

Exploring the Probiotic Potential and Safety of a Locally Sourced *Lactobacillus fermentum* Strain Isolated from Dahi, a Traditional Dairy Product

Sehar Aslam¹, Muhammad Qasim^{1*}, Mohsin Khurshid^{2*}, Usman Ali Ashfaq¹ and Muhammad Akhtar Ali³

¹Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Pakistan

²Institute of Microbiology, Government College University Faisalabad, Pakistan

³School of Biological Sciences, University of the Punjab, Lahore, Pakistan

ABSTRACT

Probiotics are live microbes that offer potential health benefits to the host, including modulation of the host immune system, improvement of anti-inflammatory response, enhancement of antibacterial and anti-allergic properties, as well as anti-proliferative properties. These beneficial microorganisms can interact with the gut microbiota to restore an impaired gut microbiome. While probiotics can be sourced from various sources, this study focuses on isolating and characterizing novel probiotic strains from dairy sources, specifically Dahi. To evaluate the probiotic potential of these isolates, various biochemical, morphological, and physiological tests were performed, followed by 16S rRNA gene sequencing to genotype the isolates. The tolerance of the isolates to pH, temperature, and bile salt, as well as their antimicrobial and adhesion ability, was evaluated. The results showed that the identified novel probiotic strains belonged to the *Lactobacillus fermentum* species, exhibited remarkable tolerance against bile salts, acidic environments, and temperature, and had excellent adhesion ability, indicating their potential as probiotic strains. Additionally, all isolates were non-hemolytic and displayed significant antimicrobial activity against antibiotic-resistant pathogens, such as *Escherichia coli* and *Staphylococcus aureus*. The anti-cancer activity of all isolates was also evaluated against the Human colorectal adenocarcinoma cell line (Caco-2), and all isolates showed significant anticancer activity. These findings validate the beneficial therapeutic values of novel probiotic strains isolated from dairy sources, specifically Dahi, and suggest that they could be used in food and drugs to treat various diseases.

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Authors' Contribution

SA, MQ and MK conceptualized the study. SA and MK conducted the experiments, while UAA provided validation and resources. SA and MQ handled data curation, formal analysis, and visualization. SA and UAA prepared the original draft. MK and MAA contributed to reviewing, editing, and finalizing the manuscript. All authors have approved the final version of manuscript.

Key words

Yogurt, Functional foods, Bile salts, Antimicrobial, Dahi

INTRODUCTION

Probiotics are live microbes that provide therapeutic effects on the host's health in addition to basic nutrient provision. They offer benefits such as inhibiting pathogenic bacterial growth, balancing gut microflora, reducing gut infections, preventing diarrhea and irritable bowel syndrome, lowering hypertension, improving immunity,

and assimilating blood cholesterol (García-Ruiz *et al.*, 2014). *Lactococcus*, yeast, *Bifidobacterium*, *Streptococcus*, and various strains of *Lactobacillus* have been widely studied as potential probiotics. Safe consumption by the host is a key aspect in the detection and application of probiotics (Nascimento *et al.*, 2019). The choice of probiotic strains is crucial for their use in the food industry because of their ability to survive harsh conditions and maintain proper functional characteristics during synthesis and storage, including freezing and spray drying (Moreno *et al.*, 2018).

Lactobacilli, comprising 7 genera including *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Leuconostoc*, are the most valuable and versatile group of Gram-positive probiotic bacteria involved in synthesizing several antibacterial peptides and volatile organic acids that provide innate resistance against pathogenic microbes (Rajoka *et al.*, 2017a). Lactobacilli have widespread applications not only in the food industry

* Corresponding author: qasimawan@gcuf.edu.pk, mohsinkhurshid@gcuf.edu.pk
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but also in the medical, pharmaceutical, and chemical industries (Bhat *et al.*, 2019). Due to their ability to synthesize bacteriocin and antimicrobial compounds, they could be recommended as bio-preservative agents (Chiu *et al.*, 2013). Additionally, Lactobacilli are commonly reported to help in the treatment of inflammation, intestinal infections, cancer, liver disorders, and bowel syndrome (Gontijo *et al.*, 2020). The potential of *Lactobacillus* for extensive use in important areas of health sciences and biotechnology is suggested by their beneficial effects (Rajoka *et al.*, 2017b).

