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RESEARCH

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Investigation of the antidiabetic and probiotic properties of lactic acid bacteria isolated from some ethnic fermented foods of Darjeeling District

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Abstract

Background Indigenous communities residing in the Darjeeling Himalayan region and its adjacent hilly areas have a deeply rooted cultural tradition of consuming a diverse range of vegetable and milk-based fermented products, believed to confer various health advantages. With this traditional knowledge, lactic acid bacteria (LAB) were isolated from popular fermented foods such as Chhurpi (derived from *Bos grunniens* milk), Gundruk (made from *Brassica juncea* leaves), Sinki (derived from *Raphanus sativus* taproots), and Kinema (produced from *Glycine max* beans). This study aimed to investigate the probiotic properties of the prevalent LABs, including aggregation properties, bile salt hydrolase activities, survival under gastro-inhibitory conditions, safety evaluations, and their potential health-promoting attributes, with a specific focus on inhibiting α-amylase and α-glucosidase enzymes.

Results Five of the LAB isolates demonstrated notable viability rates exceeding 85% when exposed to gastro-inhibitory challenges. Based on 16S rRNA gene sequencing, these isolates were identified as *Pediococcus pentosaceus* (isolate GAD), *Lactobacillus plantarum* (isolates KAD and CAD), *Lactobacillus brevis* (isolate SAD), and *Lactiplantibacillus plantarum* (isolate CMD). These LAB isolates exhibited versatile carbon source utilization, significant auto- and co-aggregation, and bile salt hydrolase (BSH) properties. Auto-aggregation capacity notably increased over time, ranging from 30 to 150 min, with percentage increments from 4.83 ± 1.92% to 67.60 ± 5.93%. *L. brevis* SAD displayed the highest co-aggregation increment (%) against *Staphylococcus aureus*, while *L. plantarum* KAD demonstrated potent antimicrobial activity. *In vitro* analyses postulated potential health benefits related to antidiabetic properties, particularly inhibiting α-amylase and α-glucosidase enzymes. *L. brevis* SAD exhibited the highest α-glucosidase inhibitory activity, while *L. plantarum* KAD displayed the most potent α-amylase inhibitory activity. Comprehensive safety assessments, including antibiotic susceptibility profiling, hemolytic activity evaluation, and *in vivo* acute toxicity studies, confirmed the suitability of these LAB isolates for human consumption.

Conclusions The isolates show promising probiotic characteristics and significant potential in addressing metabolic health. These results carry substantial scientific implications, suggesting the pharmaceutical-based applications of these traditional fermented foods. Further *in vivo* investigation is recommended to fully elucidate and exploit the health benefits of these LAB isolates, opening avenues for potential therapeutic interventions and the development of functional foods.

Keywords Antidiabetic, Dairy products, Fermented food, Lactic acid bacteria, Probiotics, Short-chain fatty acids

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Background

The beneficial effect associated with consuming fermented food products containing probiotic microorganisms is conventionally recognized. This has intrigued researchers to isolate and characterize novel probiotics from diverse sources. Fermented foods function as rich repositories of probiotics, rendering them valuable as dietary supplements for health benefits. Certain ancient societies have a longstanding tradition of incorporating fermented foods into their diets, and some are still produced through traditional methods [1, 2]. The microbial fermentation of raw ingredients in these foods facilitates the production of highly nutritious components, including bacterial metabolites, which are believed to confer health-promoting effects [3].

Research on identifying and characterizing new probiotic isolates and developing probiotic supplements with health-enhancing properties has gained increasing significance, especially in metabolic disorders such as diabetes. One strategy to mitigate the glucose load involves the delayed metabolism of carbohydrates, leading to a more balanced glucose profile. Studies have shown that inhibiting α-glucosidase and α-amylase enzymes can delay glucose absorption, resulting in a gradual increase in postprandial blood glucose levels [4, 5]. Notably, certain *Lactobacillus* spp. have demonstrated effective inhibition of α-glucosidase and α-amylase enzyme activity in vitro [6].

In the sub-Himalayan regions of West Bengal, India, particularly in the Darjeeling district, various fermented dishes are regularly consumed [7]. Several communities residing in the Darjeeling district and neighboring hilly areas of northern West Bengal traditionally prepare and consume different fermented milk and vegetable-based dishes. Examples include "Sinki" which is fermented radish (*Raphanus sativus*) taproot, "Gundruk" which is fermented leaves of certain *Brassica* species (*Brassica juncea*), "Kinema" which is fermented soybean (*Glycine max*) seeds, and "Chhurpi" which is a fermented cheese product made from the milk of Himalayan *Bos grunniens*. These preparations are reported to foster the growth of LAB isolates [8, 9]. However, further research is necessary to elucidate the antidiabetic, probiotic, and safety attributes of the LABs.

In this study, LAB strains from some popular ethnic fermented foods of Darjeeling have been isolated, and their *in vitro* antidiabetic and probiotic potentials, along with safety aspects, have been investigated.

Methods

Sample collection, processing, and isolation of LAB

Fermented food samples were collected from two different villages in the Darjeeling district of West Bengal, India, during March and April 2022. Chhurpi, a dairy-based fermented product, was collected from Tukdah Forest village, while soft Chhurpi and vegetable-based fermented preparations, namely Gundruk, Kinema, and Sinki, were collected from Bijanbari village. The samples were collected separately in sterile 50mL centrifuge tubes and transported to the laboratory in thermally insulated ice boxes to maintain their integrity. Standard procedure was followed for the collection of the samples [10].

Similar to the traditional processing for consumption, Kinema, Gundruk, and Sinki were mixed with distilled water and incubated at 37 ℃ for 20 min. Then, 200µL sample from these liquid mixtures was taken and added to separate De-Man, Rogosa, and Sharpe (MRS) (Hi-Media, India) broth and incubated at 37 ℃ for 48 h. Hard and soft Chhurpi was directly used as inoculum for MRS broth and incubated following the same process. After 48 h of incubation, all the inoculated broths were serially diluted up to 10^{-7} in phosphate buffer saline (PBS) (SRL, India) and spread on MRS agar plates separately. These plates were then incubated at 37 ℃ for 48 h. Plates with a suitable number of colonies (neither too many nor too few) were selected, and their colony numbers were counted in terms of colony-forming units (CFU). Bacterial load of all the food samples was calculated in CFU per milliliter (mL) or CFU per gram (g) unit. Colonies with distinct physical characteristics were then selected and streaked onto fresh MRS plates to make single colonies and to isolate pure cultures.

The isolated pure colonies were then transferred to MRS agar slants for experimental use and stocked in 40% glycerol for storage. The isolates were named based on the source (C for Chhurpi, G for Gundruk, K for Kinema, and S for Sinki), the month of collection (M for March, A for April), and the district of collection (D for Darjeeling), followed by sequential numbers to represent each isolate from each source. The slants were stored at 4 ℃ and sub-cultured every 15 days, not exceeding 2 passages (the transfer of organisms from an established culture to a fresh medium), while glycerol stocks were stored at − 20 ℃ and restocked every 2 months (not exceeding 5 passages) [11].

Preliminary screening of the isolates based on gastro-inhibition tolerance activity

In order to identify the most resilient LAB from the initial isolates, the isolates were exposed to conditions resembling the harsh environment of the human gastrointestinal (GI) tract. These conditions included lysozyme-mediated degradation, acidic pH, and high bile concentration [12]. After the preliminary screening,

tolerance to alkaline pH was also evaluated for the selected strains.

