Probiotic potential of lactic acid bacteria from fresh vegetables: Application in food preservation

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Fresh vegetables are potential source of lactic acid bacteria (LAB). In the present study, LAB were isolated from the fresh vegetables from Pune region. Total 266 LAB were isolated from the edible parts of fresh vegetables viz. cauliflower, gherkins, cluster beans, fenugreek, cow pea, bitter gourd, french beans, tomato, ridged gourd, cucumber and bottle gourd. On phenotypic and molecular characterization predominant genera obtained were Lactobacillus, Enterococcus and Weissella. Twenty one isolates exhibited tolerance to bile salt, acidic pH and pancreatin. Cellular extracts of several isolates with ability to survive in artificial intestinal condition additionally showed antioxidant potential and cell free supernatants exhibited antibacterial potential against selected plant and human pathogens. Bacteriocin and bacteriocin like substances (BLS) substances secreted by these isolates can be used for food preservation.

Keywords: Antioxidant activity, Antibacterial activity, Bacteriocins, LAB

The probiotics which are commercially available and routinely used for human consumption are obtained from the conventional sources, such as dairy products, human feces and human breast milk1. Isolation of lactic acid bacteria (LAB) from nonintestinal and nondairy sources can offer better health advantages2. Probiotics originating from fruits and vegetables may suit the gut environment of vegans more than those from different sources. LAB have been earlier isolated from different vegetables and fruits3 and nondairy fermented products4. Vegetable surface and phyllosphere is a natural habitat of many bacteria and LAB colonize on some vegetable and plants predominantly5. Vegetables provide nutrients, such as vitamins, minerals, high carbohydrate content and acidic microenvironment which favours the growth of LAB. Indigenously fermented vegetables have been a good source of Leuconostoc mesenteroides, Lactobacillus plantarum, Enterococcus sp., Leuconostoc lactis, Lactobacillus pentosus, Weissella hellenica, Weissella cibaria, Lactobacillus plantarum and Lactobacillus brevis which have been isolated from cabbage, cucumbers and carrots4. LAB from unconventional natural sources may provide novel, industrially important and potential strains.

LAB as whole cells and/or their antimicrobial compounds may be used for food preservation for enhancing the quality of food6. Health awareness and diet consciousness has increased the demand of fruits and fresh vegetable products all over the world. Fresh cut fruits and vegetables are in more demand due to balance diet recommendations. Process of farming, harvesting, cutting, packing and handling make these fresh vegetables more susceptible to microbial infection caused by Escherichia coli, Salmonella and several other bacteria7. Bacteriocin and bacteriocin like substances (BLS) produced by LAB found antagonistic towards closely related bacteria and pathogens8.

Different compounds, such as organic acid, ethanol, hydrogen peroxide, bacteriocin and short antimicrobial peptides secreted by LAB found application in increasing shelf life of food9. Bacteriocin produced by LAB is ribosomally synthesized antimicrobial peptides playing functional role in the food industry10.

Food preservation with biological methods is being preferred over the chemical methods due to the
hazardous effects of chemical methods on both consumers and the environment. Chemical pesticides are biomagnified in the consumers through the consumed plant material\textsuperscript{11}. LAB as plant probiotics could be an alternative for chemicals (fertilizers and pesticides) to improve the plant quality without negative health effects\textsuperscript{12}. LAB and some of their bioactive compounds (organic acid, bacteriocin) can be used in food preservation due to their ability to suppress microbial growth or due to their antimicrobial potential. Use of LAB not only controls the infection and spoilage but also increases nutritional value of the food.

Fresh fruits and vegetables get damaged by different food pathogens such as \textit{Pseudomonas} spp., \textit{Listeria monocytogenes}, etc.\textsuperscript{13-15}. Trias \textit{et al.}\textsuperscript{15} evaluated antimicrobial activity of eighteen LAB isolated from fresh vegetables and fruits against foodborne human pathogens (\textit{E. coli}, \textit{Listeria monocytogenes}, \textit{Pseudomonas aeruginosa}, \textit{Salmonella typhimurium} and \textit{Staphylococcus aureus}). Similar results of decrease in growth of \textit{Pseudomonas} sp., yeast and total coliforms were recorded in fresh-cut salads; inoculated with \textit{Lactococcus lactis} and \textit{Enterococcus faecium}\textsuperscript{14}. Selected strains of LAB inhibited the growth of \textit{L. monocytogenes} on wounded golden delicious apples and iceberg lettuce leaves\textsuperscript{15}. These studies open new avenues to explore LAB in biopreservation of fresh cut vegetables and fruits.

In the present study, LAB strains were isolated from the fresh vegetable source and were screened for probiotic potential and antimicrobial and antioxidant potential. One of the potent strain \textit{Lactobacillus plantarum} AG40V was tested for biopreservation ability on fresh vegetables and sprouts.