Dahi is a widely used dairy product and a simple form of yogurt that has been known for centuries and is globally accepted for its health and nutritional benefits. It is a nutrient-dense food that is a good source of dairy proteins, magnesium, vitamin B12, and other essential fatty acids. Dahi provides health benefits by enhancing nutrient digestion and absorption. Additionally, it contains beneficial bacterial cultures, making it a potential source of probiotics, including LAB (lactic acid-producing bacteria) and many other bacterial strains (Lavrentev *et al.*, 2021). Dahi is considered a nutritious food due to the bioavailability of its nutrients and its high digestibility. It could be recommended to patients suffering from metabolic disorders and gastrointestinal disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease, and can also promote immune function, lactose intolerance, and weight control (Aryana and Olson, 2017).

The aim of this study was to isolate probiotic strains from Dahi, a traditional dairy product, and evaluate their probiotic potential and safety. The probiotic potential was assessed by testing the strain's tolerance to different concentrations of phenol and bile salts, while safety evaluation was performed by evaluating the strain's antioxidant and antibacterial effects, antibiotic susceptibility, and hemolytic activity. This comprehensive characterization of the locally isolated potential probiotic strain will contribute to our understanding of their potential as a probiotic. Research on exploring the probiotic potential and safety of a locally sourced strain can potentially have a positive impact on the local industry by providing new opportunities for the development of probiotic products, improving consumer confidence, and promoting traditional dairy products.

MATERIALS AND METHODS

Isolation and identification of probiotic strain

Dahi samples were collected from local dairy sources in Punjab, Pakistan, and was placed in the sterilized box. The sample was homogenized (20% w/v) in PBS (phosphate buffered saline) using a stomacher. Different

dilutions were prepared in saline and the sample was cultivated in MRS (DeMan-Rogosa-Sharpe agar, pH 6.3, HiMedia® Laboratories, India). The medium was supplemented with 0.04% L-cysteine (Sigma Aldrich®, USA) and was incubated for 2-3 days anaerobically at 37°C. Various colonies appeared after incubation and were re-streaked on MRS agar to isolate pure strains.

The isolated strains were examined and differentiated using Gram staining, morphology, catalase test, endospore, and motility test, etc. After screening analysis, the pure isolates were preserved at -80°C in glycerol as a stock. These isolates were further analyzed to evaluate their safety and probiotic potential. The genomic DNA of all bacterial strains was extracted using Bacteria genomicPrep Mini Spin Kit (Buckinghamshire, UK) and 16S universal primers (27F and 1492R) were used to amplify the rDNA. The genome sequencing was performed in Macrogen™ (South Korea). The obtained sequences were identified and molecularly analyzed using the in-silico tool BLAST available at NCBI Genbank. Phylogenetic analysis of the most suitable isolate was identified by using the MEGA X tool and a phylogenetic tree was constructed using the neighbor-joining method.

Evaluation of probiotic potential of isolated strains

The isolated strains were evaluated for pH tolerance, heat stability, phenol tolerance, autoaggregation and tolerance to H₂O₂.

For determining the capability of isolates to survive under different low pH (acidic) conditions was tested by cultivating isolates in fresh MRS broth at pH ranging from 2 to 8. The bacterial cultures were incubated for 24 h at 37°C. Bacterial density was measured by using a spectrophotometer in the range of 600 nm to estimate bacterial growth. The experiment was performed in triplicate to evaluate the accuracy of results.

For evaluation of heat stability, isolates were cultivated in MRS broth and incubated at a different range of temperature i.e., from 15°C to 45°C to calculate the optimum temperature. Then, the bacterial density was observed at 620 nm through U.V spectrophotometer to measure the growth of bacteria.

The salt tolerance of isolates was observed by cultivating on MRS agar plates, the medium was supplemented with different concentrations of sodium deoxycholate (C₂₄H₃₉NaO₄) salt (Sigma Aldrich®, USA) ranging from 0.5 to 2.0%. The resistance of isolates against salt was estimated by measuring the number of colonies of isolates on MRS agar plates. Similarly, the bacterial cell-free cultural suspension was inoculated, having 10⁸ CFU/ml in the test tubes of MRS broth. The phenol (0.5%w/v) was added to each test tube for estimating the phenol

tolerance after the incubation at 0h, 12h, and 24h. The resistance against phenol was estimated by counting the viable count after plating. All experiments were performed in triplicates to evaluate the accuracy of results.

The auto aggregation analysis was performed by following the previously described technique of Xu *et al.* (2009). The overnight culture of all isolates was centrifuged at 4200×g, for 10min at 37°C. The cells of isolates were washed twice with phosphate buffer saline (PBS) and re-suspended in PBS. The initial absorbance was measured at 605 nm at 0 h $a_{(0h)}$. 3ml of each bacterial culture suspension was vortexed for 8 sec and incubated at room temperature (37 °C) for 2, 4, 6, 8, 10 and 12 h. Then the absorbance of the supernatant was measured at 605 nm using a UV spectrophotometer after these h of incubation $a_{(2xh)}$. The equation for the calculation of auto-aggregation is:

$$\text{Auto-aggregation (\%)} = 1 - (a_{(2xh)} / a_{(0h)}) \times 100$$

Where, a_{0h} = absorbance at 0 h and a_{2h} = absorbance after 2, 4, 6, 8, 10 and 12 h of incubation.