To assess lysozyme tolerance, a standard protocol was followed [13]. Briefly, overnight-grown bacterial cells were subjected to lysozyme tolerance testing. The cells were harvested, washed, and resuspended in Ringer's solution. A suspension of 10 µL was inoculated into a sterile electrolyte solution containing lysozyme at a concentration of 100 mg/L (SRL, India). A control broth without lysozyme was also prepared. The samples were then incubated at 37 °C, and after 2 h, the viable cell count was determined as a measure of lysozyme tolerance (in terms of Log CFU/mL).

For the analysis of acid and bile tolerance, standard protocols with certain experimental adjustments were followed [14, 15]. To assess acid tolerance, the isolates were cultured in acidic MRS broths (Test) adjusted to pH 3.0 with 1 M hydrochloric acid, alongside control MRS broth with normal pH. Here, a direct endpoint method was employed. This involved determining the survival percentage by comparing the bacterial count (expressed as log CFU/mL) in the control broth (not subjected to acidification) with that in the test broth (acidified to pH 3.0). For the experimental assessment of bile, acid, and lysozyme tolerance, *L. plantarum* MCC 2156 served as the positive reference strain [16]. The assessment was performed after a fixed incubation time of 3 h, utilizing an overnight-grown culture as the inoculum at a concentration of 1% V/V. Similarly, for the bile tolerance assay, a test broth containing 2% (w/v) deoxycholic acid (SRL, India) was used, and again, Log CFU/mL values of the 'Test' broth were compared with those of the 'Control' broth involving the same incubation parameters. The survivability of the isolates was calculated by comparing the Log CFU/mL values of the 'Test' and the 'Control' broths, and the survivability for each isolate was expressed as 'Percent Survival' (% Survival) using the following formula:

$$\text{Percent Survival (\% Survival)} = \frac{\text{Log CFU per mL of Text}}{\text{Log CFU per mL of Control}} \times 100$$

Isolates showing survival values $\geq 85\%$ for the GI conditions (lysozyme, acid, and bile tolerance) were selected for further analysis. The chosen cut-off value of "85%" was selected because scoring more than 85% of all three parameters creates a probability of success ($85\% \times 85\% \times 85\%$) and reinforces the strains' potential to thrive in the harsh GI environment, making them promising candidates for probiotic use.

After preliminary screening, survival of the selected isolates in alkaline pH was further assessed, taking alkaline-tolerant *Escherichia coli* K12 strain as control. Briefly, broths with pH levels of 8.5, reflecting the maximum pH detected in the human gastrointestinal tract [17], were prepared alongside control broths with unaltered pH. Inoculums were incubated for 12 h at 37 °C, and the % OD change of the individual broths was measured at 600 nm in a spectrophotometer (Shimadzu UV-1900I, Japan) indicating the extent of growth inhibition due to alkaline pH.

Molecular identification of the LAB isolates

The selected LAB isolates were identified by analyzing the partial sequences of their 16S rRNA genes through molecular phylogeny. The genomic DNA of each isolate was separately extracted following standard protocols outlined by De et al. [18]. The partial 16S rRNA gene was then amplified using universal primers 27F (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTAC CTTGTTACGACTT-3′) as described in a previous study by Frank et al. [19]. For amplification, reaction mixture was prepared using reagents from Promega (USA) and Eurofins Genomics, India (primers) following the manufacturer's instructions. Thermal cycling was carried out in a Mastercycler® Nexus GX2 thermal cycler (Eppendorf instrumente gmbh, Germany). The PCR products were visualized on an ethidium bromide-agarose gel under a UV transilluminator (Hi-Media, India). The PCR products were purified and sequenced using Sanger's method at Heredity Biosciences, India. The resulting sequences were aligned in the NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/blast.cgi) for similarity analysis and to infer functional and evolutionary relationships between sequences. The raw sequences obtained were trimmed and aligned using the MEGA 11 version 11.0.13 package with similar sequences retrieved from the database for drawing the phylogenetic relationship [20]. Finally, the curated sequences were deposited in the GenBank (https://www.ncbi.nlm.nih.gov/websub/) for public access. Phylogenetic tree and evolutionary relationships were constructed using the neighbor-joining method in the MEGA 11 (v. 11.0.13) package, with *Leuconostoc gasicomitatum* strain Tb1-10 serving as the outgroup [20]. To assess the accuracy of the tree topologies, bootstrap analysis with 100 repetitions was performed [21].

Assessment of probiotics properties

Carbohydrate fermentation profiling and esculin hydrolysis

A good probiotic strain should have the ability to utilize multiple carbohydrate sources, which ensures its survival in the host's digestive tract [22]. To identify the patterns of sugar fermentation, and esculin hydrolysis of the LAB isolates, a standard carbohydrate fermentation kit (Hi-Lacto Identification Kit, KB020-10KT, Hi-Media, India)

was used. For the assessment, 50 µL of overnight-grown cultures was aseptically applied to each of the specific designated wells of the kit by surface inoculation method, and then, the kits were again sealed off and incubated at 37 °C for 24 h, and after the incubation, the results were interpreted as per the instruction manual (as positive, + or negative, −).

Auto-aggregation assay

Modified version of the auto-aggregation assay developed by Del Re et al. [23] was used to test the capacity of the isolated LAB isolates to auto-aggregate. Briefly, 2 mL of bacterial suspension from overnight-grown cultures was vortexed, and then, the mixture was allowed to stand in static condition at 37 °C, and at regular intervals (30 min, 60 min, 90 min, 120 min, and 150 min) 100 µL of liquid from the upper surface from each bacterial culture was taken and combined with 900 µL of PBS (0.1 M), and then, the absorbance was measured using a microplate reader (SPECTROstarNano, BMG Labtech, Germany) at 600 nm (A_{600}). The auto-aggregation (%) of all the LAB isolates was then calculated by the formula:

$$\text{Auto} - \text{aggregation}\,(\%) = \frac{A_0 - A_T}{A_0} \times 100$$

where A_0 is A_{600} at 0 h and A_T represents the A_{600} of cell suspension at different time intervals (30 min, 60 min, 90 min, 120 min and 150 min). For comparison and analysis, the percentage value of each time frame was compared with the previous time frame for individual LAB isolates.

Co-aggregation assay

For co-aggregation assay, four commonly used laboratory strains were used as control. These were *Escherichia coli* K12 ATCC® 29425™, *Pseudomonas aeruginosa* CCEB-481 ATCC® 10145™, *Staphylococcus aureus* NCTC-8532 ATCC® 12600™, *Bacillus cereus* CCM-2010 ATCC® 14579™. For assessing the co-aggregation properties, standard protocols were followed with some modifications [24]. For this assay, overnight cultures of LAB isolates were mixed with the overnight cultures of the above-mentioned laboratory strains in equal volumes to form a cell suspension of a definite bacterial count (10^9 CFU/mL). The mixed suspensions were then incubated at 37 °C for 2 and 8 h. 2 mL of pure bacterial suspensions (for each bacterium) was taken as control. After the defined time duration, the optical density was measured at 600 nm. The following formula calculated the percentage values of co-aggregation of all the LAB isolates:

$$\text{Co - aggregation}(\%) = 100 \times \left[\frac{\left(\frac{A_{LS}+A_{LAB}}{2}\right) - A_{mix}}{\left[\frac{A_{LS}+A_{LAB}}{2}\right]}\right]$$

where A_{LS} and A_{LAB} stand for the OD_{600} of the pure cultures of the laboratory strains (LS) and LAB (LAB) isolates, respectively. A_{mix} is the OD of the mixed suspension different LAB and LS. Percent co-aggregation of the 8th h was compared with that of the 4th h (for each LAB to each LS).