**Materials and Methods**

Dehydrated media were from HiMedia Laboratories (Mumbai, India). Other chemicals and reagents were from Thermo Fischer, India. LAB isolates were maintained on MRS (de Man Rogosa and Sharpe) agar for regular use. Reference strains \textit{Lactobacillus plantarum} MCC 2156 and other bacteria \textit{viz.}, \textit{Klebsiella pneumoniae} MCC2570, \textit{Staphylococcus epidermidis} MCC2044, \textit{E. coli} MCC2079, \textit{Enterobacter cloacae} MCC2303, \textit{Pseudomonas aeruginosa} MCC2081, \textit{Ralstonia solanacearum} NAIMCC-B-00419, \textit{Xanthomonas campestris} NAIMCC-B-00496, \textit{Erwinia carovotora} NAIMCC-B-00295 were obtained from Microbial culture collection, National Centre for Cell Science (NCCS), Pune, India and National Agriculturally Important Microbial Culture Collection (NAIMCC), Uttar Pradesh, India were maintained on nutrient agar. Long term preservation of all the bacteria was done in glycerol (20% w/v) at ultralow temperature \(-80^\circ\text{C}\).

**Isolation of lactic acid bacteria**

The fresh vegetable samples like cauliflower, gherkins, cluster beans, fenugreek, cow pea, bitter gourd, french beans, tomato, ridged gourd, cucumber and bottle gourd were collected from the fields near Pune region. Three locations Junnar, Ambegaon and Khed were chosen target location for sampling based on the productivity. The edible part of selected vegetables was used for the isolation of LAB. In detail, 1.0 g of vegetable was minced and suspended in tube containing 1.0 mL sterile saline; the debris was allowed to settle briefly and the upper layer of saline was mixed with 15 mL of MRS agar (molten) and poured in a sterile Petri dish. In enrichment method, 1.0 g of vegetable minced into small pieces was suspended in flask containing 9 mL MRS broth\textsuperscript{3}. This broth was incubated at 30\textdegree C under static microaerophilic conditions for 48 h. The appropriate dilution was plated on MRS agar and incubated under same conditions as above. Morphologically distinct colonies were further streak on another media plate and selected for biochemical characterization.

**Identification of lactic acid bacteria**

The selected cultures were identified using metabolic fingerprints with Biolog; proteome analysis by MALDI-TOF and using 16S rRNA gene sequencing. For metabolic characterization by Biolog standard guidelines and protocol were followed (Biolog Inc., CA).

Molecular identification by partial 16S rDNA gene sequencing was done as described by\textsuperscript{16}. Genomic DNA isolation was done from freshly grown culture in exponential growth phase. DNA pellet obtained was washed with ethanol and re-suspended in Tris EDTA buffer. PCR amplification of partial 16S rDNA was done using domain bacteria specific universal primers\textsuperscript{17}. The primers 5'- AGAG TTTGATCTGCTGCTAG 3' and 5' GGACTACHVGGTWTCTAAT 3', was used in a PCR reaction with an annealing temperature of 57\textdegree C. The DNA sequencing reaction included Terminator Ready Reaction Mix (BigDye® Terminator v3.1 Cycle
Sequencing Kit, Applied Biosystems) (0.5 µL), BigDye® Sequencing Buffer (1.8 µL), One sequencing primer 806R (3.2 pmol), and template DNA (2 µL) with standard milliQ (SMQ) water (4.7 µL) to make up the volume of 10 µL. The cycle sequencing protocol was as follows: Initial denaturation at 96ºC for 1 min, followed by 25 cycles of 96ºC for 10 s, annealing at 50ºC for 5 s, and elongation at 60ºC for 4 min. The PCR amplified products were kept on rapid thermal temperature to 4ºC and hold until ready to purify. After amplification, products were purified by using a GeneO-spin PCR product Purification kit (GeneOmbio technologies, Pune; India). Purified PCR product was directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Bio systems, USA), 3130 Genetic Analyzer Automated DNA sequencing machine and analyzed using Sequencing Analysis and ChromasProv3.1. The sequences were analyzed using sequencing analysis software (5.2). BLAST analysis was performed at BlastN site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment using BLAST tool was performed to find closest match in Genbank database. Using BLAST it was confirmed that sequences belong to 16S rRNA gene. Phylogenetic tree was made with CLC sequence viewer 7.5 using the UPGMA method with Kimura 80 as nucleotide distance measure, bootstrap analysis was done with 1000 replicates.

Screening of LAB for probiotic properties

All the LAB were tested for acid, bile and pancreatin tolerance using turbidity as a growth measurement at 600 nm for percent viability. Selected isolates showing tolerance to artificial intestinal conditions were further confirmed by total viable cell count18.

Acid tolerance

For acid tolerance, 1% (v/v) of overnight culture (10^7 CFU/mL) was suspended in phosphate buffer saline (PBS) at pH 7.2 (control) and pH 3 (adjusted with 1N HCL) (acidic condition) and incubated anaerobically at 37C for 3 h. After incubation, cells were serially diluted in PBS and suitable dilutions were spread plated on MRS agar or inoculated in broth (1% v/v inoculum). Incubation was carried out under static microaerophilic conditions at 37C for 3 h. These cell suspensions were serially diluted in PBS and suitable dilutions were spread plated on MRS agar or inoculated in broth (1% v/v inoculum). Incubation was carried out at 37°C for 48 h under static microaerophilic conditions. After incubation, viable cell count was recorded and percent viability to bile salt was estimated by comparison of the viable count of test with control.