For determining tolerance to H_2O_2 , freshly prepared cultures of isolates were exposed to different concentration levels of hydrogen peroxide (0.25, 0.5, 0.75, 1.0, 1.25mM) in test tubes with 5ml (0.9%w/v) NaCl. Then test tubes were incubated for 60 min and the viable count of isolates was determined.

Biofilm formation

The capability of all *Lactobacillus* isolates to produce bio-film was evaluated, as described previously (Pérez Ibarreche *et al.*, 2014). After one day of incubation at 37°C, the biofilm rings were obtained that were washed with double distilled water (ddW). The wells were treated with 1 mL of a solution containing 0.05% (v/v) of crystal violet and subsequently rinsed twice with ddW. Next, 1 mL of 96% ethanol (v/v) was introduced into each well, and an ELISA reader was used to measure the optical density at 595 nm. The comparison of isolates with the blank (PBS, MRS) identified the capability of isolates in bio-film production. The test was performed in a triplet experiment and the final value was suggested as weak bio-film production ($OD_{control} < OD \leq 2 \times OD_{control}$), strong biofilm production ($4 \times OD_{control} < OD$), moderate biofilm production ($2 \times OD_{control} < OD \leq 4 \times OD_{control}$) and non-biofilm production ($OD \leq OD_{control}$) (Borges *et al.*, 2012).

DPPH scavenging activity assay

Antioxidant potential was estimated from the scavenging activity of isolates towards stable free radicals of DPPH as explained by (Chen *et al.*, 2016). 1.5mL of (0.1mM) DPPH solution was added into 0.1mL freshly prepared cells of isolates. Then the mixture of *Lactobacillus* isolates was placed in a dark place for

half an hour. The absorbance was calculated using a spectrophotometer at 517 nm. The equation used for the calculation of scavenging activity is:

$$\text{DPPH scavenging activity (\%)} = 1 - (A_{\text{sample}} - A_{\text{blank}} / A_{\text{control}}) \times 100$$

where A_{sample} is solution of bacterial isolates cells and DPPH; A_{blank} is mixture of bacterial isolate cells and methanol and A_{control} is solution of DPPH

Adhesion ability of isolates

The human colorectal adenocarcinoma cell line (Caco-2) was allowed to grow in a higher glucose DMEM (Dulbecco's Modified Eagle's Medium; DNAbiotech Co, Iran) supplemented with 1.5% penicillin-streptomycin and 10% FBS, with 4-5% anaerobic atmosphere at 37°C. The medium was replaced after every next day until the confluency of cells was reached 75-85%. 0.5 McFarland absorbance of freshly prepared *Lactobacillus* cultures was settled using DMEM. Afterward, 100µl *Lactobacillus* suspension was added to each well of the plate and incubated for 2 h at 37°C. Then cells of *Lactobacillus* isolates were washed 2 times with PBS, fixed with methanol, and afterward stained with crystal violet for 5-6min. The adherence ability of cell members was quantified by using the method described previously (Fernández *et al.*, 2003).

Anti-bacterial activity

The anti-bacterial potential of *Lactobacillus* isolates was evaluated against two human pathogenic microbial strains i.e., *S. aureus* and *E. coli* using agar well diffusion method. 1.5mL of freshly prepared cells of all isolates of lactobacilli were filtered through a 0.2-micron syringe filter paper. Each pathogenic strain was allowed to grow on Muller Hinton (MHA) agar plates. The wells of 5mm diameter were created with borer and 50 µL filtrate of lactobacilli isolates were poured into the wells. Furthermore, plates were incubated for 24 h at 37 °C and the diameter of the bacterial growth zone was measured.

Anti-cancer activity

The anti-cancer potential of *Lactobacillus* isolates was identified through the already described procedure (Chen *et al.*, 2017) with fine modifications. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the anti-cancer effects of isolated *Lactobacillus* strains against human colorectal adenocarcinoma cell line (Caco-2). This MTT assay quantified the production of blue formazan because of MTT reduction by oxoglutarate dehydrogenase complex of mitochondria, which indicates cell viability and healthy mitochondrial functioning. Briefly, 10^4

Caco-2 cells/well were added to a 96-wells microtiter plate and were cultured for 24 h. Then, the culture-free supernatants of *Lactobacillus* isolates were added in different concentrations to each well and were subjected to incubation for 24, 48, and 72 h at room temperature with anaerobic condition (5% CO₂), MTT solution (100µl/ml)/well was added and the mixtures were further incubated for 4-6 h. The 100µl of DMSO solution was added to each well to dissolve the blue crystal.