For the auto- and co-aggregation assays too, *L. plantarum* MCC 2156 served as the reference strain [16] for comparing the performance of the isolated LABs.

Bile salt hydrolase activity (BSH)

One of the most potent health-promoting features of any LAB is the BSH activity, which determines its ability to break down conjugated bile salts [25]. For the assessment of BSH activity, one of the standard protocols laid down by Hernández-gómez et al. [26] was used with few modifications. Briefly, bile salt (Oxbile, Hi-Media) and $CaCl_2$ were added to MRS agar at 0.5% (w/v) and 0.37 g/L, respectively. Sterile disks made from filter paper were spot inoculated with 10 µl of overnight-grown LAB cultures in MRS broth, and finally, the disks were laid over modified MRS agar plates and then incubated at 37 °C for 48 h (to give ample amount of time for the deposition of detectable amount of precipitate). It was considered positive when bile acid precipitations started to form surrounding the disk in a diffused manner.

Cell surface enzyme characteristics

Extracellular enzyme activities like proteolytic, lipolytic, and amylolytic properties were evaluated following standard protocol [27].

For proteolytic property assessment, skim milk powder (SRL, India) was dissolved in 100 mL of bacteriological agar media to create skim milk agar plates. Then fresh overnight cultures of LAB isolates were inoculated onto skim milk agar plates, which were then incubated for 24 h at 37 ℃. The bacteria showing translucent haloes around the colonies were thought to have proteolytic properties.

Tributyrin supplement (SRL, India) was employed as a source of lipids to assess extracellular lipase activity. Tributyrin was autoclaved at 120 ℃ for 15 min. It was then diluted by 1/100 mL and added to Tributyrin Supplement Agar (TSA; SRL, India). The resultant colloidal medium was plated, allowed to dry, streaked with the LAB isolates, and then incubated for 24 h at 37 °C. Positive result was interpreted by the presence of a clear zone and the emergence of broken lipid droplets around the colonies.

For amylolytic activity assessment, 1 g starch is added in 100 mL of sterile nutrient agar media (SRL, India) and autoclaved at 121 ℃ for 15 min. The starch agar plates were streaked with cultures of the LAB isolates, incubated for 24 h at 37 °C, and after that, the plates were flooded with 1% iodine solution. Clear zones around the streaked lines indicated the presence of extracellular amylase enzyme, while the absence of such zones indicated negative results.

Antimicrobial properties

In order to check the antimicrobial activity, indicator strains, viz. *E. coli* K12ATCC® 29425™, *E. coli* HB101 ATCC® 33694™, *P. aeruginosa* CCEB-481 ATCC® 10145™, *S. aureus* NCTC-8532 ATCC® 12600™, *B. cereus* CCM-2010 ATCC® 14579™, were used and the experiment was conducted following a standard protocol [15] with experimental modifications. The overnight cultures of LAB isolates were centrifuged for 5 min at 8000×*g* (Eppendorf 5430R, Eppendorf Instrumente GmbH, Germany), the cells were pelleted out, and the cell-free supernatant was utilized to assess the antibacterial activity. A sterile borer was used to make 6-mm-diameter wells in Muller Hinton Agar (MHA) plates (SRL, India) that had been surface inoculated with overnight-grown culture suspensions of the indicator organisms (100 mL). Each well received 150 mL of cell-free supernatant of all LAB isolates, after which the plates were kept at 37 °C for a day and checked for zones of inhibition (ZOI). Results were interpreted as no inhibition = −, '+' = ZOI: 1–3 mm; '++' = ZOI: 3–8 mm; '+++' = ZOI: ≥ 8 mm (the ZOI was calculated after subtracting the well diameter size, i.e., 6 mm).

Cell surface hydrophobicity (CSH) assessment

One of the most commonly used methods to evaluate CSH activity is to find the hydrocarbon attachment propensities of the bacteria in question. CSH of the LAB isolates was determined using a standard protocol [28] with certain modifications. Briefly, after 24 h of incubation bacterial cultures were washed twice in PBS and then resuspended to a concentration of ~ 10^9 CFU/mL, and the initial absorbance at 600 nm value was determined (A_0). A two-phase system was then created by adding 3 mL of cell suspension with 1 mL of solvent (Xylene, Ethyl acetate, Acetone). It was then vortexed for 2 min. After 20 min of incubation, the aqueous phase was removed, and its absorbance at 600 nm was measured (A_1). CSH or '% adhesion' or hydrophobicity was calculated according to following formula:

$$\text{CSH or Adhesion (\%)} = \frac{A_0 - A_a}{A_0} \times 100$$

where A_0 is the OD_{600} of bacterial culture before solvent mixing and A_1 is the OD_{600} of bacterial culture after solvent mixing.

In vitro antidiabetic assessment

Partial or complete inhibition of carbohydrate-hydrolyzing enzymes determines the glucose-lowering activity of any LAB isolates because it reduces the amount of available free glucose in peripheral circulation.

α -amylase inhibitory activity

The ability of the isolated LAB strains to inhibit α-amylase was assessed following standard protocol [29]. Briefly, 250 µL of cell-free supernatant of each overnight LAB culture was mixed with 250 µL of 0.5 mg/mL α-amylase (Merck, India) solution and then incubated for 10 min at 25 °C. After that, 250 µL of starch solution (1% w/v in 0.02 M sodium phosphate buffer) was added to the reaction mixture and again incubated at 25 °C for 10 min. The reaction was then terminated with the addition of 500 µL of 3,5-dinitrosalicylic acid (DNS) color reagent (Merck, India) (96 mM DNS and 5.31 M sodium potassium tartrate in 2 M sodium hydroxide solution). The reaction mixture was heated for 5 min, cooled at room temperature, and diluted four times, and then, the absorbance was measured at 540 nm. The reaction mixture without bacterial supernatant was taken as a control. The inhibition propensity (%) was calculated according to the following formula:where A_C is the absorbance of the control reaction mixture and A_B is the absorbance of the reaction mixture with bacterial supernatant.

$$\alpha - \text{amylase inhibitory activity (\%)} = \left[\frac{(A_C - A_B)}{A_C}\right] \times 100$$

α -glucosidase inhibitory activity

The α-glucosidase inhibition activity was carried out following standard protocol [30]. Briefly, 25 µL of cell-free supernatant from each overnight LAB culture was mixed with 150 µL 0.01 M of potassium phosphate buffer (pH 6.8) and incubated for 10 min. To this mixture, 50 µL of α -glucosidase (Merck, India) enzyme was added and again incubated for 15 min at 37 °C. Then to this reaction mixture, 75 µL of 5 mM p-nitrophenol-D-glucopyranoside (pNPG) (Merck, India) substrate was added and incubated for 30 min at 37 °C; the enzymatic reaction was stopped with the addition of 1 mL of 0.1 M Na_2CO_3. The absorbance of the reaction mixture was measured at

540 nm. The reaction mixture without bacterial supernatant was taken as a control. The inhibition propensity (%) was calculated according to the following formula:

$$\alpha - \text{glucosidase inhibitory activity (\%)} = \left[1 - \frac{A_B}{A_C}\right] \times 100$$

where A_C is the absorbance of the control reaction mixture and A_B is the absorbance of the reaction mixture with bacterial supernatant.

