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Isolation and Characterization of Potential Probiotic Lactic Acid Bacteria (LAB) from *Centella asiatica*

V.L.P. Amarasinghe[1]*, N.S. Weerakkody[2], T. Madhujith[3]

[1] *Postgraduate Institute of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka*
[2] *Department of Agricultural and Plantation Engineering, The Open University of Sri Lanka, Nawala, Nugegoda, Sri Lanka*
[3] *Department of Food Science and Technology, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka*

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*Corresponding author:
lakminiamarasinghe222@gmail.com

ABSTRACT

Background: Lactic acid bacteria (LAB) represent a diverse group of probiotic microorganisms widely utilized across the food industry, as well as in clinical and agricultural sectors.*Centella asiatica* (Gotu Kola) is a green leafy vegetable (GLV) delivering promising nutritional and medicinal values. Although probiotic properties of many GLVs have been widely studied, probiotic properties of LAB isolated from *C. asiatica* are not reported abundant in literature.

Objective: The aim of this study was to isolate and evaluate probiotic characteristics of LAB isolated from *C. asiatica*.

Materials & Methods: LAB isolation was performed in de Man Rogosa Sharpe medium anaerobically. After morphological and biochemical characterizations, probiotic properties were evaluated using low pH resistance, autoaggregation ability, hydrophobicity, antimicrobial attributes and bile salt tolerance. One-way ANOVA and Tukey's Mean comparison statistics were used in data analysis. All the tests were performed in triplicates.

Results: Twenty creamy white isolates were separated. Among them, ten were gram-positive and catalase-negative. Among them, significantly highest survival rate of 134.89% at pH 3 and 242.46% at pH 7 were demonstrated by I006 and I001respectively. After 2 h of incubation, the significantly highest auto-aggregation showed by I009. After 4 h I009 and I002 strains showed significantly higher ($p<0.05$) auto-aggregation 31.80% and 24.16%, respectively. The strain I005 showed the significantly highest hydrophobicity of 33.97%. The significantly highest Diameter of Inhibition Zones (DIZ) against *Staphylococcus aureus* and *E.coli*. were demonstrated by I001 and I006 and the values were 7.16 mm and 7.50 mm respectively. Among the selected best five isolates, I001 showed the highest survival rate (100.00%) in 0.3% (w/v) bile salt concentration after 2 and 4 h of incubation. All selected best five isolates formed gas in glucose fermentation.

Conclusions: It can be concluded that *C. asiatica* is a natural source for potential probiotic LAB and genetic characterization and molecular identification are highly recommended.

INTRODUCTION

Probiotics are live microorganisms used as food supplements, which provide health benefits, by improving the intestinal microbial balance of the host. Lactic acid bacteria (LAB) which comprise a large number of genera is a promising probiotic that can enhance gut health and have other health benefits like removal of carcinogens, lowering plasma cholesterol, immune stimulation, and allergy lowering effects (Shuhadha, Panagoda, Madhujith, & Jayawardana, 2017). In general, LAB is considered as gram-positive, non-spore-forming and anaerobic or microaerophilic rods or cocci in shape. LAB produces lactic acid as the major end product of carbohydrate fermentation (Bintsis, 2018).

One of the first techniques for processing food is the use of LAB as a microbial culture in the production of fermented meat, dairy, and fermented vegetables. In conventional to modern food industry, lactic acid fermentation is used to extend shelf-life and to enhance the organoleptic qualities of food (Soltan Dallal, Zamaniahari, Davoodabadi, Hosseini, & Rajabi, 2017). Food bio-preservation is an alternative and novel method of preservation with increasing special interest from consumers as a green and safe post-harvest treatment. With the significant antimicrobial properties of LAB, they can be used as a bio-preservative and natural inhibitor for food decaying and pathogenic microorganisms (Agriopoulou, Stamatelopoulou, Sachadyn-Król, & Varzakas, 2020).