Finally, the absorbance at 570nm was measured to determine the viability of cancer cells (Caco₂) after being treated with culture free supernatant from the *Lactobacillus* isolates via following equation.

$$C.V (\%) = (OD_{\text{treat}} / OD_{\text{control}}) \times 100$$

Where C.V = cell viability. The experiments were performed in triplicates to estimate the accuracy and reproducibility of results.

Safety evaluation of isolated strains

Hemolytic activity and antibiogram analysis were used for determination of safety evaluation of isolated strains.

For determination of hemolytic activity, the *Lactobacillus* isolates were cultured on Columbia blood Agar having 5% (v/v) sheep blood and were incubated for 2 days at room temperature. After incubation, the plates were examined/analyzed, and the hemolytic activity was evaluated based on the lysis of RBCs (red blood cells). The plates were identified as β-hemolysis having complete hemolysis of cells, α-hemolysis having partial hemolysis of cells, and γ-hemolysis having no hemolysis of cells. Only isolates having no hemolysis i.e., γ-hemolysis are regarded as safe.

The antibiogram analysis was investigated by the Kirby-Bauer Disc Diffusion method. In this method, the anti-microbial susceptibility of *Lactobacilli* isolates was analyzed against 10 different antibiotics i.e., amoxicillin, penicillin, gentamicin, ampicillin, streptomycin, rifampicin, tetracycline, and ciprofloxacin. The overnight culture of *Lactobacillus* isolates was swabbed on the plates of MRS agar, and the antibiotic disc was placed on the plates. Then the plates were subjected to incubation for 48 h at 37 °C. After incubation, the inhibition zones were measured for all antibiotics, and resistance was interpreted according to CLSI guidelines (CLSI, 2019).

RESULTS AND DISCUSSION

Lactobacillus isolates

The analysis of Dahi led to the identification of five different isolates, all of which were found to resemble *Lactobacillus* based on their morphological, physiological,

biochemical, and microscopic characteristics. On MRS agar plates, all isolates formed shiny, rod-shaped, white colonies. Furthermore, the initial characterization of the isolates indicated that they were Gram-positive, rod-shaped, catalase-negative, and non-motile, as well as non-spore-forming. Based on these findings, all the isolates were identified as *Lactobacillus fermentum*. While several studies have attempted to identify new and promising probiotics from dairy products, further research is necessary to investigate their unique species-specific characteristics (Terai *et al.*, 2015). It is worth noting that probiotics can be sourced from both dairy and non-dairy sources. Nevertheless, probiotics from dairy products have unique attributes that distinguish them from non-dairy probiotics. For instance, they exhibit greater resistance to lower pH levels and higher concentrations of bile salts, and they demonstrate more remarkable adherence capabilities (O'Toole *et al.*, 2017).

Probiotic potential

All the *Lactobacillus* isolates exhibited resistance to bile salt and were able to survive in varying pH conditions. The L-1 and L-2 isolates demonstrated maximum growth at a pH of 2, with survival rates of 85% and 77%, respectively, after 2 h of incubation. The other isolates, L-3, L-4, and L-5 also exhibited high resistance at pH 2, with survival rates of 40%, 63%, and 65%, respectively, after 2 h of incubation (Fig. 1). Regarding temperature, all isolates except L-1 exhibited minimal growth at 15°C, while all isolates were able to grow efficiently at a higher temperature of 45°C, except for L-3 (Table I). The pH level is a crucial factor that significantly influences the viability and growth of probiotic isolates during intestinal transit. The survival rates of the *Lactobacillus* strains used in this study are similar to those reported in previous studies (Bengoa *et al.*, 2018).

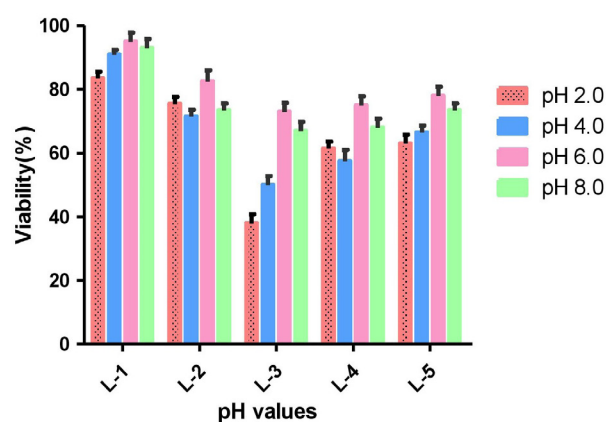


Fig. 1. Percentage viability of *Lactobacillus* isolates at different pH values.