Several strains *of L. plantarum* have been documented for their significant anti-diabetic properties, alongside notable *in vitro* inhibition of α -amylase and α -glucosidase enzymes. Hence, *L. plantarum* MCC 2156 has been employed as a reference strain to validate the experimental protocols and to assess the performance of the isolated LABs in these assays [31, 32].

Safety assessment

Antibiotic susceptibility profiling

Antibiotic susceptibility of a potent probiotic is linked with host safety. Antibiotic susceptibility of the selected isolates was carried out on MHA plates using antibiotic discs (Hi-Media Combi IV Octadisc, Hi-Media, India) of standard antibiotics, ampicillin (amp) 10 µg, cephalothin (cep) 30 µg, chloramphenicol (c) 30 µg, clindamycin (cd) 2 µg, erythromycin (e) 15 µg, gentamicin (gen) 10 µg, oxacillin (ox) 1 µg, vancomycin (va) 30 µg, streptomycin (str) 10 µg, kanamycin (kana) 15 µg, and tetracyclin (tet) 20 µg standard antibiotics. The results obtained were interpreted according the standard methods [33]. Briefly, the obtained ZOI diameter values were grouped into 2 subsection, "+" (having ZOI ≤ 8) and "−" (having ZOI ≥ 8) and the result was interpreted in a table.

Hemolytic activity

The hemolytic activity of bacteria is its capacity to degrade red blood cells and release hemoglobin, and it is required for the safety assessment of a putative probiotic. For this assay, standard protocol with slight modification was used [34]. Briefly, overnight cultures of LAB isolates were spread onto Blood Agar Plates (tryptone soy agar plate containing 5% human blood) and incubated for 24 h. The plates were then examined for patterns of hemolysis that is whether complete (beta), partial (alpha), or absence of visible (gamma) destruction of RBCs indicated [35] by a clear zone around the colonies. *E. coli* K12 was taken as positive hemolytic indicator strain to define the experiment's accuracy.

In vivo acute toxicity test

To ensure the safety of the LAB (probiotic isolates), acute oral toxicity study was conducted on Swiss albino mice (20 ± 2 g) aged 6–8 weeks. The mice were sourced from a licensed animal dealer (Chakraborty Enterprise, Kolkata, India; Regd. No. 1443/PO/Bt/s/11/CPCSEA) and housed in controlled conditions with specific temperature, humidity, and light–dark cycle. They were acclimatized for two weeks in the animal house keeping them in cages and with free access to pellet diet and water. The study was approved by the Institutional Animal Ethical Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the University as per rules, under the animal ethical approval number IAEC/NBU/2022/27. The mice were randomly divided into six groups a vehicle group and five groups with dietary LAB supplementation. Each group received a single dose of bacterial suspension (6×10^9 CFU/Kg body weight). Observations of various parameters such as aggression, food and drink intake, sedation, diarrhea, fur loss, and lethargy were recorded at regular intervals over 2 weeks. Particular attention was given to identifying any late signs of toxicological effects within the first four hours of each day. The investigation followed the OECD guideline for acute oral toxicity testing [36, 37].

Statistical analysis and graphical interpretation

All *in vitro* experiments were repeated three times. The data were represented by the replicate value's mean and standard deviation. GraphPad prism software package Version 8.0.2 was used for statistical analysis for data normality (Shapiro–Wilk test), statistical differences (ANOVA), multiple-comparison post hoc testing (Dunnet's or Tukey's test for significance test), and data visualization. $p \leq 0.05$ were considered significant.

Results

Isolation and screening of the isolates for tolerance to gastro-inhibitory conditions

Colony counts with respect to the weight of the source sample led to the enumeration of bacterial load in the food samples. In Gundruk it was 3.83×10^7 CFU/g, in Kinema it was 6.52×10^8 CFU/g, in Soft Chhurpi it was 1.65×10^7 CFU/g, in Hard Chhurpi it was 1.51×10^7 CFU/g, and in Sinki it was 6.03×10^8 CFU/g. A

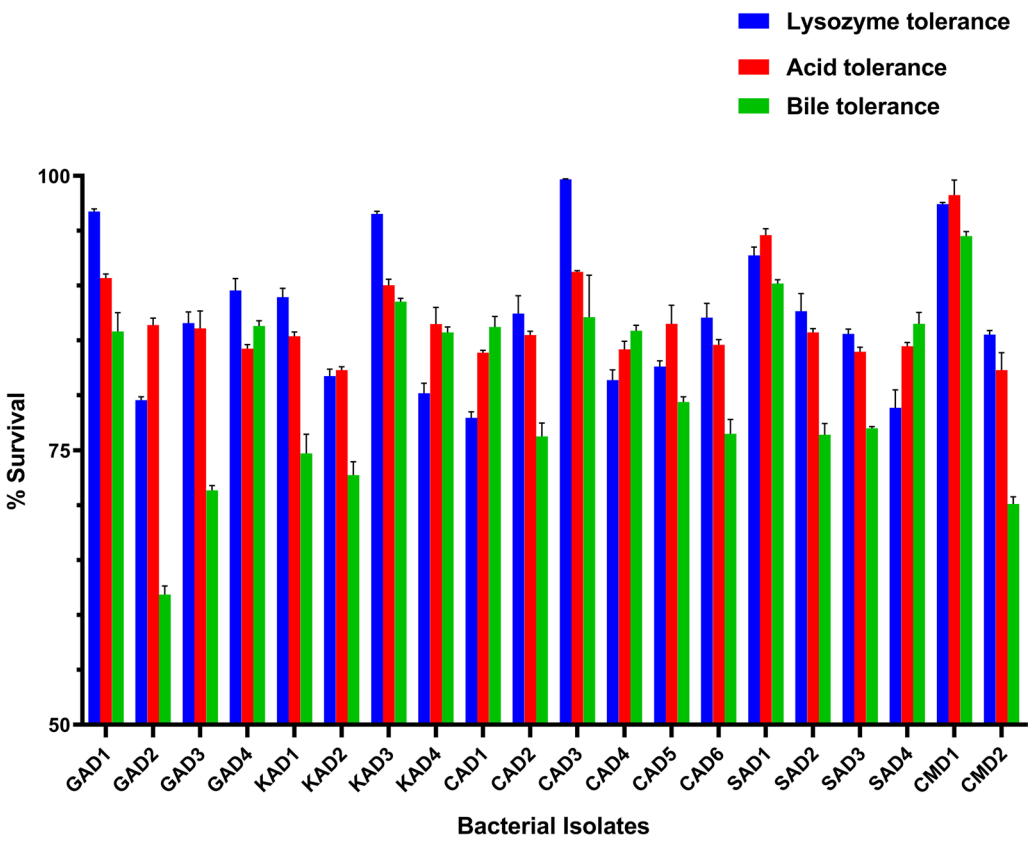

Fig. 1 Lysozyme, acid, and bile tolerance (in terms of % survival) of 20 bacterial isolates from fermented foods of Darjeeling

total number of 20 isolates were obtained from different food sources. From Gundruk, 4 isolates, from Kinema 4 isolates, from Soft Chhurpi 6 isolates, from Sinki 4 isolates, and from Hard Chhurpi 2 isolates were acquired. All the 20 isolates were subjected to screening based on their gastro resistant properties. The results are depicted in Fig. 1.