Pancreatin tolerance

For pancreatin tolerance LAB cells were suspended in PBS to a final concentration of 10^7 CFU/mL and were inoculated (1% v/v) in 5 mL of the freshly prepared pancreatin solution [PBS containing 150 mM NaHCO₃ and 1.9 mg/mL pancreatin (Sigma, USA), pH 8] and control solution (PBS, pH 7.2). The cultures were incubated anaerobically at 37C for 3 h. These cell suspensions were serially diluted in PBS and suitable dilutions were spread plated on MRS agar or inoculated in broth (1% v/v inoculum). Incubation was carried out at 37°C for 48 h under static microaerophilic conditions. After incubation, viable cell count was recorded and percent viability to acidic conditions was estimated by comparing the viable counts at pH 3.0 and pH 7.2.

Screening of LAB for antioxidant potential

The isolates showing the probiotic potential were further screened for additional beneficial properties like antioxidant activity19, 21. LAB were inoculated in MRS medium and incubated under microaerophilic condition at 37C for 32 h. Cell free supernatant (CFS) was obtained by centrifugation at 5000 g for 5 min at 4°C. The CFS was neutralized to pH 7.0 with NaOH (1M) and heated at 95°C for 5 min. The lipid oxidation reaction mixture was composed of ultrapure water, extra virgin olive oil (500 µL) with 100 µM ferrous sulphate (500 µL) and this was incubated in a water bath at 80°C for 10 min. 500 µL of CFS was added with 250 µL of sodium dodecyl sulphate (SDS, 81 mg/mL) buffered with acetic acid at pH 3.4 and 500 µL of thiobarbituric acid (TBA, 6mg/mL). The reaction mixture was incubated in a water bath at 100°C for 1 h. Quercetin (200 µM) was used as standard. The absorbance was measured at 532 nm. The results were expressed as mean ± standard deviation (SD) of three independent measurements (n=3). The
the concentration of TBARS was determined using a standard curve with 1,1,3,3-tetramethoxypropane and the results were expressed as nmol of malonaldehyde (MDA) per mL of the sample.

Screening of LAB for antimicrobial properties

The antimicrobial activity of LAB was determined against representative plant and human pathogens. The plant pathogens used were *Ralstonia solanacearum* (NAIMCC-B-00419), *Xanthomonas campestris* (NAIMCC-B-00496), *Erwinia carotovora* (NAIMCC-B-00295) and *Pseudomonas aeruginosa* (MCC 208). The human pathogens used were *Klebsiella pneumoniae* (MCC 2570), *Staphylococcus epidermidis* (MCC2044), *Escherichia coli* (MCC2079), *Enterobacter cloacae* (MCC 2303), *Citrobacter freundii* (MCC 2078) and *Bacillus cereus* (MCC 2039). The testing of antimicrobial activity was done according to the European Committee of Antimicrobial Susceptibility Testing guidelines (EUCAST, 2015). The active LAB cells (1×10⁷ CFU/mL) were inoculated in MRS broth which was then incubated at 30°C in microaerophilic condition for 38 h (log phase). Cell free supernatant (CFS) was obtained by centrifugation at 10000 rpm 4°C for 5 min and subsequent filtration through membrane filter (0.22 µm). Actively growing plant and animal pathogens (1×10⁷ CFU/mL) were seeded on Muller-Hinton agar which received CFS (100 µL) in agar wells of 7 mm diameter. The plates were incubated at 30 /37°C under aerobic conditions. The diameter of zone of inhibition was measured to check the antimicrobial activity. The assay was performed in triplicate and the mean diameter of zone of inhibition was determined from three independent measurements (n=3).

Antibiotic susceptibility of LAB

Antibiotic susceptibility test for the LAB strains was carried out using disc diffusion. The sensitivity of LAB was tested towards amikacin (30 µg), gentamicin (10 µg), cefepime (30 µg), ticarcillin (75 µg), piperacillin (100 µg), imipenam (10 µg), bacitracin (10 Units), chloramphenicol (30 µg), penicillin G (10 Units), polymyxin B (300 µg), gentamicin (10 µg) and neomycin (30 µg). All antibiotics disks were obtained from Himedia. LAB cells (1×10⁵ cells/mL) were spread plated on MRS agar and the antibiotic discs were placed; followed by incubation in microaerophilic condition at 37°C for 48 h. The zone of inhibition was measured and the LAB were expressed as susceptible (S), intermediate (I), and resistant (R) as per CLSI standards.

Prevention of spoilage of cut vegetables/sprouts by LAB

The spoilage prevention ability of LAB on the fresh cut vegetables and sprouts was tested. 5×10⁵ cells/mL of freshly grown *Lactobacillus plantarum* AG40V were re-suspended in sterile distilled water (D/W) and cell free supernatant (CFS) was sprayed on the inner sides of the container and the wrap film and dried in laminar air flow under sterile conditions. The vegetables were washed with sterile D/W, air dried and placed in the container coated with LAB cell suspension whereas control was sprayed with sterile D/W. The containers were wrapped with the LAB coated wrap and kept at room temperature for 72 h. The indigenous microbial growth was enumerated from test and control samples every after 0, 24, 48 and 72 h of incubation at room temperature. The densities of naturally occurring indigenous LAB, *Pseudomonas* sp., yeasts and molds, and total viable bacteria was enumerated using conventional culture methods. LAB were cultured anaerobically on MRS at 37°C for 48 h, *Pseudomonas* sp. on cetrimide agar (Himedia) at 30°C for 48 h; yeast and molds on potato dextrose agar (Himedia) at 30°C for 48 h and standard plate count agar (SPC; Himedia) at 37°C for 24 h was used for enumeration of total viable bacteria. The effect of LAB on the vegetables and sprouted mung were observed for microbial growth and decay. The experiment was performed in triplicate with fresh salad and sprouts (n=3).