C. asiatica commonly known as 'Gotu Kola' belongs to the family Apiaceous. It is a well-known herb plant (perennial plant) in the areas of South Africa, China, Sri Lanka, Colombia, Venezuela, Mexico, and the eastern regions of South America (Ganie et al., 2022). As a nutrition-rich edible plant, *C. asiatica* is consumed in the form of Green Leafy Vegetables (GLVs) and in the preparation of juice, drink, raw salad and other food products, therapeutic agents and elements for cosmetic products for centuries. Furthermore, its welldocumented medicinal effects, including neuroprotective, antidepressant, cardio-protective, anticancer, antimicrobial, anti-inflammatory, gastro-protective, and antioxidant properties, provide a compelling rationale for investigating its potential probiotic properties. (Ganie et al., 2022). This extensive array of reported health benefits suggests that *C. asiatica* may harbor microbial strains beneficial for gut health, thus warranting scientific investigation into its probiotic potential.

Although the nutritional and microbiological profiles of GLVs have been well studied, less focus has been placed on assessing *C. asiatica'*s associated LAB probiotic qualities. To address this gap, our research suggests that *C. asiatica* as well as other GLVs may be beneficial sources of probiotics. Therefore, the objective of this research is to isolate and evaluate the probiotic characteristics of potential LAB from *C. asiatica.*

MATERIALS & METHODS

Plant material collection and preparation

Freshly harvested soil-grown *C. asiatica* samples were randomly collected from local supermarkets in Colombo, Sri Lanka. They were directly transported to the Food Microbiology Laboratory, Department of Agricultural and Plantation Engineering, Faculty of Engineering, The Open University of Sri Lanka. Samples were gently washed with running tap water to remove extraneous matter on the surface.

Extraction and isolation of LAB

C. asiatica samples (25.0 g) were blended in autoclaved (121 °C, 20 min) saline solution (0.85%, 225.0 mL) using sterile stomacher (BIOBASE, BK-SHG04, 200W/220V, 50Hz). Sample (1.0 mL) was inoculated on de Man Rogosa Sharpe (MRS) agar media plates using the spread plate method and incubated anaerobically at 37 °C for 48 h in CO_2 incubator (JLab, JLab109/07, CO280ll1901027). Single colonies were counted using a digital colony

counter (HYC-560, 220V, 30W). Twenty distinct colonies were picked from MRS plates for purification by 16s streaking. Pure cultures of LAB were stored in Glycerol (80%) at -20 °C in a deep freezer (DW-25L300). Isolation was done in three trials and colony selection was done in the last trial.

Identification and characterization of LAB

Gram staining:

Gram staining was performed as described in Ismail, Yulvizar, and Mazhitov (2018). Slide was observed under a light microscope (MOTIC B series, ×10).

Catalase test:

A loop of bacterial culture was suspended on a drop of distilled water. Hydrogen peroxide (3%) was added to it and allowed to react. It was observed for non-bubble-forming isolates (Shuhadha, Panagoda, Madhujith, & Jayawardana, 2017).

Characterization of probiotic properties of selected isolates

A total of ten isolates were selected after the biochemical characterization. Characterization of probiotic properties was applied to them.

Low pH resistance:

The low pH resistance test was carried out according to the method described in Sadeghi, Panahi, Mazlumi, Hejazi, and Nami (2022) with slight modifications. In brief, 1.0 mL of a fresh MRS broth culture containing 10^8 CFU/mL of LAB strains were added to 9.0 mL of MRS broth of pH 3.0 and 7.0 and inoculated at 37 °C for 3h. Solution pH was adjusted with NaOH (0.1M) and HCl (0.1 M) using pH meter (MP103). After the incubation time, spectrophotometer (JENWAY 6300, 230/115 V, 13VA, 50/60 Hz) was used to measure the optical density (OD) of the samples at 600 nm. Low pH resistance was estimated by determining the survival rate (%) using the following equation:

Survival Rate (%) = [OD (After treatment)/OD (Before treatment)] × 100%

Auto-aggregation assay

Aggregation abilities of isolates were tested according to the method described by Zommiti, Connil, Hamida, and Ferchichi (2017) with slight modifications. Overnight cultures of bacterial cells were harvested by centrifugation (8000rpm, 10 min), and the cells were washed twice with phosphate-buffered saline (pH 7.1) and re-suspended in the same buffer. Absorbance at 600 nm (A600) of the upper suspension was measured after 0, 2 and 4 h of incubation time using spectrophotometer (JENWAY 6300, 230/115 V, 13VA, 50/60 Hz). Aggregation ability was measured as Aggregation % after 4 h of incubation:

Agg% = [1 − (A Time / A 0) × 100] where A Time represents the absorbance of the mixture at 0, 2 and 4 h and A 0, absorbance at time 0.

Cell surface hydrophobicity:

Activated LAB strains were inoculated into MRS agar and cultured at 37 °C under an anaerobic condition for 18 h. The bacteria were collected by centrifugation (8000 r/min, 10 min) and washed twice with phosphate buffer solution (PBS). A suitable amount of PBS was added to create a bacterial suspension, and 2.0 mL of the suspension was mixed with 0.4 mL of xylene by vortexing for 120 s. The water phase was separated, and the absorbance at a wavelength of 600 nm was measured. The absorbance of the water phase was used as a measure of the surface hydrophobicity (H %). The following formula was used to calculate the hydrophobicity; where 'A_0' and 'A' are the absorbance before and after extraction with xylene respectively (Liu et al., 2021).

H= (1-A/A 0) ×100%

Antimicrobial Activity:

The antimicrobial activity of isolates was tested using Agar Spot test method. LAB strains were cultured in MRS broth (5.0 mL) at 37 °C for 16 h. Aliquots (10 µL) of the culture were spotted

onto agar plates containing MRS medium (10.0 mL). After 18 h at 37 °C, the plates were overlaid with the appropriate soft agar (Nutrient Agar, 5.0 mL) inoculated with the cell suspension of the indicator strain at a final concentration of 10^5 CFU/mL. The plates were incubated for 24–72 h, depending on the growth of the indicator strain, and the appearance of inhibitory zones was observed. Used indicator strains were *Escherichia coli* ATCC 1858, *Staphylococcus aureus* SA113, and *Listeria monocytogenes* Scott A (4b). Indicator strains were obtained from the library of the Food Microbiology Laboratory, Department of Agricultural and Plantation Engineering, The Open University of Sri Lanka (Hernández, Cardell, & Zárate, 2005).

Bile Salt Tolerance:

LABs were grown in MRS broth at 37 °C overnight. Saturated bile solution was prepared separately by dissolving powdered bile extract. Bile solution was added to two of the cultures having 10^{-5} CFU/ml to achieve a final concentration of 0.3 % and the second culture with 0 % bile served as a control sample. The cultures were incubated at 37 °C for 4 h and every 2 h for 4 h viable counts were determined by pour plate. (Hassanzadazar, Ehsani, Mardani, & Hesari, 2012).

Characterization of the isolates using Gas Formation from Glucose

CO_2 production from glucose was determined in modified MRS broth containing inverted Durham tubes with glucose (1%). MRS broth (9.0 mL) in separate tubes containing glucose (1%) with inverted Durham tubes were prepared and inoculated separately with overnight fresh cultures of LAB (50 µL). Tubes were incubated at 37 °C for 5 days. The presence of gas in Durham tubes during 5 days of observation indicates CO2 production from glucose. MRS broth containing no LAB inoculation was used as the control (Mulaw, Sisay Tessema, Muleta, & Tesfaye, 2019).