Table I. Percentage of DPPH free radical scavenging activity and phenol tolerance of *Lactobacillus* isolates.

Iso-lates	Phenol tolerance			DPPH free radical scavenging assay (%)
	0 h	12 h	24 h	
L-1	8.75±0.24	8.50±0.17	8.20±2.6	89.53%
L-2	8.40±1.9	8.25±3.0	7.8±0.5	82.62%
L-3	8.21±0.11	7.9±0.14	7.3±1.2	79.89%
L-4	7.89±0.21	7.4±0.56	7.0±0.80	75.45%
L-5	8.12±0.39	7.7±1.5	7.1±2.1	69.93%

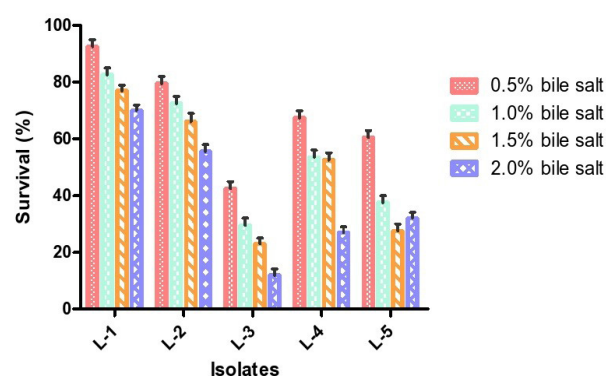
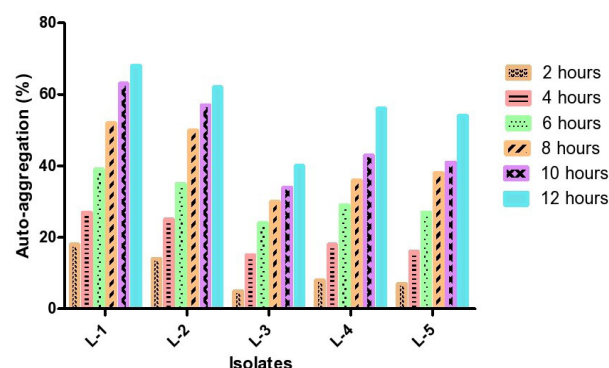
Table II. Antagonistic activity of *Lactobacillus* isolates against pathogenic bacterial strains.

Isolates	Zone of inhibition (mm)	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
L-1	15 ± 0.35	10 ± 0.15
L-2	11 ± 2.5	9 ± 3.5
L-3	10 ± 4.3	8 ± 2.0
L-4	8 ± 3.7	7 ± 4.5
L-5	6 ± 0.5	5 ± 2.5

All the *Lactobacillus* isolates exhibited excellent tolerance to lethal concentrations of bile salt and phenol, with isolate L-1 proving to be the most persistent even at high concentrations of bile salt (2.0% w/v) (Fig. 2) and phenol (0.5%) (Table II). All isolates exhibited robust growth and maximum viability. These results suggest that all *Lactobacillus* isolates can tolerate high concentrations of bile salt and phenol, indicating their potential as probiotics. All the strains exhibited excellent survival rates, except for L-3, even at a concentration of 2.0% (w/v) of bile salt. The isolates L-1 and L-2 showed survival rates of almost 70% and 58%, respectively, at high concentrations of bile salt. However, the viability of the *Lactobacillus* isolates decreased as the concentration of bile salt increased. Other reports have identified that mutant strains exhibit more viability at high concentrations of bile salt than wild-type strains (Bujnakova and Strakova, 2017; Melia *et al.*, 2022). A study investigating the resistance of *Lactobacillus* to bile salt found that *L. fermentum* exhibited tolerance to bile salt even after four h of incubation. Furthermore, some probiotic isolates showed tolerance to more than 2.0% (w/v) bile salt concentration, which is about three times higher than the concentration of bile salt in the human intestine (Chiu *et al.*, 2013).