Results and Discussion

**Distribution of lactic acid bacteria on vegetables**

Fresh vegetable samples were collected from the fields of three regions namely Junnar, Ambegaon and Khed talukas of Pune district. From the edible parts of fresh vegetables, total 266 LAB were isolated using the conventional methods. Fig. 1 (A, Junnar; B, Ambegaon and C-Khed) shows the number of LAB isolates obtained from different vegetables. In Junnar, maximum number of isolates were obtained from french beans (21 isolates) and cow pea (21 isolates); followed by cauliflower and gherkins (17 isolates each) and from cluster beans 15 isolates while least number of isolates were obtained from cucumber (8 isolates). From Ambegaon, highest number of LAB were obtained from gherkins (17), followed by cauliflower (16 isolates), fenugreek
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(10 isolates), tomato (10 isolates) and cabbage (10 isolates). From Khed, maximum number of isolates were obtained from tomato (22 isolates) followed by the cauliflower (11 isolates) and least were from bitter gourd (7 isolates). The commonly preferred vegetables for LAB colonization appeared to be tomato, cauliflower and gherkins whereas bottle gourd and bitter gourd did not seem to be favourable for colonization of LAB. The difference in the count of lactic acid bacteria from each plant is due to the change in the nutritive composition of plant.

The isolates were identified by determination of their morphological and biochemical properties using BIOLOG AN Microplate® (Biolog, Inc., Hayward, CA, USA) system. Besides this, MALDI-TOF identification and 16S rRNA gene sequencing was done for confirmation of representative isolates. All the isolates were Gram positive, non-motile, catalase negative and oxidase negative. LAB isolates belonged to the genera Lactobacillus, Lactococcus, Pediococcus, Enterococcus, Leuconostoc and Weissella and others. The isolates under this study could be classified to the genera Lactobacillus, Enterococcus, Lactococcus, Pediococcus, Leuconostoc and Weissella. However, members of the genus Lactobacillus were predominant on the vegetables used in this study. Lactobacillus plantarum MCC2156 was used as a reference strain. The identity of the isolate was confirmed further by 16S rRNA gene sequencing. Table 14 shows the source and identification details of all the LAB isolates.

Fig. 2 is a cladogram showing phylogenetic relationship of the LAB isolates with other related genera based on the 16S rRNA gene sequences. Weissella sp. ID10V closely related to the Weissella halotolerans but it diverges differently from Leuconostoc and other LAB genera. Lactobacillus sp. LAB8V and J32V were closely related to L. plantarum and standard strain L. plantarum MCC2156. Although other Lactobacillus sp. diverged from L. plantarum MCC2156 but closely related to L. plantarum AG40V and other standard L. plantarum UIGOA137; only Lactobacillus sp. KG16V diverged from others. All isolates from the present study diverged differently from the genera Pediococcus clearly showing that they were unrelated to the members of this genus. Enterococcus sp. ID17V was closely related to Enterococcus hirae and Enterococcus sp. ID19V, ID11V and ID6V were closely related to Enterococcus faecium.

Screening of isolates for tolerance to acid, bile and pancreatin

According to the guidelines of Food Safety Standards Act of India (FSSAI), ICMR–DBT and World Health Organization (WHO) for probiotics, the strains intended for use as probiotics should be confirmed for the tolerance to acidic pH, inhibitory bile salts and pancreatic enzymes. These parameters are considered as an indicator for the survival of bacterial strains in the gastrointestinal (GI) tract. Isolates exhibiting more than 75% tolerance to acid,
bile and pancreatin were selected for further study. Table 1 shows the tolerance of LAB isolates towards acid, bile and pancreatin respectively. *Lactobacillus plantarum* AG40V maintained high cell viability in presence of acid, bile and pancreatin. The cell viability of many lactic acid bacteria was at par with the standard probiotic strain MCC2156. Besides, several *Lactobacillus* isolates, *Enterococcus* sp. ID11V, *Enterococcus* sp. ID19V and *Weissella* sp. ID10V exhibited more than 80% tolerance to acid, bile and pancreatin. *Lactobacillus* sp. ID18V, *Enterococcus* sp. ID19V, *Lactobacillus* sp. AG1V, *Lactobacillus* sp. AG40V, *Lactobacillus* sp. LAB5V and *Lactobacillus* sp. J88V, *Lactobacillus* sp. ID12V, *Lactobacillus* sp. J131V, *Lactobacillus* sp. LAB 8V and *Lactobacillus* sp. J129V were more tolerant than other isolates. Seventeen isolates from different vegetables were superior in tolerance as compared to the standard *L. plantarum* MCC2156. Maximum number of isolates showing tolerance to gastric conditions were obtained from the leafy vegetable fenugreek; followed by gherkins, cauliflower and tomato. *Weissella* sp. ID 10V isolated from ridged gourd also exhibited potential tolerance to the gastric conditions.

**Screening of lactic acid bacteria for bioactive properties**

All the LAB strains were further selected for antioxidant properties. Antioxidant potential of LAB isolates was screened by their ability to scavenge reactive oxygen species particularly those involved in lipid peroxidation with the intention of using these strains for food preservation.