Isolates GAD1, KAD3, CAD3, SAD1, and CMD1 showed ≥ 85% survival in all the tolerance tests involving lysozyme, acid, and bile. These five isolates have no significant differences in comparison with the control strain for the gastro-inhibitory (bile, acid and lysozyme tolerance) tests and their influence on host body weight (refer Additional file 1: Fig. S1 and S2 for the results of gastro-inhibitory parameters and influence on host body weight respectively). For tolerance to alkaline pH, four of these isolates except CAD3 showed noteworthy reduction in survival rates compared to the reference *E. coli* K12 strain (refer Additional file 1: Fig. S3 for the results of alkaline tolerance of the isolates). These five isolates, subjected to further analysis were subsequently named as GAD, KAD, CAD, SAD, and CMD for easy understanding.

Molecular identification and phylogenetic analysis

The concentration of the isolated DNA from the five isolates was ~ 44 µg/mL. 16S rRNA gene amplicons of the five isolates were compared to those of the strains listed in GenBank using NCBI-BLAST. Isolate GAD (Acc. No. OQ306562) showed highest percent identity (99.05%) with *Pediococcus pentosaceus* isolate 4412 (Acc. No. MT544941.1); isolate KAD (Acc. No. OQ306564) showed highest percent identity (98.18%) with *Lactobacillus plantarum* strain 6415 (Acc. No. MT515856.1); isolate CAD (Acc. No. OQ306566) showed highest percent identity (99.39%) with *L. plantarum* strain 1887 (Acc. No. MT597711.1); isolate SAD (Acc. No. OQ306549) showed highest percent identity (99.45%) with *L. brevis* isolate gp71 (Acc. No. KM495920.1); and isolate CMD (Acc. No. OQ306547) showed highest percent identity (99.52%) with *Lactiplantibacillus plantarum* strain thankcomeLP1 (Acc. No. MZ045749.1).

A phylogenetic tree constructed using the neighbor-joining approach with a bootstrap test (100 repetitions) depicts the proportion of duplicate trees in which the linked taxa clustered together (Fig. 2).

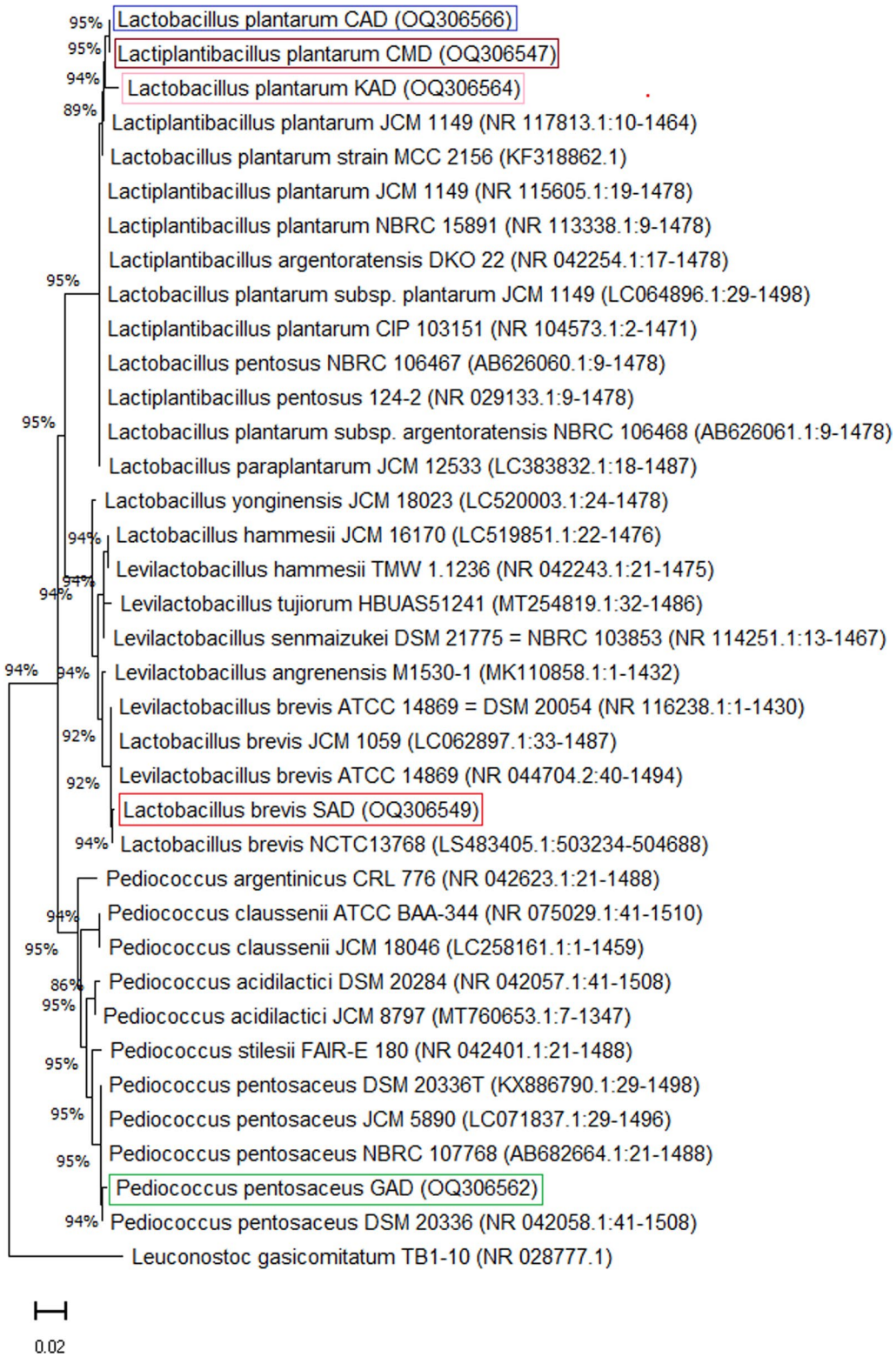

Fig. 2 Phylogenetic tree demonstrating the evolutionary relationship of isolated five LAB strains from fermented foods of Darjeeling, as inferred by the neighbor-joining approach based on the 16S rRNA gene sequences in MEGA11. The evolutionary distances were computed using the Maximum Composite Likelihood method. This analysis involved 37 nucleotide sequences and 1551 positions

