

Functionality of Potential Probiotic Bacteria Isolated From Vegetables

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Abstract

This study aimed to identify and characterize the lactic acid bacteria having probiotic properties from non-dairy sources. Morphological and biochemical characterization of bacteria isolated from vegetables was performed. Isolates were examined for acid, bile, antimicrobial activity, thermotolerance and gas production. The best isolate was subjected to molecular identification. The extrusion technique was performed to encapsulate the strain and encapsulation efficiency was determined. Out of ten isolates only 101 showed significantly ($p < 0.05$) higher survival percentages at pH 3, no loss of viability at 0.3% and 0.7% bile salt and a diameter of inhibition zone 11.5 mm and 10.73 mm against *Salmonella* Typhimurium and *Staphylococcus aureus*, respectively. The best selected strain 101 was identified as a new strain and deposited as *Lacticaseibacillus rhamnosus* OUSLNIMADU accession no PX050959.1. A 91% encapsulation efficiency was reported. The study discovered that lactic acid bacteria isolated from carrot, exhibited functional properties that make them potential probiotics.

INTRODUCTION

Probiotics are live microorganisms that exert health benefits by improving the balance of gut bacteria and boosting the individual's health when administered in adequate amounts, (FAO/WHO, 2002). However, the term 'gut health' is increasingly used in research relating with probiotics. The International Scientific Association for Probiotics and Prebiotics (ISAPP) proposed a definition of gut health as "a state of normal gastrointestinal function without active gastrointestinal disease and gut-related symptoms that affect quality of life" could achieved by co-culturing healthy populations, including diet, probiotics, prebiotics and other biotic substances (Marco et al. 2026). The *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Weissella*, *Leuconostoc*, and *Pediococcus* genera are the most prominent members of this group of microorganisms. To be classified as a probiotic, bacteria must exhibit specific functional characteristics, such as a tolerance to gastrointestinal conditions, should not cause infections or diseases, ability to attach to epithelial cells, bile salt hydrolysis, resistance to antibiotics, survival during the processing and storage, and antimicrobial properties (Khan, 2014).

The primary source of probiotics consumed by humans is dairy products. Some bacteria species associated with non-dairy foods may possess strong probiotic properties. Therefore, there is a need to identify and characterize probiotic strains from "unconventional sources," or alternative sources such as fresh fruits, vegetables, and traditional fermented foods that can perform well in milk or non-milk-based products. The molecular identification and chemical characterization are the primary methods to identify LAB. The 16s RNA gene sequencing has been used to determine the genetic diversity of LAB associated with food using an array of genetic tools (Liu et al. 2026).

The probiotics derived from fruits and vegetables often contain various strains of LAB involved in various fermentation processes. *Leuconostoc mesenteroides* is frequently identified in tomatoes, whereas *Lactobacillus plantarum* has been recognized in many fruit juices, including citrus fruits (Naeem et al. 2012). Fresh fruits and vegetables such as guava, star fruit, dragon fruit, papaya, durian, ginger, and

carrots can be used to identify beneficial LAB that produce antimicrobial compounds like hydrogen peroxide, lactic acid, propionic acid, and bacteriocin. These LABs can inhibit pathogenic fungi and bacteria that lead to diseases in tomatoes (El-Mabrok et al. 2012).

Most food products containing probiotic bacteria in the market have a very short shelf-life was identified as the limitation. The viability of probiotics is compromised due to harsh conditions involved in food processing and long-term storage conditions. Moreover, the effects of bile salt, stomach acid, and enzyme secretions in the gastrointestinal tract after ingesting decrease the lifespan of probiotic bacteria. Therefore, improving the viability of probiotics under these conditions is important. For these improvements various strategies such as encapsulation can be used. Encapsulation is the covering of a material around the cells of microorganisms to trap and separate them from the environment. Which increase viability at different environmental conditions and, targeted release of probiotics at the appropriate time and location (Foroutan et al. 2017). Various encapsulation techniques such as extrusion, spray-drying, emulsion and phase separation can be used with alginate, Maltodextrin, chitosan, carrageenan, gums, gelatin, and whey protein and starch (Latif et al. 2023).

One of the common materials for encapsulating microorganisms is alginate developed from brown algae, or kelp, and is primarily made up of β -L and β -D guluronic acids (Bevilacqua et al. 2019). The extrusion procedure form bacteria with polymer droplets to gel and form beads to protect bacteria (Petraitytė and Šipailienė, 2019). Encapsulation improves survival of probiotics during food processing and protects them during passing through the human gastrointestinal tract. LAB isolated from non-dairy based sources and encapsulated probiotic viability were not much studied and reported as to dairy based isolates. The aim of this study was to identify and molecular characterization of lactic acid bacteria having probiotic properties from non-dairy sources to use as a starter culture.