**Antioxidant potential of lactic acid bacteria**

Lipid oxidation in foods limits the shelf life and compromises the palatability of many food products. The foods rich in lipids, sea foods, meat, etc. are more susceptible to spoilage; besides many emulsified...
foods face a similar problem leading to rancidity and off flavours. Lipid oxidation can be prevented by antioxidants, thus probiotic bacteria with ability to prevent lipid oxidation can be useful in enhancing the shelf life of lipid-rich foods. Besides this, LAB with antioxidant potential can be helpful in alleviating inflammation and other pathological or disease conditions where reactive oxygen species (ROS) are known to aggravate the symptoms. ROS have been implicated in cancer, Alzheimer's disease, Parkinson's disease, aging and several other diseases.

The inhibition of lipid peroxidation was recorded as decrease in concentration of MDA Fig. 4. The isolates showing more than 50% reduction in MDA content as compared to MRS which showed a potential inhibitors of lipid peroxidation. L. plantarum AG40V, Lactobacillus sp. LAB5V, Lactobacillus sp. ID2V, Lactobacillus sp. J23V, Lactobacillus sp.
LAB4V, Lactobacillus sp. ID12V. Enterococcus sp. ID8V, Enterococcus sp. ID11V, Enterococcus sp. ID19V and Weissella sp. ID10V displayed the potential efficacy to inhibit lipid peroxidation. Weissella sp. ID10V and Enterococcus sp. ID11V from ridged gourd and fenugreek, respectively displayed maximum ability to inhibit lipid oxidation.

**Antimicrobial activity of lactic acid bacteria**

In addition to successful passage through gastric condition, every potential probiotic strain is also expected to show antagonistic activity against pathogens. LAB are well known for production of antimicrobial substances like bacteriocins, organic acids and short chain fatty acids. Table 2 and 3 shows the antimicrobial activity of LAB against plant and human pathogens.

CFS was used to test the antibacterial activity of the isolates against the selected plant pathogens. Table 2 shows the extent of growth inhibition

![Fig. 4—Antioxidant activity of Lactic acid bacteria (n=3)](image)

<table>
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<tr>
<th>Source</th>
<th>Genus</th>
<th>Code</th>
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<th>Ralstonia solanacearum</th>
<th>Pseudomonas aeruginosa</th>
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<td>J129V</td>
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</table>

[Results expressed as mean diameter of zone of inhibition of three readings (n=3)]
recorded for common plant pathogens. *Erwinia carovotora* is a well-known plant pathogen, a causative agent of bacterial rot and infects several vegetables and plants like tomato, cucumber, lettuce, potatoes and several others (Bhat et al., 2010). *L. plantarum* AG40V, *Lactobacillus* sp. ID12V and *Enterococcus* sp. ID8V, *Enterococcus* sp. ID11V potentially inhibit *E. carovotora*. Besides this, *Weissella* sp. ID10V also inhibited *E. carovotora*. *X. campestris*, known to cause black rot, was inhibited by *L. plantarum* AG40V, *Enterococcus* sp. ID8V, *Enterococcus* sp. ID11V, *Lactobacillus* sp. J129V and ID13V and *Weissella* sp. ID10V. Members of the genus *Enterococcus* were better in their antimicrobial activity against *Xanthomonas* than others. *R. solanocearum*, the causative agent of brown rot and several other plant diseases was inhibited by *L. plantarum* AG40V, *Lactobacillus* sp. ID12V, *Enterococcus* sp. ID11V and *Weissella* sp. ID10V. *P. aeruginosa* was inhibited by *Lactobacillus* sp. J129V, *Enterococcus* sp. ID11V and *Weissella* sp. ID10V. It was observed that isolates from fenugreek and tomato were superior in their antimicrobial potential. Representative human pathogens namely *E. coli*, *K. pneumoniae*, *S. epidermidis*, *B. cereus*, *Citrobacter freundii* and *Enterobacter cloacae* were used as test strains to determine antimicrobial activity (Table 3). *Lactobacillus* sp. J129V exhibited antimicrobial activity against *E. coli*, *K. pneumoniae*, *S. epidermidis*, *B. cereus* and *E. cloacae*. Isolates from tomato, *Lactobacillus* sp. J129V and *Lactobacillus* sp. ID12V were more potent inhibitors of human pathogens. Isolates of the genus *Enterococcus* did not inhibit *E. coli* and few isolates like ID7V, ID8V, LAB8V and ID18V of the genus *Lactobacillus* did not inhibit *K. pneumoniae*. *Enterococcus* sp. ID8V and

![Table 3 — Antibacterial activity of LAB against representative human pathogens](https://example.com/table3.png)