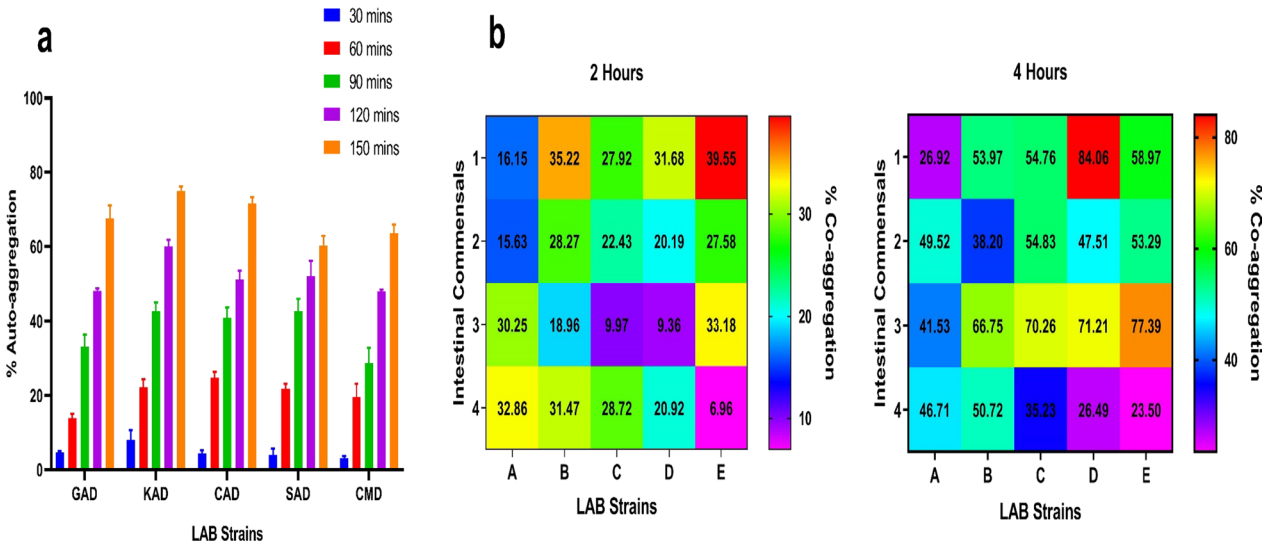

Fig. 3 Aggregation properties of isolated five LAB strains. **a** Auto-aggregation measured at successive intervals of 30 min reveal significant increase in all strains (adjusted *p* value ≤ 0.0001). **b** Heatmap showing co-aggregation properties of the strains against known intestinal commensals. Key: A, GAD; B, KAD; C, CAD; D, SAD; E, CMD; 1, *E. coli* K12; 2, *P. aeruginosa*; 3, *S. aureus*; 4, *B. cereus*

Probiotic assessment

Carbohydrate fermentation pattern and esculin hydrolysis

All the isolates could ferment carbohydrate sources present in the kit, viz. xylose, cellobiose, arabinose, maltose, galactose, mannose, mellibiose, raffinose, sucrose, and trehalose. For esculin hydrolysis, all showed positive properties.

Auto-aggregation assay

Auto-aggregation values of all five LAB isolates increased significantly starting from 30 min up to 150 min (from 4.83 ± 1.92 to 67.60 ± 5.93%). Most isolates tended not to collapse after forming clumps during the incubation period. Longer incubation times resulted in higher and significant auto-aggregation percentages (Fig. 3a). *L. plantarum* strain KAD showed highest percent aggregation amongst these five isolates. Overall, it can be seen that auto-aggregation values of all the LAB isolates increased significantly in each successive time frame after initial dissociation.

Co-aggregation assay

All the five LAB isolates have shown the ability to co-aggregate with the intestinal commensal strains tested. Highest percent co-aggregation at 2nd hr was seen in the case of CMD (39.55 ± 1.16%) with *E. coli* K12, and the least was seen in the case of SAD (9.35 ± 4.80%) with *S. aureus*., whereas in the case of the 4th hr, the isolate SAD showed the highest co-aggregation percentage (84.76 ± 0.44%) with *E. coli* K12, and during the same time frame, the least percent co-aggregation value was seen in the case of CMD (23.50 ± 2.22%) with *B. cereus*. Maximum percent co-aggregation increment (2nd–4th h) is seen in association with *S. aureus* with the isolates KAD (18.96 ± 6.46–66.74 ± 0.37%), CAD (9.97 ± 0.74–70.26 ± 1.05%) and SAD (9.35 ± 4.80–71.21 ± 1.82%) (Fig. 3b).

The five LABs exhibited no significant overall variances compared to the positive strain for the auto-aggregation and co-aggregation properties (Additional file 1: Fig. S1). However, in three specific instances (% auto-aggregation at 90, 120, and 150 min), positive strain yielded higher values.

Bile salt hydrolase (BSH) activity

All five LAB isolates displayed BSH-positive results, which was consistent with the isolates' earlier documented bile salt tolerance results. SAD showed the highest precipitation zone (6.66 ± 2.51 mm), while CMD showed the lowest (2 ± 1 mm). All of the other isolates displayed values that are more or less in the middle, falling between 2.33 mm and 3.66 mm (Fig. 4a, b).

Cell surface enzyme characteristics

The five LAB isolates displayed cell surface enzyme features to varied degrees (Table 1). GAD displayed the highest levels of proteolytic activity; CAD and SAD showed highest lipolytic activities; GAD and KAD showed highest amylolytic activities. None of the LAB isolates showed positive results in all the three parameters.

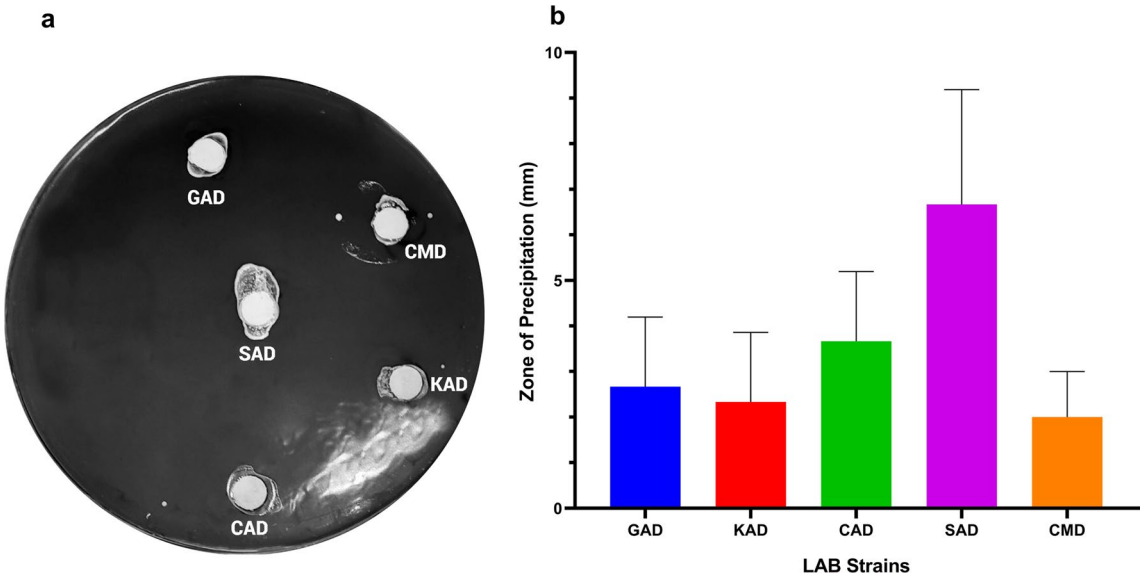

Fig. 4 Bile Salt Hydrolysis (BSH) assay of isolated five LAB strains **a** MRS medium containing bile salt showing BSH activity of the LAB strains as evidenced by the development of a precipitation zone around the colony. **b** Graphical representation of the BSH activity of isolated five LAB strains. The diameters of the precipitation zones are given in millimeters (excluding the diameter of the disc)