Statistical Analysis

One-way ANOVA was applied to determine whether the differences between LAB isolates had a significant influence on the quantitative parameters ($p < 0.05$). Tukey's test was used to detect differences between mean values with a p-value of $p < 0.05$. To calculate the mean values and standard deviations, all tests were performed at least three times. Software Minitab 17.0 was used in data analysis.

RESULTS

Morphological and biochemical characterization of pure cultures

Bacterial colonies with distinct morphological appearances on MRS medium were selected for further experiments. Selected twenty isolates were purified by double streaking on MRS agar plates and incubated anaerobically at 37 °C for 48 h. They were numbered as I001 to I020 and morphological and biochemical properties are shown in Table 1.

LAB are considered as Gram positive and are stained in purple color in Gram staining (Figure 1). Two isolates were gram negative with red color in staining and they were not selected for further characterizations.

Under microscopic observations, the majority of isolates could be identified in spherical shape while 20% of isolates were observed in rod shape. Most of the colony surfaces were smooth while the surface characteristics of B is rough. Catalase test was used as a biochemical characterization test for isolates. A total 14 isolates were catalase-negative in the experiment. A total of ten Gram-positive, catalase-negative and well-grown isolates (I001-I010) were further investigated for their probiotic characteristics.

Probiotic characteristics

Biochemical tests used for probiotic characterization were resistance to low pH, auto-aggregation capacity, hydrophobicity, bile salt tolerance and antimicrobial activity.

Low pH resistance:

Under pH 7 experimental condition I001, I003, I004, I006, I008 and I010 demonstrated more than 100% survival rate and I001 showed significantly highest survival rate of 242.46 ± 5.39 % (Table 2). In the extreme intestinal condition of pH 3, selected isolates demonstrated a survival rate between 38.34 ± 1.24 % and 134.89 ± 9.87 % and I006 showed significant and the highest survival rate in low pH.

Table 1. Biochemical and morphological characteristics of selected isolates

Isolate	Gram staining	Catalase test	Shape
I001	+ ve	- ve	Spherical
I002	+ ve	+ ve	Spherical
I003	+ ve	- ve	Spherical
I004	+ ve	- ve	Rod-shaped
I005	+ ve	- ve	Spherical
I006	+ ve	- ve	Rod-shaped
I007	+ ve	- ve	Spherical
I008	+ ve	- ve	Spherical
I009	+ ve	- ve	Spherical
I010	+ ve	- ve	Spherical
I011	+ ve	- ve	Spherical
I012	+ ve	+ ve	Rod shaped
I013	+ ve	+ ve	Spherical
I014	- ve	- ve	Spherical
I015	+ ve	- ve	Spherical
I016	+ ve	+ ve	Spherical
I017	+ ve	+ ve	Spherical
I018	+ ve	- ve	Spherical
I019	- ve	+ ve	Spherical
I020	+ ve	+ ve	Rod shaped

All isolates were creamy-white in color with circular colony shapes and raised elevations. According to Ramadhanti, Melia, Hellyward, and Purwati (2021), circular, creamy white colonies raised in MRS agar could be characterized as LAB.

Auto-aggregation ability:

After 2 h of incubation, the highest auto-aggregation ability was shown by I009. Values for auto-aggregation ranged from 1.19 ± 1.07% to 29.38 ± 4.61%. Auto-aggregation values of

isolates after the incubation time of 4 h is greater than auto-aggregation values after 2 h of incubation. I009 and I002 showed the highest auto aggregation ability after 4 h and the values are 31.80 ± 5.72% and 24.16 ± 0.13% respectively are shown in Table 2.

Hydrophobicity:

Hydrophobicity of isolates varies from 0.49 ± 0.32% to 33.97± 3.50%. I005 showed the significantly highest hydrophobicity ($p < 0.05$) compared to other isolates.