<table>
<thead>
<tr>
<th>Source</th>
<th>Genus</th>
<th>Code</th>
<th>Antimicrobial activity against Human pathogens Zone of inhibition in (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>NCCS</td>
<td><em>Lactobacillus</em></td>
<td>MCC-2156</td>
<td>-</td>
</tr>
<tr>
<td>Bitter Gourd</td>
<td><em>Lactobacillus</em></td>
<td>LAB8V</td>
<td>-</td>
</tr>
<tr>
<td>Cabbage</td>
<td><em>Lactobacillus</em></td>
<td>LAB6V</td>
<td>10-14</td>
</tr>
<tr>
<td>Cabbage</td>
<td><em>Lactobacillus</em></td>
<td>AG31V</td>
<td>15-19</td>
</tr>
<tr>
<td>Cabbage</td>
<td><em>Lactobacillus</em></td>
<td>KG28V</td>
<td>-</td>
</tr>
<tr>
<td>Cauliflower</td>
<td><em>L. plantarum</em></td>
<td>AG40V</td>
<td>10-14</td>
</tr>
<tr>
<td>Cauliflower</td>
<td><em>Lactobacillus</em></td>
<td>LAB5V</td>
<td>10-14</td>
</tr>
<tr>
<td>Cauliflower</td>
<td><em>Lactobacillus</em></td>
<td>J88V</td>
<td>15-19</td>
</tr>
<tr>
<td>Cluster bean</td>
<td><em>Lactobacillus</em></td>
<td>ID13V</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Cluster Bean</td>
<td><em>Lactobacillus</em></td>
<td>J36V</td>
<td>-</td>
</tr>
<tr>
<td>Fenugreek</td>
<td><em>Lactobacillus</em></td>
<td>ID2V</td>
<td>-</td>
</tr>
<tr>
<td>Fenugreek</td>
<td><em>Enterococcus</em></td>
<td>ID8V</td>
<td>-</td>
</tr>
<tr>
<td>Fenugreek</td>
<td><em>Enterococcus</em></td>
<td>ID11V</td>
<td>-</td>
</tr>
<tr>
<td>Fenugreek</td>
<td><em>Lactobacillus</em></td>
<td>ID18V</td>
<td>-</td>
</tr>
<tr>
<td>Fenugreek</td>
<td><em>Enterococcus</em></td>
<td>ID19V</td>
<td>-</td>
</tr>
<tr>
<td>Fenugreek</td>
<td><em>Lactobacillus</em></td>
<td>AG1V</td>
<td>15-19</td>
</tr>
<tr>
<td>French Beans</td>
<td><em>Lactobacillus</em></td>
<td>J23V</td>
<td>&gt;20</td>
</tr>
<tr>
<td>French beans</td>
<td><em>Lactobacillus</em></td>
<td>J87V</td>
<td>15-19</td>
</tr>
<tr>
<td>Gherkins</td>
<td><em>Lactobacillus</em></td>
<td>ID7V</td>
<td>15-19</td>
</tr>
<tr>
<td>Gherkins</td>
<td><em>Lactobacillus</em></td>
<td>LAB4V</td>
<td>15-19</td>
</tr>
<tr>
<td>Ridged gourd</td>
<td><em>Weissella</em></td>
<td>ID10V</td>
<td>15-19</td>
</tr>
<tr>
<td>Ridged gourd</td>
<td><em>Lactobacillus</em></td>
<td>J122V</td>
<td>-</td>
</tr>
<tr>
<td>Tomato</td>
<td><em>Lactobacillus</em></td>
<td>ID12V</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Tomato</td>
<td><em>Lactobacillus</em></td>
<td>J131V</td>
<td>-</td>
</tr>
<tr>
<td>Tomato</td>
<td><em>Lactobacillus</em></td>
<td>J129V</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

[Results expressed as mean diameter of zone of inhibition of three readings (n=3)]
Enterococcus sp. ID11V exhibited maximum activity against C. freundii, a causative agent of several opportunistic and nosocomial infections. Interestingly both isolates are from fenugreek. The isolates from tomato and fenugreek seem to display better antimicrobial potential against human pathogens also as was found for plant pathogens. The antimicrobial activity observed in the study indicates the potential of the isolate in bacteriocin or bacteriocin like substance production. These bacteriocins can be used as safe alternative for vegetable preservation and to prolong the shelf life.

Detection of antibiotic susceptibility of LAB

Newly isolated strains before use in the probiotic fortified foods should be known for their sensitivity pattern against different antibiotics. The susceptibility of isolates to different antibiotics was assessed by antibiotic sensitivity testing as per the criteria of FSSAI. In the present study antibiotic sensitivity pattern of three strains was done as these were the best performer representing the genus. Lactobacillus plantarum AG40V, Enterococcus sp. ID11V and Enterococcus sp. ID19V were tested against different antibiotics to determine their sensitivity. Antibiotics used for sensitivity study were amikacin, gentamicin, cefepime, ticarcillin, piperacillin, imipenam, bacitracin, chloramphenicol, penicillin G, polymyxin B, gentamicin and neomycin. Depending on the mean of the diameter of zone of inhibition, the results were expressed as susceptible (S), intermediate (I), and resistant (R) as per Clinical and Laboratory Standards Institute CLSI standards24 as shown in Table 4.

Among all tested antibiotics Lactobacillus plantarum AG40V strain was sensitive to β-lactam antibiotics and chloramphenicol; this strain exhibited resistance mainly towards aminoglycoside group of antibiotics. Enterococcus sp. ID11V was sensitive to β-lactam antibiotics and aminoglycoside group antibiotics. Enterococcus sp. ID19V also exhibited sensitivity towards β-lactam group and aminoglycoside group except neomycin.