Table 1 Cell surface enzyme characteristics and antimicrobial profiling of isolated five LAB isolates

Isolates	Cell surface enzyme characteristics			Antimicrobial profiling against pathogens				
	Proteolytic activity	Lipolytic activity	Amylolytic activity	*E. coli* K12	*E. coli* HB101	*P. aeruginosa*	*S. aureus*	*B. cereus*
GAD	+++	–	++	++	++	–	++	++
KAD	++	–	++	+	++	+	++	++
CAD	–	++	–	+	++	+	+	++
SAD	–	++	–	–	+++	+	++	++
CMD	++	+	–	++	+++	–	++	++

[a] '+++' = highly positive; '++' = moderately positive; '+' = weekly positive; '–' = negative

[b] '–' = no inhibition; '+' = ZOI: 1–3 mm; '++' = ZOI: 3–8 mm; '+++' = ZOI: ≥ 8 mm

Antimicrobial properties

In the case of antimicrobial properties, it was seen that all the five LAB isolates showed the highest antimicrobial properties against *E. coli* HB101, whereas the least antimicrobial activity against *P. aeruginosa.* Among LAB isolates, KAD and CAD showed antimicrobial activity against all the strains tested. The complete absence of antimicrobial activity was seen in the case of GAD (against *P. aeruginosa*), SAD (against *E. coli* K12), and CMD against (*P. aeruginosa*) (Table 1).

Cell surface hydrophobicity (CSH) assessment

Microbial adhesion to solvents (MATS) serves as an illustration of the association between surface hydrophobicity and adhesion nature. The highest hydrophobicity (adhesion/CSH %) for all the LAB isolates was

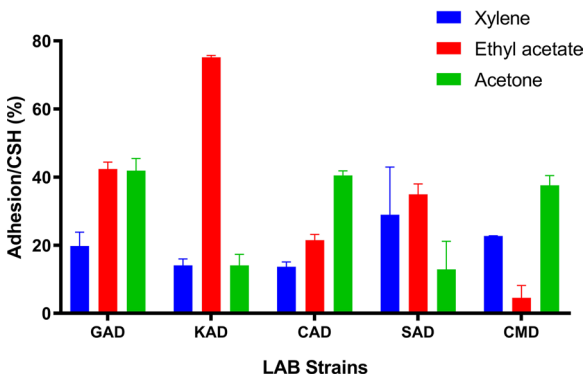

Fig. 5 Graph showing cell surface hydrophobicity (Adhesion/CSH %) of isolated five LAB strains with various solvents

seen in the case of ethyl acetate, followed by acetone and then by xylene. On the other hand, among all the isolates tested with all the solvents, isolate KAD showed the highest adhesion/CSH % (75.15 ± 0.57%) with ethyl acetate, whereas the lowest was seen in the case of CMD (4.53 ± 3.64) with ethyl acetate. Overall, on the basis of solvents, SAD showed the highest adhesion/CSH % with xylene (28.97 ± 13.96%), isolate KAD with ethyl acetate (75.15 ± 0.574%), and isolate GAD with acetone (41.87 ± 3.56) (Fig. 5).

In vitro antidiabetic assessment

α-amylase and α -glucosidase inhibitory activity

In the present study, it was found that the α-amylase inhibitory activity for all five isolates ranged from 53 to 63%. Where KAD showed highest inhibition (63.16 ± 1.91%) followed by isolate SAD (62.45 ± 0.53). Isolates GAD and CAD showed similar inhibitory pattern (58.42 ± 0.41% and 58.38 ± 1.64%, respectively). The least inhibition was shown by isolate CMD (53.37 ± 1.47%) (Fig. 6a). In the case of α -glucosidase inhibitory activity, it was seen that the values ranged from 34 to 39%. The highest inhibition was seen in the case of isolate SAD (39.75 ± 2.78%) followed by isolate CMD (37.30 ± 2.40%); isolate GAD and KAD showed similar pattern of inhibition (36.54 ± 3.50% and 35.47 ± 2.06%, respectively). The least activity was seen in the case of isolate CAD (34.86 ± 2.78%) (Fig. 6b).

All the five LABs exhibited negligible variances in the % inhibition in α-amylase and α-glucosidase activities compared to the positive strain (Additional File 1: Fig. S1).

Safety assessment

Antibiotic susceptibility profiling and hemolytic activity

All the selected LAB isolates showed varying degrees of susceptibility against a panel of standard antibiotics. Isolate-wise resistance was observed against certain antibiotics. Isolate GAD showed resistance against gentamicin, vancomycin, streptomycin, and kanamycin; isolate KAD showed resistance against erythromycin, oxacillin,

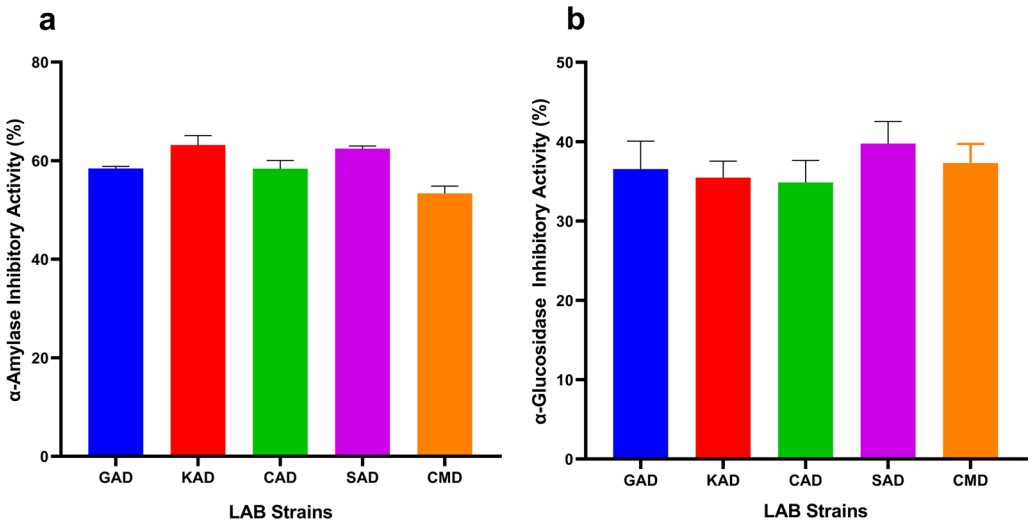

Fig. 6 α-Amylase and α -glucosidase inhibitory activity of isolated five LAB strains

Table 2 Antibiotic susceptibility assessment of the isolated five LAB isolates

Isolate	Antibiotics[a,b]										
	Amp	Cep	C	CD	E	Gen	Ox	Va	Str	Kana	Tet
GAD	–	–	–	–	–	+	–	+	+	+	–
KAD	–	–	–	–	+	–	+	+	+	+	–
CAD	+	+	+	+	+	+	–	+	+	+	–
SAD	–	–	+	+	–	–	–	–	+	–	+
CMD	+	+	–	–	–	–	+	+	–	+	+

[a] *Amp* ampicillin; *Cep* cephalothin; *C* chloramphenicol; *CD* clindamycin; *E* erythromycin; *Gen* gentamicin; *Ox* oxacillin; *Va* vancomycin; *Str* streptomycin; *Kana* kanamycin; *Tet* tetracycline