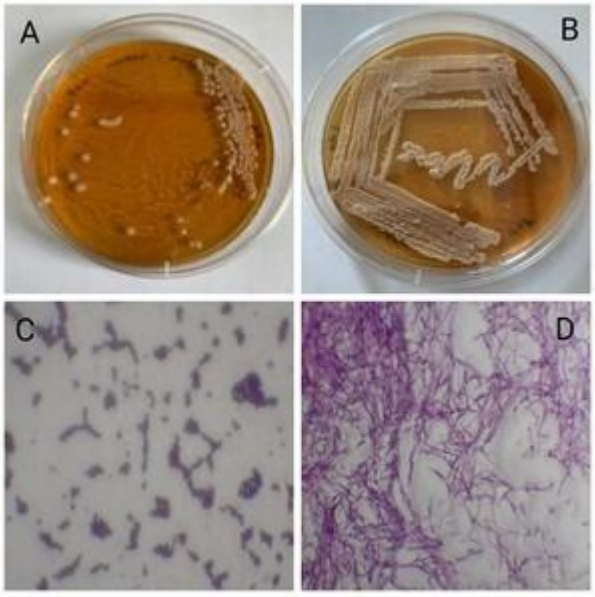

Figure 1. Morphological and biochemical characterization of isolates. A and B: Creamy white colonies on MRS medium; C: Cocci shapes; D: Rod-shaped bacteria stained by Gram staining

Antimicrobial activity:

The antimicrobial activity of selected isolates was tested against *S. aureus*, *E. coli* and *L. monocytogenes*. Figure 2 shows the inhibitory effect of different isolates against *S. aureus*.

According to the results, I003 did not show inhibitory activity against any of the pathogenic strains used in the experiment. The highest significantly different DIZ for *S. aureus* was demonstrated by I001 and the value is 7.16 mm. I006 showed the greatest significantly different inhibition of 7.50 mm for *E.coli* while 6.50 mm DIZ was demonstrated by I004 and I006 against *L. monocytogenes* (Supplementary file 1).

Bile Salt tolerance:

After considering the above probiotic attributes, the best five isolates that demonstrate significantly different probiotic characteristics, I001, I003, I005, I006 and I010 were selected for screening bile salt tolerance (Table 3).

Bile salt tolerance of all five LAB isolates was reduced with time. Surprisingly, isolate I001 showed the highest viable colony counts after 2 h and 4 h of incubation in 0.3% (w/v) bile salt concentration with the values of 8.46 ± 0.0 logcfu/mL and 8.45 ± 0.01 logcfu/mL respectively.

Table 2. Survival rate (%) under pH 3 and 7, autoaggregation (%), and hydrophobicity (%) of isolates

LAB Isolate	Survival rate (%) at p H=3	Survival rate (%) at p H=7	Auto aggregation (%) after 2 h	Auto aggregation (%) after 4 h	Hydrophobicity (%)
I001	120.05 ± 0.41^{b†}	242.46 ±5.39^{a}	6.77 ± 7.89^{bc}	12.17 ± 9.99^{b†}	19.58± 0.20^{b}
I002	57.72 ± 6.45^{d}	26.00 ± 3.06^{h}	15.97 ± 7.62^{b}	24.16 ± 0.13^{a}	0.49 ± 0.32^{c}
I003	96.62 ± 2.29^{c}	123.18±2.80^{cd}	4.90 ± 1.17^{bc}	6.68 ± 1.98^{b}	7.98 ± 2.33^{c}
I004	38.34 ± 1.24^{e}	105.89± 3.92^{e}	4.24 ± 1.84^{c}	7.69 ± 0.15^{b}	2.64 ± 0.95^{c}
I005	95.81 ± 2.64^{c}	51.73± 1.03^{g}	2.55 ± 1.63^{c}	3.56 ± 1.03^{b}	33.97± 3.50^{a}
I006	134.89 ± 9.87^{a}	158.17±13.3^{b}	6.37 ± 2.05^{bc}	8.59 ± 1.90^{b}	20.62± 0.46^{b}
I007	58.80 ± 1.62^{d}	77.46 ± 2.14^{f}	4.31 ± 0.794^{c}	5.18 ± 0.49^{b}	0.89 ± 0.59^{c}
I008	57.86 ± 9.09^{d}	134.11± 3.30^{c}	1.18 ± 1.07^{c}	3.07 ± 2.50^{b}	0.66 ± 0.21^{c}
I009	53.47 ± 2.14^{d}	43.45 ± 2.28^{g}	29.38 ± 4.61^{a}	31.80 ± 5.72^{a}	2.29 ± 2.21^{c}
I010	113.29 ± 1.14^{b}	115.02 ± 3.10^{de}	11.90 ± 0.37^{bc}	12.81 ± 0.75^{b}	15.94 ± 3.77^{b}