Prevention of infection/decay of cut vegetables/sprouts by Lactobacilli

We carried out a first approach of preservation of vegetables and sprouts in intact or cut forms to cater to the fast-food market. For the purpose of biopreservation of vegetables, we used LAB strain isolated from fresh vegetables Lactobacillus plantarum AG40V. The cut vegetables and sprouted mung beans were packed in food wraps and containers coated with Lactobacillus plantarum AG40V suspension post drying of the cells, and these containers were kept at room temperature (37-38°C). It was found that there was initiation of decay and infection after 24 h, in the control sample in which L. plantarum AG40V was not sprayed. In containers which were sprayed or coated with Lactobacillus AG40V, no spoilage was seen in vegetables until 48 h as (Fig. 4) and in sprouted mung beans [Fig. 5 (A-D)]. This indicates that L. plantarum AG40V can be a vital weapon for post-harvest biopreservation of vegetables and fruits thus increasing their shelf life. We have demonstrated that the antagonism shown by Lactobacillus prevents the proliferation of naturally associated pathogens.

L. plantarum AG40V when inoculated on the fresh cut opened tomatoes and lettuce inhibited the growth of naturally associated pathogens and other microflora like Pseudomonas sp., yeast and molds and total viable indigenous bacteria present naturally during 72 h storage at room temperature compared to controls as shown in Fig. 5 A-E, G and H, respectively. After 72 h, the yeasts and molds and total viable bacteria increased to 4.9 cfu/g and 2.83 cfu/g respectively on control samples; whereas in the test samples sprayed with L. plantarum AG40V. The count of yeasts and molds and bacteria was 2.15 cfu/g and 0.5 log cfu/g, respectively.

### Table 4—Antibiotic sensitivity testing of lactic acid bacteria

<table>
<thead>
<tr>
<th>Isolates</th>
<th>AK 30 µg</th>
<th>GEN 10 µg</th>
<th>CPM 30 µg</th>
<th>TI 75 µg</th>
<th>PI 100 µg</th>
<th>IPM 10 µg</th>
<th>B 10 Units</th>
<th>C 30 µg</th>
<th>P 10 Units</th>
<th>PB 300 µg</th>
<th>N 30 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus plantarum AG40V</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Enterococcus sp. ID11V</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Enterococcus sp. ID19V</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

[Amikacin (AK 30 µg) Gentamicin (GEN 10 µg) Cefepime (CPM 30 µg) Ticarcillin (TI 75 µg) Piperacillin (PI 100 µg) Imipenem (IPM 10 µg) Bacitracin (B 10 Units) Chloramphenicol (C 30 µg) Penicillin G (P 10 Units) Polymyxin B (PB 300 µg) Gentamicin (GEN 10 µg) Neomycin (N 30 µg). Susceptibility breakpoints are as per CLSI guidelines; ≥20 mm-sensitive (S); 15-19 mm Intermediate (I); ≤14 mm Resistant (R)]
The biopreservation ability of the *L. plantarum* AG40V was tested on other perishable food like mung bean sprouts (commonly known as matki). During the growth at 72 h the *Pseudomonas* sp., yeast and molds and total viable bacterial count increased to 4.35, 1.47 and 2.19 log cfu/g respectively in control that was not sprayed with *L. plantarum* AG40V; and the count of *Pseudomonas*, yeast and molds and total bacteria was 2.05, 1.21 and 1.04 cfu/g in test sample treated with *L. plantarum* AG40V. The strain found inhibitory on vegetables and sprouts as compared to non-treated control samples to prolong the shelf life.

No substantial decrease was observed in the LAB count in the test fresh to mato and lettuce as well as sprouts (Matki) treated with *L. plantarum* AG40V as shown in Fig. 5 B and F. Human or animal intestinal origin probiotics are widely studied whereas the probiotics from non-intestinal and non-dairy fermented foods are less explored. The plant phyllosphere is densely colonized by a complex and highly diverse microbial population exhibiting pronounced differences with respect to difference in plant species. One of the key influencing factors determining the type and density of microbes colonizing the plant phyllosphere is phytochemical concentration and composition. Diverse species of LAB, such as *Enterococcus munditii*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Lactococcus lactis* and *Streptococcus* sp. populated the plants but *Lactobacillus plantarum* stably dominated both epiphyte and endophyte throughout all the phonalological stages. In agreement with this, in the present study, *L. plantarum* was the predominant species isolated from fresh vegetables. Fresh vegetables harbor LAB and the predominant genera in the identification were found as *Lactobacillus*, *Enterococcus* and *Weissella*. LAB colonization appeared to preferably occur on tomato, cauliflower, fenugreek, and gherkins. Interestingly, all these vegetables are rich source of dietary fibers and antioxidants. The nutritional contents, plants secondary metabolites and phyllosphere provide favorable conditions for the survival and growth of LAB on plants.

One of the primary selection criteria for new probiotic functionality is its ability to survive and colonize in the stressful gut environment with tolerance to acidic pH, bile toxicity and pancreatic enzymes. LAB isolates in this study showed potential probiotic than the reference strain *L. plantarum* MCC2156. The isolates predominantly belonging to the genera *Lactobacillus* and *Enterococcus* were more tolerant and one isolate *Weissella* sp. ID10V had also exhibited significant tolerance to gastric conditions; reflecting their suitability of the plant originated isolates as a probiotic culture. These results are in accordance with the studies revealing the ability of the *Lactobacillus* strains isolated from dairy products to be viable when exposed to low pH. One study...
reports the probiotic potential of *Lactobacilli* isolated from fermented olives. In our study, *L. plantarum* AG40V exhibited significant viability when exposed to low pH and bile toxicity, whereas other research suggests about strong bile tolerance but considerably lower viability at acidic pH of *L. plantarum* isolated from spontaneous fermented wheat bran. The common resistance mechanisms in *Lactobacillus* species, to withstand the effect of bile acids on cell physiology, are found to be active bile efflux system, bile salt hydrolysis, changes in central metabolic pathways and changes in the design/composition of cell membrane and cell wall. Our results are in agreement with the previous study where majority of the *Lactobacillus* isolates obtained from traditional Greek dairy products and meat products were found to be highly resistant to bile salts even after 4 h of exposure. This shows that isolates from unconventional sources particularly non-intestinal isolates are equally potent in their probiotic properties as compared to the isolates of intestinal origin. Besides these basic probiotic abilities, isolates were also assessed for bioactive properties such as antioxidant activity and antagonism against human and plant pathogenic bacteria.