All the *Lactobacillus* isolates demonstrated a high degree of auto-aggregation activity, but L-1 and L-4 isolates exhibited the highest levels of auto-aggregation

activity, at 68% and 62%, respectively. In contrast, the L-3 isolate exhibited the lowest level of auto-aggregation activity, at 40%. However, it is worth noting that auto-aggregation activity increased in all isolates with increasing incubation time, as shown in Figure 3. Auto-aggregation activity is an important characteristic of bacterial cells that is closely related to their ability to attach and colonize the gastrointestinal tract. The results of the present study indicate that L-1 and L-4 isolates have a high capacity for auto-aggregation after one day of incubation, while L-3 isolate demonstrated relatively low auto-aggregation ability. The ability of probiotic isolates to bind to the intestinal mucosa is crucial for excluding pathogens from the gut and for immunomodulation (García-Ruiz *et al.*, 2014) *in vitro* analysis of cell surface properties is insufficient to fully understand the interaction of isolates within the host. *In vivo* studies are necessary to gain a better understanding of these interactions. Nonetheless, the results of this study demonstrate that all the *Lactobacillus* isolates exhibited a high degree of auto-aggregation activity, indicating their potential for use as probiotics.

**Fig. 2. Percentage of survival rate of *Lactobacillus* isolates at various bile salts concentrations.****Fig. 3. Percentage auto-aggregation of *Lactobacillus* isolates at different time intervals.**

The results have shown that all the isolates were resistant to hydrogen peroxide and the concentration of H_2O_2 is inversely proportional to the viable count of isolates. As the concentration of H_2O_2 increased, a decrease in viable cell count was observed. However, the L-1 isolate showed maximum survival (8.5 log CFU/ml) and a minimum viable cell count (5.05 log CFU/ml) was observed in L-3 isolates as shown in Figure 4.

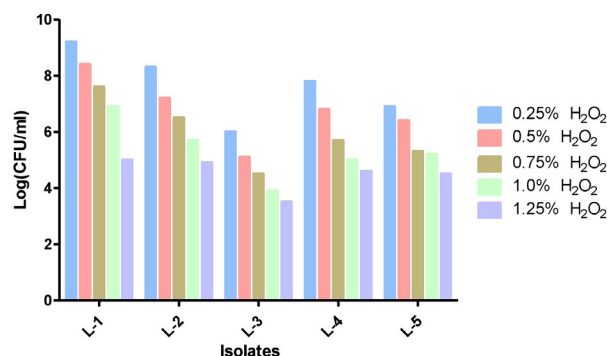


Fig. 4. Count of *Lactobacillus* isolates at different concentrations of H_2O_2 .

Antioxidant properties

The antioxidant activity of *Lactobacillus* is well-known, as it can quench free radicals of oxygen. In this study, we investigated the cell-free supernatant (CFS) of five *Lactobacillus* isolates (L-1 to L-5) for their antioxidant activity. Our results showed that L-1 (89.53%), L-2 (82.62%), L-3 (79.89%), L-4 (75.45%), and L-5 (69.93%) exhibited effective antioxidant activity, surpassing the values reported in previous studies (Table I). A study reported a DPPH free radical scavenging activity of 75.16% for the culture supernatant of Lactobacilli isolates (Shen *et al.*, 2011). Another study demonstrated that *Lactobacillus*-fermented soymilk had a higher antioxidant ability compared to unfermented soymilk (Moreno *et al.*, 2018). However, these studies only reported *in vitro* analyses of antioxidant ability, and it is crucial to perform *in vivo* antioxidant activity to confirm the therapeutic potential of these compounds. The use of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene can lead to serious health issues, particularly during long-term use, owing to their carcinogenic and toxic effects (Haghshenas *et al.*, 2014). Therefore, it is crucial to identify non-toxic, low-cost, and natural antioxidants that can serve as a substitute for all synthetic antioxidants used in the food and medicinal industries (Soubra *et al.*, 2007).

Adhesion potential

A crucial factor in selecting a probiotic is its ability

to adhere to epithelial cells and mucosal surfaces, as it indicates the organism's colonization and survival in the human gastrointestinal mucosa. The results showed that all isolates except L-3 and L-5 exhibited strong adherence ability, while L-3 and L-5 showed moderate adherence. Another critical characteristic of *Lactobacillus* isolates is their ability to survive and colonize the gut mucosa, which can be evaluated through an adhesion assay using a human colorectal adenocarcinoma cell line (Caco₂) (Kozak *et al.*, 2015). In this study, the probiotic isolates showed a range of adherence abilities, from moderate to strong adhesion. The results of the adhesion assay indicated the capability of the isolated *Lactobacillus* strains to colonize the intestinal epithelial cells. Adhesion ability is a significant feature of a suitable probiotic, as it inhibits the colonization of pathogenic bacteria in the human gastrointestinal tract (Abushelaibi *et al.*, 2017; Somashekaraiah *et al.*, 2019).

