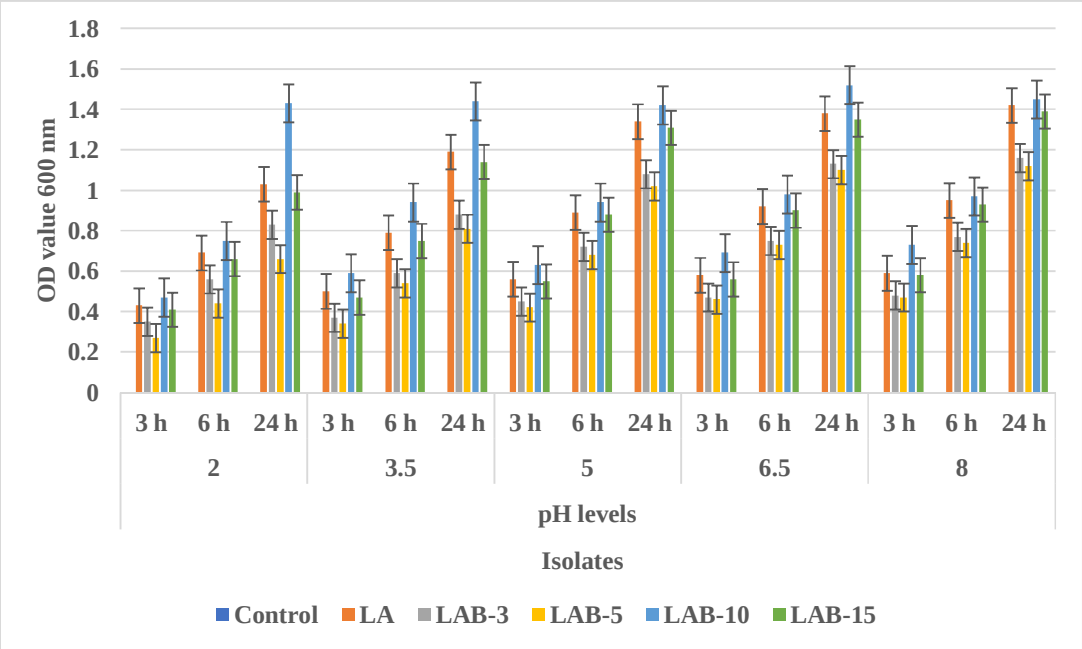

Figure 2: Effect of pH on growth of LA bacterial isolates in MRS broth at intervals

3.9. **Bile Salt Tolerance**

Bile tolerance is necessary for colonization and metabolic activity in the intestine. The isolates were tested at 0.5–2.0% bile salt concentrations over 3, 6, and 24 hours. All isolates were bile-tolerant, with LAB-10 exhibiting the highest tolerance, followed by *Lactobacillus acidophilus*. LAB-5 showed the lowest (**Figure 3**). Similar bile resistance was reported by Chen et al. (2020) and Menconi et al. (2014), who noted viability reduction with increasing bile concentrations, but confirmed overall tolerance of the strains tested (44,46).

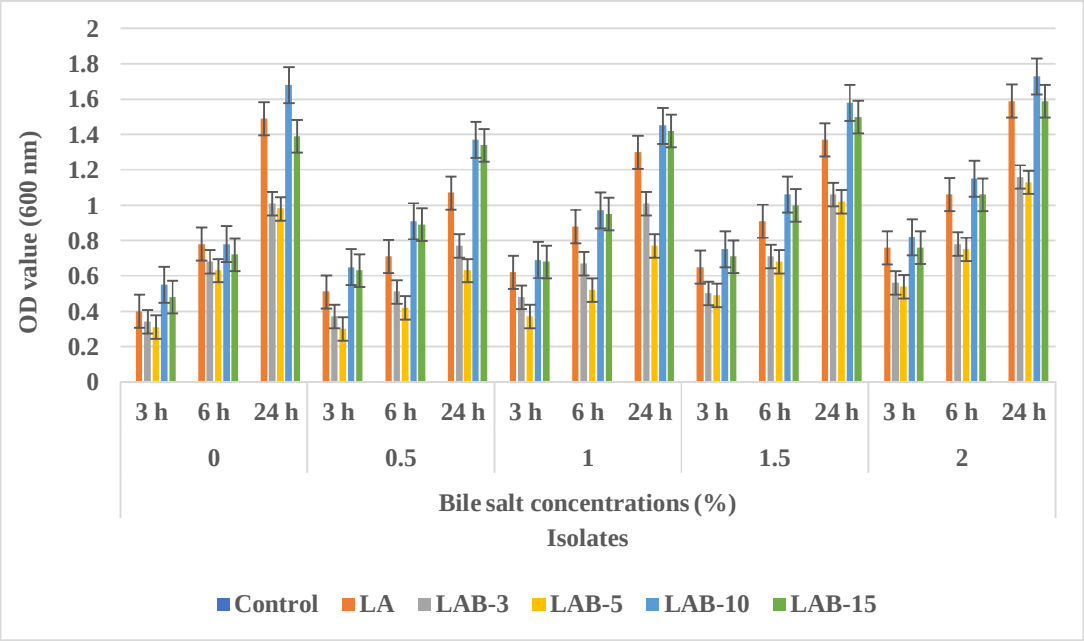

Figure 3: Effect of bile salt concentrations on growth of LA bacterial isolates in MRS broth at intervals

4. Conclusion

The present study demonstrates that lactic acid bacteria (LAB) isolated from the phylloplanes of raw edible and culinary leaves possess promising probiotic attributes. Selected LAB isolates that initially exhibited notable antibacterial and antifungal activity were further evaluated for their tolerance to gastrointestinal stress factors and their potential health-promoting properties. All isolates were non-haemolytic, confirming their safety and exhibited varying degrees of resistance to commonly used antibiotics and antifungal agents. Among them, *Lactobacillus acidophilus* and isolate LAB-10 showed superior probiotic potential by demonstrating high cell surface hydrophobicity, significant auto-aggregation ability, and strong tolerance to NaCl, phenol, bile salts, and acidic pH conditions. These isolates also exhibited optimal growth at temperatures simulating the human body. The ability of these strains to survive under gastrointestinal-like conditions highlights their potential as effective probiotic candidates. Further *in vivo* studies and functional assessments are warranted to confirm their application in food and health industries.

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Declaration of Competing Interest

The authors report no conflict of interest.

Ethics Statement

This research did not involve any human participants or animal subjects, and therefore, no ethical approval was required.

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ORCID

Shashank S: ***0009-0008-3799-0497***

Uday Kumar B V: ***0009-0006-8208-4675***

Uddalak Das: **0009-0004-0007-5691**

Author Contributions

Shashank S: Conceptualization, Methodology, Formal Analysis, Writing—Original Draft, Writing—Review & Editing. ***Uday Kumar B V***: Writing—Review & Editing. ***Suvarna V C***: Formal Analysis. ***Uddalak Das***: Project administration. All the authors have read and agreed to the published version of the manuscript.

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