[b] "+" denotes resistance & "–" denotes susceptibility

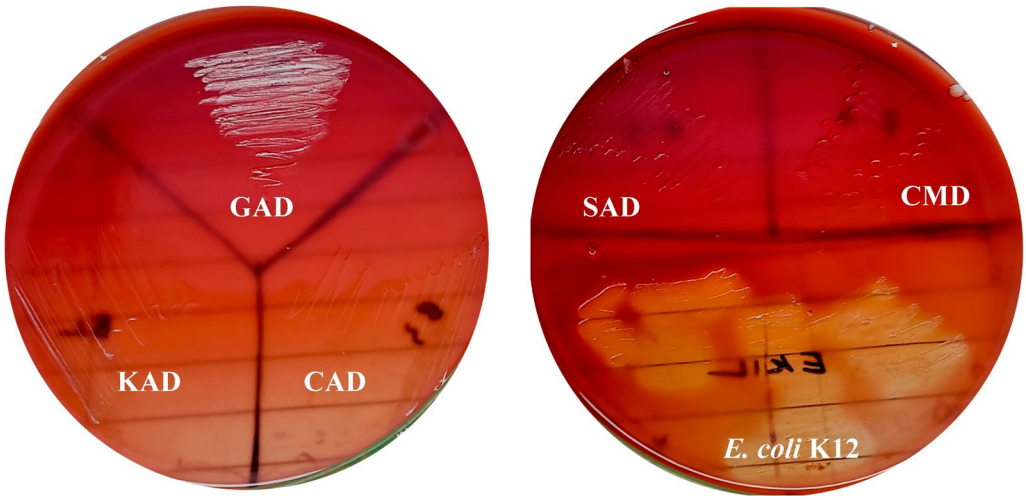

Fig. 7 Hemolytic activity assessment of isolated five LAB strains (compared with hemolysis-positive *E. coli* K12)

vancomycin, streptomycin, kanamycin; isolate CAD showed resistance against all antibiotics except oxacillin and tetracycline; isolate SAD showed resistance against chloramphenicol, clindamycin, streptomycin, and tetracycline; isolate CMD showed resistance against ampicillin, cephalothin, oxacillin, vancomycin, kanamycin, and tetracycline (Table 2; Additional file 1: Table S1 for the antibiotic susceptibility assessment of LABs in terms of zone of inhibition).

In case of hemolytic activity, all the five LAB isolates displayed a pattern of gamma hemolysis which indicates lack of hemolysis as in this case there should be no reaction in the surrounding medium, whereas the *E. coli* K12 strain showed beta hemolysis (Fig. 7).

In vivo acute toxicity test

After oral treatment of a predetermined high dosage of bacterial suspension (6×10^9 CFU/Kg body weight) of each isolate to separate male and female mice, there was no mortality or side effects noted during the first 24 h. During the subsequent 14-day observation period in each group, there was no mortality or deviation in the way the animals appeared or behaved or changes in their feeding habits or body weight gain (Additional file 1: Fig. S2).

Discussion

All five LAB isolates have primary probiotic attributes like gastro-tolerant activities, tolerance to alkaline pH which may enable them to survive in the harsh intestinal microenvironment [38]. Their ability to utilize multiple carbon sources enhances their persistence in conditions like food deprivation or dietary changes. Furthermore, the capacity of LAB isolates to auto and co-aggregate with similar and other bacterial strains provides protection against external pressures, including host immune attacks, ensuring prolonged persistence in the GI tract [39]. In the present study, all LAB isolates showed BSH-positive results, indicating their capability to degrade conjugated bile salts, contributing to the lowering of serum cholesterol levels [24]. Notably, the LABs' production of deconjugated bile salts suggests reduced reabsorption and increased excretion of free bile acids in the stool. Additionally, all the LAB isolates exhibited cell surface enzyme characteristics needed for the breakdown of proteins, lipids, and carbohydrates. This may be linked to improved food digestibility and nutritional quality associated with health benefits of consuming these fermented foods.

Phylogenetic analysis of these isolates revealed that they group with the members of the family *Lactobacillaceae.* Such bacteria are known for producing short-chain fatty acids (SCFAs) which are reported to have beneficial effects on physiological and metabolic homeostasis of mammals [40–43]. These LAB isolates also demonstrated potent antimicrobial properties against common pathogens, producing organic acids, bacteriocins, and hydrogen peroxide to resist the growth of pathogenic microbes [44–46]. The ability of LAB isolates to bind to the intestinal mucosa enhances their survival and growth in the GI tract. Higher CSH% (cell surface hydrophobicity) indicates strong adhesion abilities and competitive exclusion of other microbes, fostering interactions with bacterial communities in the GI tract [47]. Moreover, CSH activity determines the ability of cells to attach and separate from surfaces. A probiotic strain's intestinal colonization, or adherence and persistence, is measured by the hydrophobicity of its cell surface. High hydrophobicity indicates more colonization capability

[48]. The LAB isolates also fulfil these criteria by showing higher CSH% values. In respect with the of the antidiabetic activity, the isolated LAB displayed significant inhibitory activity against α-glucosidase and α-amylase enzymes, which can potentially slowdown glucose absorption and reduce postprandial blood sugar levels. This highlights their potential as therapeutic hypoglycemic agents as well as hyperglycemia-preventive supplements. Regarding antibiotic resistance, some LAB isolates showed resistance to vancomycin, streptomycin, and kanamycin [49]. Moreover, lack of hemolytic activity of the isolates demonstrated their safety. The results from acute toxicity tests reaffirmed the safety of these LAB isolates, establishing their status as "Generally Recognized as Safe" [50].

Conclusion

This investigation highlights the probiotic attributes of five LAB isolates, namely *P. pentosaceus* isolate GAD, *L. plantarum* isolate KAD, *L. plantarum* isolate CAD, *L. brevis* isolate SAD, and *L. plantarum* isolate CMD. All the five isolates show potential for probiotic applications and as naturally derived pharmaceutical agents, owing to their gastro-tolerance, metabolic activities, adhesion capabilities, antimicrobial properties, and hypoglycaemic effects. These findings support their safety as health-beneficial LABs. However, further *in vivo* investigations are essential to validate and establish their suitability to be used as dietary supplements.

Abbreviations

BSH	Bile salt hydrolase
CLSI	Clinical and Laboratory Standards Institute
CSH	Cell surface hydrophobicity
GI	Gastrointestinal
LAB	Lactic acid bacteria
LS	Laboratory strains
MATS	Microbial adhesion to solvents
MHA	Mueller Hinton Agar
MRS	De-Man, Rogosa, and Sharpe
OD	Optical density
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate buffer saline
SCFA	Short-chain fatty acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43094-024-00630-4.

Additional file 1. Fig. S1 and S2 for the results of gastro-inhibitory parameters and influence on host body weight respectively. Fig. S3 for the results of alkaline tolerance of the isolates. Table S1 for the antibiotic susceptibility assessment of LABs in terms of zone of inhibition.

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Author contributions

TS conceptualized and supervised the project. AJG and SG performed the experiments, and prepared the first draft of the original manuscript. TS, MDC, AJG, and SG analyzed and interpreted the data and curated the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The information created and analyzed during the current investigation is available upon reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate

The *in vivo* animal experimentation was approved by the Institutional Animal Ethical Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the University of North Bengal in West Bengal, India (Vide ref no. IAEC/NBU/2022/27 Dated. 23.09.2022).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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