†*Within a column, mean values followed by the same lowercase letter are not significantly different ($p < 0.05$) as determined by Turkey's mean comparison test.*

Figure 2. Inhibitory effects of different isolates against *S. aureus*

Table 3. The viable colony count (log cfu/mL) of LAB isolates incubated in bile salt (0.3%) containing medium with time: 0 h, 2 h, and 4 h

LAB Isolate	Number of Colony Forming Units of LAB isolates with time (log cfu/mL)		
	0 h	**2 h**	**4 h**
I003	$8.16 \pm 0.01^{b\dagger}$	8.10 ± 0.02^{c}	7.92 ± 0.02^{c}
I005	8.45 ± 0.01^{a}	8.09 ± 0.01^{c}	7.84 ± 0.02^{c}
I006	8.41 ± 0.00^{a}	8.35 ± 0.01^{b}	8.30 ± 0.01^{b}
I010	7.26 ± 0.04^{c}	7.01 ± 0.02^{d}	6.66 ± 0.10^{d}
I001	8.46 ± 0.00^{a}	8.46 ± 0.00^{a}	8.45 ± 0.01^{a}

†*Within a column, mean values followed by the same lowercase letter are not significantly different ($p < 0.05$) as determined by Turkey's mean comparison test.*

Gas formation:

Five isolates I001, I003, I005, I006, and I010 produced gas by glucose metabolism in 48 h. LAB are generally considered as non-gas-forming bacteria in a sugar medium. With the time of inoculation, LAB produces different metabolites and some metabolites can influence gas formation. Selected LAB isolates in this experiment produce gas in a glucose medium and the cause for gas formation should be further studied.

DISCUSSION

The discussion section, it analyzes the results of morphological and biochemical characteristics of potential probiotic LAB isolated from *C. asiatica*. Our findings revealed distinct morphological features, with colonies appearing creamy white and exhibiting convex, circular shapes on MRS agar plates. Microscopic examination indicated Gram-positive, cocci and rod-shaped cells, a characteristic trait of LAB. Moreover, biochemical tests demonstrated positive reactions for catalase, consistent with LAB profiles. Importantly, strains exhibited notable acid and bile salt tolerance, with survival rates of >90% highlighting their potential suitability for gastrointestinal probiotic applications. With high auto-aggregation (31.80%), hydrophobicity (33.97%), and antimicrobial activity (7.50 mm), specific strains underscore the promising probiotic attributes of LAB strains derived from *C. asiatica*, suggesting their potential therapeutic value in promoting gut health and warranting further investigation into their functional properties and sustainable food industry applications.

The catalase test is used to detect enzyme catalase in bacteria cells. In the presence of Catalase, bacteria break down bactericidal H_2O_2 into water and Oxygen. LAB are considered as Catalase negative and no air bubble formation in catalase (Reiner, 2010)**.** In the current study, some isolates produced air bubbles and some were not. Only the isolates that did not produce air bubbles were considered as LAB and selected for further experiments.