Antibacterial potential

The study results showed that all *Lactobacillus* isolates exhibited remarkable antimicrobial activity against pathogens such as *E. coli* and *S. aureus*. The only exception was L-3, which did not exhibit any inhibition against *E. coli* or *S. aureus* (Table III). Probiotics are effective in controlling various gastrointestinal infections and provide beneficial effects against rotavirus, traveler's diarrhea, and antibiotic-associated diarrhea. *Lactobacillus* produces antimicrobial substances, particularly proteinases. A study reported that the antimicrobial peptides synthesized by *Lactobacillus* exhibited strong antagonistic effects against antibiotic-resistant and antibiotic-sensitive pathogenic strains. These antibacterial peptides have the potential to be more therapeutically effective in ameliorating various inflammatory disorders (Wang *et al.*, 2018).

Table III. Antibiotic susceptibility profile of *Lactobacillus* isolates.

S. No.	Antibiotics disc	Isolate L-1	Isolate L-2	Isolate L-3	Isolate L-4	Isolate L-5
1.	Amoxicillin	R	S	S	S	R
2.	Penicillin	S	S	S	S	S
3.	Gentamicin	R	R	R	R	R
4.	Ampicillin	S	S	S	S	S
5.	Streptomycin	R	R	S	R	R
6.	Rifampicin	R	R	R	R	R
7.	Tetracycline	S	S	S	S	S
8.	Ciprofloxacin	R	R	S	S	S

Safety assessment

All the isolates were identified as non-hemolytic (γ -hemolysis). Antibiotic susceptibility testing was performed using the disc diffusion method, and all isolates were found to be resistant to different antibiotics, including amoxicillin, penicillin, gentamycin, ampicillin, streptomycin, rifampicin, tetracycline, and ciprofloxacin, except for L-3, which was susceptible to ciprofloxacin. Additionally, L-2 and L-4 showed sensitivity to amoxicillin, while L-1 showed greater resistance than all other isolates (Table III). Before suggesting any probiotic strain for therapeutic purposes or for use in the food industry, its safety must be assessed through *in vivo* and *in vitro* experimental studies. In this regard, the key experimental analyses include antibiotic susceptibility tests and hemolysis (Oh and Jung, 2015). None of the *Lactobacillus* isolates exhibited hemolytic activity (γ -hemolytic) in this study.

In this study, most of the isolates showed resistance to commonly used antibiotics, except for L-3, which was susceptible to streptomycin. Previous studies have reported that LAB strains, including *Leuconostoc*, *Lactobacillus*, and *Pediococcus*, were resistant to vancomycin (Tarrah *et al.*, 2019). The antibiotic resistance in the *Lactobacillus* group is chromosomally encoded and is non-inducible and non-transferable (Wong *et al.*, 2015).

According to the European Food Safety Authority (EFSA), if the isolated bacteria are intended for use in the food industry, the estimation of their hemolytic activity is mandatory, even if the isolated strains belong to probiotics that are generally recognized as safe (GRAS) (Oh and Jung, 2015). In the current study, the hemolytic potential of all *Lactobacillus* isolates was evaluated on Columbia blood agar plates (5-6% sheep blood), and none of the isolates exhibited hemolytic activity, including partial hemolysis (Nami *et al.*, 2018). This finding is consistent with previous reports that identified the probiotic potential of *Lactobacillus* from fermented foods and found no hemolytic effects of the isolated probiotics. The lack of virulence factors is an important criterion for selecting and evaluating probiotic isolates, making isolates without hemolytic activity ideal candidates (Bujnakova and Strakova, 2017).

Anti-cancer activity

The study investigated the anti-cancer potential of the isolated *Lactobacillus fermentum* strains on the human colon cancer cell line Caco₂. The results showed that the antiproliferative potential varied among the different isolates, ranging from 40% to 90% at a concentration of 30% (v/v) after 1 day of incubation. The anti-cancer potential increased with the concentration and incubation

time of the cell supernatants, and the L-1 and L-4 isolates exhibited the best anti-cancer activity after 24 h of incubation (Fig. 5). The cell-free culture supernatants of these isolates prevented the exponential growth of Caco-2 cells up to 90% after 72 h of incubation at a concentration of 30% (v/v). The study also highlighted the earlier research findings that revealed the anti-cancer activity of the cell-free supernatant of *Lactobacillus fermentum* against colon cancer. The culture-free supernatant was found to induce apoptosis, which inhibited the exponential growth of cells in colorectal cell lines. This suggests that *Lactobacillus fermentum* CFS has the potential to be used as a good multi-target antiproliferative chemotherapeutic agent (Lee *et al.*, 2019).