Lipid oxidation reduces nutritional value and causes development of rancidity in lipid rich foods. The antioxidant ability of CFS of *Lactobacilli* was determined for their possible applications in preservation of foods rich in fatty acids. It is noteworthy that antioxidant activity of LAB isolated from fenugreek was higher than LAB isolated from other vegetables used in the study. This can be attributed to their origin, where fenugreek is a rich source of dietary fibers and antioxidants. Fenugreek leaves contains different phytochemicals; saponins, alkaloids, flavonoids which helps to enhance its antioxidant capacity. Ultraviolet radiation has been reported to cause lipid peroxidation. In natural conditions due to presence of UV radiation in the sunlight, high oxidative stress might be generated as a result of lipid peroxidation and this may be the reason why LAB isolated from fenugreek might have evolved with maximum antioxidant potential in response to this increased oxidative stress. One of the study reports about high antioxidant ability of culture supernatant and intracellular extract of probiotic strain *Enterococcus durans* LAB18s. Our study showed similar results with respect to antioxidant properties of *Enterococcus* isolates.

LAB synthesizes different metabolites such as organic acids, hydrogen peroxide, diacetyl, ethanol, acetaldehyde, bacteriocins, etc. LAB having potent antimicrobial activity can be an effective alternative for antibiotics and can be useful in urogenital and gastrointestinal infection therapies. Probiotics also serve to enhance the intestinal barrier function through competitive exclusion and antagonism against pathogenic microorganisms. The antimicrobial activity of LAB isolates was tested against representative human pathogens such as *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Citrobacter freundii* and *Enterobacter cloacae*. Antimicrobial activity of *Enterococcus* sp. ID11V and ID19V was found to be higher amongst all *Enterococcus* isolates, whereas *Lactobacillus plantarum* AG40V, *Lactobacillus* sp. J129V, J23V and ID12V were found to be inhibitory when tested against the above mentioned representative human pathogens. *L. plantarum* AG40V and *Lactobacillus* sp. J129V showed maximum inhibitory activity against *K. pneumoniae*– one of the important nosocomial pathogen showing multi-drug resistant phenotype. Cell free supernatant of LAB from vegetable origin was found inhibitory against all the tested clinically important human pathogens.

In addition, antagonism was also tested against representative plant pathogens like *R. solanacearum*, *E. carotovora*, *X. campestris* and *P. aeruginosa*. These pathogens are responsible for crop damage ultimately resulting in severe economic loss. There is need of control on chemical pesticides in agricultural sectors. Chemical pesticides create negative effects on health and environment. It was observed that LAB isolated from fenugreek and tomato were superior in their antimicrobial potential. This study highlights their potential application as biocontrol agents against phytopathogens.

Several physical and chemical techniques are routinely used for preserving foodstuffs, agricultural products, and pharmaceuticals, etc. Biological materials, however, can be irreversibly damaged during these treatments. Therefore, it is essential to design protective agents to preserve food nutritional quality and texture. LAB being natural flora on vegetables; their prospective application for prevention of vegetable spoilage explores a new upcoming interest in the biopreservation technology. In the present study *L. plantarum* AG40V was tested for in vivo applicability in food preservation. This
isolate successfully reduced the growth of *Pseudomonas* sp., Yeast and molds during the storage at room temperature on fresh salads (tomato and lettuce) and mung bean sprouts (matki). Similar results were reported\textsuperscript{20}, when tested *Enterococcus faecium*, *Streptococcus thermophilus* and *Lactobacillus casei* on fresh-cut yellow onions. Another study with *Lactococcus lactis* and *Enterococcus faecium* on fresh-cut salads remarkable reduced the growth of *Pseudomonas* sp., yeasts and total coliforms\textsuperscript{43}. The usefulness of the LAB in preventing the spoilage of fruits, vegetables and other food products was also explored\textsuperscript{3,11}. Overall, this study indicates the applicability of the *Lactobacillus plantarum* AG40V to increase the shelf-life of ready-to-eat cut salads and sprouts besides conferring possible probiotic benefits as demonstrated in this study. This extends the application of the probiotic bacteria in different areas of food technology.

**Conclusion**

Vegetable surfaces provided an unconventional source of lactic acid bacteria. The lactic acid bacteria potentially survived in artificial intestinal conditions proving their suitability to be used as probiotics. These lactic acid bacteria successfully inhibited the plant and human pathogens indicating their future applications in food preservation.

**Acknowledgement**

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**Conflict of Interest**

The authors declare that there is no conflict of interest.

**References**