Low pH resistance is an essential probiotic property because probiotic strains should survive under acidic pH in the gastric juice (below pH 3) and to colonize and secrete beneficial compounds to the host. At the same time, probiotic strains need to withstand high pH 7 mimic to the small intestine due to bile salts. According to the observations in this experiment (Table 2) and the classification of the previous study, under pH 7 experimental conditions and after the incubation time of 3 h, I001, I003, I004, I006, I008 and I010 showed

very good resistance (survival rate greater than 80%) to the acidic environment while I002, I005 and I009 showed moderate resistance (survival rate between 10% and 60%) and I007 demonstrated a good resistance (survival rate between 60% and 80%) (Sadeghi, Panahi, Mazlumi, Hejazi, & Nami, 2022). Five isolates showed a very good resistance at pH 3 after 3 hours of incubation and they are I001, I003, I005, I006 and I010. Also, I006 showed the highest survival rate of 134.89 ± 9.87% under pH 3. Strain 1005 showed the ability to withstand pH 3 and 7. Similarly, another study shows that the survival rate of *L. paracasei* remained relatively high, above 76% after exposure to pH 3 for 2 h and 61% after 3 h (M'hamed et al., 2022). Supporting the above results, a study reported that *L. paracasei* and *L. casei* isolates exhibited a high survival rate (>96%) in the gastrointestinal condition (Kumari et al., 2022). Thirteen of the *Lactobacillus* and *Lactococcus* isolates that were tested (Feriala & Saif, 2016), survived incubation times of 1-3 hours at pH 3.0; however, the percentage of isolates that survived decreased as the exposure duration increased.

Auto-aggregation shows the ability of the strain to self-aggregate to achieve a high cell density in the gastrointestinal tract and avoid possible colonization of pathogenic microorganisms. In line with current study findings, it has been reported that auto-aggregation values of 8.4% for *L. plantarum*, 21.4% for *L. acidophilus* strain isolated from probiotic products and the auto-aggregation of *L. rhamnosus* was 13.1%, which is a value on a similar level as *Lacticaseibacillus, Lactiplantibacillus and L. acidophilus* (Zawistowska-Rojek, Kośmider, Stępień, & Tyski, 2022). After 4 hours of incubation, the *L. paracasei* L2 aggregation percentage was determined to be 4.25%, the greatest auto-aggregation reaching 47.69% after 24 hours of incubation reported in a previous study in line with the current study (M'hamed et al., 2022). More or less similar findings were reported in another study reporting auto-aggregation percentage of LAB ranged from 43.8 ± 1.05% to 55.73 ± 0.96% for LAB from soybean (Kharnaior & Tamang, 2023).

The ability of isolates to bind to epithelial cells is correlated with their hydrophobicity towards different hydrocarbons. The hydrophobic elements in the outer membrane enable this ability. Our findings on hydrophobicity are more or less in accordance with the previous study findings with hydrophobicity values of 6.9% to 77.1% for Xylene from LAB isolates recovered from fresh vegetables (Alameri et al., 2022). However, in a different research reported a maximum of 76.28% and a minimum of 52.13% hydrophobicity values for LAB sp. of *L. rhamnosus, L. plantarum, L. pentosus, L. paracasei and L. casei.* (Huligere et al., 2023).But, in line to current study results, L. paracasei L2's cell surface hydrophobicity test findings revealed that the ethyl acetate solvent produced the greatest percentage of hydrophobicity, 22.8% while 15% hydrophobicity showed in Xylene (M'hamed et al., 2022).

Metabolites produced by probiotic LAB such as bacteriocins, peptides, organic acids, and volatile compounds are highly associated with antimicrobial activity while cytoplasm and plasma membrane of killed LAB act as barriers against foodborne pathogens (Alameri et al., 2022). According to another study, *Lactobacillus plantarum* has been successfully used for the reduction of pathogenic potential of *L. monocytogenes* in cabbages (Dong et al., 2020). Also, LAB isolated from fresh vegetables demonstrated antimicrobial activity against *S. aureus, E. coli* and *L. monocytogenes* (Alameri et al., 2022). In line with current study results of DIZ between 5-8 mm, antagonistic activity of genus *L. plantarum, L. brevis, Pediococcus sp.* and *Leuconostoc* sp. isolated from fresh fruits peach, pashmala, apricot, grape, banana, yellow and red apple, Kiwi, guava and orange and some vegetables as cucumber, tomato and strawberry against *S. aureus* ATCC6538, *E. coli* ATCC8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella typhimurium* ATCC22 bacteria were identified as the inhibition area not