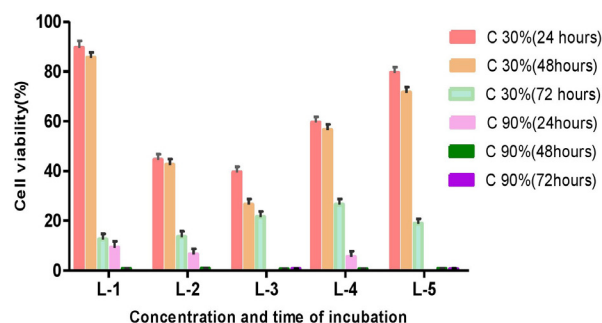


Fig. 5. Anti-cancer effects of culture-free supernatants of *Lactobacillus* isolates against human colorectal adenocarcinoma cell line at different concentrations of supernatants and incubation times.

Phylogenetic analysis

The isolated bacteria were identified as *Lactobacillus fermentum* through in-silico analysis with the highest homology (99.99%) with other *L. fermentum* strains, as shown in Figure 6. The 16s rRNA sequences of L-1 having the best probiotic potential was submitted to GenBank NCBI and assigned the accession number (MW397206). The phylogenetic analysis of this isolate revealed the highest homology (99.99%) with *L. fermentum* strain SABA5 and (99.32%) with other *L. fermentum* strains. The phylogenetic tree was constructed using the MEGAX tool following the neighbor-joining method (Gontijo *et al.*, 2020). Based on these results, *L. fermentum* strain SEHAR1 (L-1) can be considered a potential probiotic strain for further evaluation and *in-vivo* studies.

CONCLUSIONS

The current study has successfully identified and characterized five potential probiotic isolates with multifaceted probiotic properties, all of which were

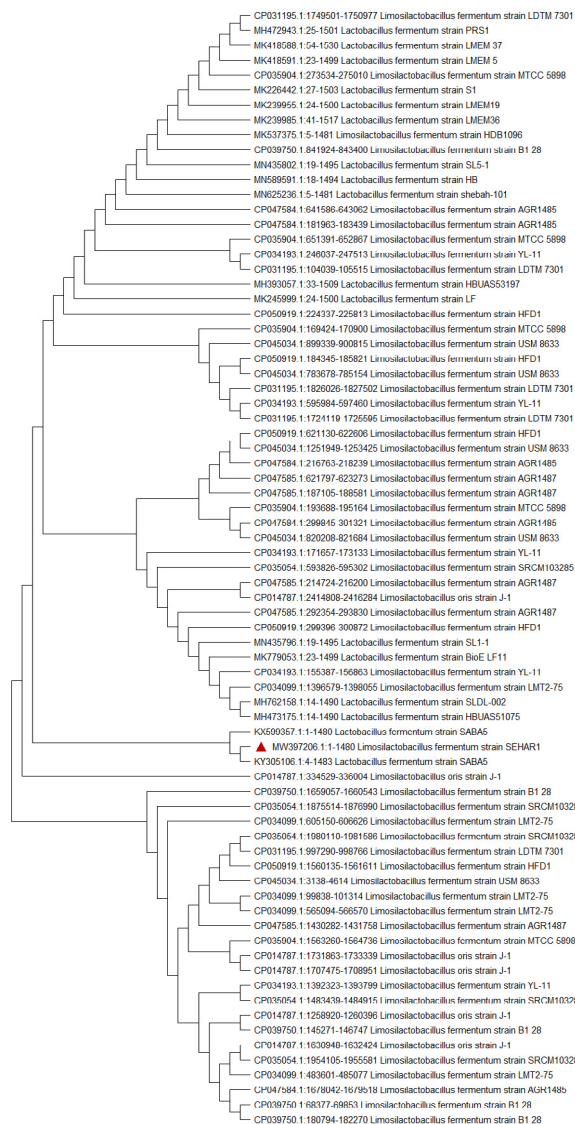


Fig. 6. Phylogenetic analysis using the Neighbor-joining tree building method based on 16S rRNA sequences of *Lactobacillus* isolates, indicating the homological association of *L. fermentum* strain SEHAR1.

identified as *L. fermentum* species. These indigenously isolated probiotic strains possess novel properties that could have significant therapeutic benefits in the cosmetics, pharmaceutical, and food industries. The study also confirms that dairy products can serve as a valuable source of unique potential probiotics with essential functional characteristics. These potential probiotic strains can be safely used to develop functional food products. However, further evaluation of these potential probiotics is required, particularly through *in vivo* studies using mouse models, to assess their immunomodulatory effects and safety for

human consumption.

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IRB approval and ethical statement

Since the study did not involve human or animal subjects, ethical approval was not required.

Statement of conflict of interest

The authors have declared no conflict of interest.

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