exceeding 5mm (Feriala and Saif, 2016). With contrast values to the current study values, another study showed that LAB isolates from fresh vegetables displayed the highest antagonistic activity against *S. aureus* ATCC 25923, *L. monocytogenes* (clinical isolate), *E. coli* ATCC 25922, and *Salmonella enterica Typhimurium* with the inhibition zone ranged from 19.33 to 21 mm in diameters.

The findings of the current study are in line with the findings of a previous study that shows LAB strains; *L. reuteri, Pediococcus acidilactici, P. acidilactici, P. pentosaceus*, and *Enterococcus faecium* isolated from chicken epithelium demonstrating viability between 8.925 ± 0.055 to 9.245 ± 0.077 logcfu/mL for intestine while 8.847 ± 0.048 to 9.131 ± 0.029 log CFU/mL for the crop after 6 h incubation in 0.3% bile salt (Reuben, Roy, Sarkar, Alam, & Jahid, 2019). Previous study findings demonstrated that, after 24 and 48 h of incubation, the *L. paracasei* L2 isolate could survive at a concentration of 1% oxgall and when the amounts of bile salts increased, the growth rate dropped (M'hamed et al., 2022). Another study based on the probiotic properties of LAB isolated from fermented soybeans indicated that the tested LAB had a survival rate of >52% in pH 3.0 and >66% in bile salt. Of these, the two species with the highest survival rates were *Pediococcus acidilactici* Ph32 (peha) and *Enterococcus faecium* Kn19 (kinema), with 79.71 ± 0.13% low pH resistance and 73.67 ± 1.05% in 3% bile salt respectively (Kharnaior & Tamang, 2023).

Gas formation serves as an indicator of metabolic activity and may influence gastrointestinal conditions. Monitoring gas production in glucose medium aids in assessing probiotic functionality and potential effects on gut health, facilitating research into their therapeutic applications. The primary feature of homofermentative LAB is their inability to ferment glucose and other carbohydrates into gas. But, with the production of acids and other chemicals LAB can produce gas. In line with current research, *L. casei, L.s plantarum*, and *S.lactis* create acid and a considerable amount of gas when they are cultured on broth media containing glucose, galactose, lactose, and sucrose along with aflatoxin (Sutić & Banina, 1990).

LAB is a large and diverse group of bacteria and different conditions should be considered in the identification of LAB. Since certain desirable and unattractive traits are shared by several species, identification is a crucial and first step in any future applications. Traditionally, phenotypic characteristics are used for identification; however, DNA sequence-based methods are now commonly used. The most common application of 16S rRNA gene sequencing is identification with housekeeping gene sequencing employed when necessary (Endo, Tanizawa, & Arita, 2018). Therefore, in this research genetically identification of selected isolates was highly recommended.

CONCLUSIONS

Lactic acid bacteria were obtained from an unconventional source of leafy vegetable surfaces: *C. asiatica*. It appears that LAB are suitable for use as probiotics because they were able to survive under artificial intestinal conditions. In the current research, twenty isolated bacterial colonies from *C. asiatica* were characterized morphologically and biochemically. Ten Gram-positive and catalase-negative isolates demonstrated probiotic properties demonstrated probiotic properties like low pH resistance, auto-aggregation ability, hydrophobicity and antimicrobial attributes. The best five isolates: I001, I003, I005, I006 and I010 showed bile salt tolerance with gas formation. Further investigations are recommended for molecular identification of selected isolates.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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