MATERIAL AND METHODS

Sample Preparation and Isolation of LAB

Fresh vegetable samples Carrot (*Daucus carota L*), Beet, Gotukola(*Centella asiatica*), and Lettuce were collected. The sample preparation and isolation of LAB was conducted according to Schifano et al. (2021) with some modifications. Each vegetable (25 g Carrot, Beet, Gotukola, and Lettuce) were washed with distilled water and then these samples were homogenized by stomacher (BK- SHG04, China) and diluted in 225 ml of 0.85% saline solution. An aliquot of 100 μ l of sample was inoculated on MRSA (HiMedia Laboratories Pvt.Ltd, India) medium for 24 h at 37°C anaerobically in a plastic anaerobic jar. Then, morphologically different colonies were streaked on MRSA and incubated at 37°C for 24 h to isolate the purified strain. Again one single colony of each isolated strain was inoculated in MRS broth (HiMedia Laboratories Pvt.Ltd, India) for 24 h at 37°C anaerobically. After growth, bacterial stock was preserved in 40% glycerol and stored at -20° C.

Biochemical characterization of the isolate

Isolated bacteria were identified as LAB using morphological features; Gram staining and biochemical methods such as catalase and oxidase tests according to Rahayu and Setiadi (2023).

Evaluation of probiotic properties

Acid tolerance

Acid tolerance assay was done according to Foroutan et al. (2017) with slight modifications. Overnight grown cultures were centrifuged (Centurion, Scientific Ltd, UK) at 8000 rpm for 10 min to obtain cellular pellets. Bacterial pellets were re-suspended in 1 mL sterile 0.85% saline. MRS broth (9 mL) with pH 3 and 7 (control) were prepared separately and inoculated with previously prepared 1 mL of bacterial samples. After 3 h incubation, 1 mL of each sample was pipetted to 9 mL of sterile 0.85% saline solution to obtain dilution factor range from 10¹ up to 10⁵. Each dilution was cultured on MRS agar plate for 48 h and viable bacterial colonies were counted.

Bile tolerance

Bile tolerance test was done according to Foroutan et al. (2017) with slight modifications. One single colony of isolated bacterial strains were inoculated into 2 mL MRS broth and incubated overnight. Then cultures were centrifuged at 8000 rpm for 10 min using the laboratory centrifuge to obtain cellular pellets. Bacterial pellets were re-suspended in 1 mL sterile 0.85% saline. Prepared bacterial samples were placed in tubes containing 9 mL of MRS broth containing the 0.3% and 0.7% concentrations of Bile salt (Oxoid Ltd., UK). Each sample was incubated for 8 h at 37°C and after 8 h incubation, 1 mL of aliquot was serially diluted with saline and inoculated onto MRS agar plate and incubated for 48 h under anaerobic conditions.

Antimicrobial activity

Antibiotic susceptibility was determined by disc diffusion method according to the protocol suggested by Weerakkody et al. (2011). Antimicrobial activity assays was carried out using the disk diffusion method with *Escherichia coli* ATCC1858, *Salmonella* Typhimurium, and *Staphylococcus aureus* ATCC 25923 bacteria.

Thermo tolerant capacity determination

According to the methods described by Anderson and Johnston (1991) the thermotolerant capacity of isolated LAB strains was assessed in water bath at various temperatures.

Gas formation from glucose

MRS broth (9 mL) media were prepared with glucose (1%) and Durham tubes were inserted into each tube. Tubes were inoculated with 1 mL overnight fresh LAB cultures. Tubes were kept at 37°C for 5 days. MRS broth containing no LAB inoculation was used as the control.

Molecular characterization of the isolate

Total DNA was extracted from a well grown 101 isolate and 5 µl of extracted DNA was subjected to the Polymerase chain reaction (PCR) using 27F/1492R primers. Amplified DNA was subjected to DNA sequencing using the same primers, and the obtained DNA sequences were compared with already existing DNA sequences in the National Center for Biotechnology Information database.

A total of 21 sequences of 16S rRNA gene including 8 closely related sequences (maximum score $\geq$ 1273), 10 sequences from other known diverse *Lacticaseibacillus rhamnosus* strains, and 3 outgroup sequences (*Lactobacillus casei*: NR_041893.1, *Lacticaseibacillus zeae*: NR_037122.1 and *Lacticaseibacillus chiayiensis*: NR_179712.1) were accessed from GenBank for the phylogenetic analysis (supplementary data). The sequences along with the unknown sequence, were aligned using ClustalW multiple alignment program with default parameters in BioEdit. The alignment was manually edited by truncating all the sequences to the size of the smallest sequence (1,006 bp). Final alignment was saved in PHYLIP format for downstream analysis. DNADist Distance Matrix program of the PHYLIP software package (version 3.63) was used to calculate genetic distances. Phylogenetic tree was constructed using DNADist Neighbor-Joining method (version 3.63) with 3 outgroup species and the tree was drawn with PhyloDendron – Phylogenetic Tree Printer Online Software (<http://iubioarchive.bio.net/treeapp/treeprint-form.html>, accessed on 04th January, 2025) Sequence identity matrix of all sequences were also computed using BioEdit (Version 7.0.1).

Statistical support for the resulted tree was given by bootstrapping 100 times using the SEQBOOT program (version 3.63). DNADist Distance Matrix program (version 3.63) was used to calculate genetic distances in multiple datasets. Multiple dendrograms were recreated with DNADist Neighbor-Joining method (version 3.63) using the generated bootstrapped data.

Encapsulation of probiotic strain using extrusion technique

The extrusion technique was used to encapsulate probiotic strain according to Sohail et al., (2011). A single colony was taken from the isolated bacterial culture and inoculated into 100 mL of MRS broth, which was then incubated for 24 h at 37°C. After being centrifuged for 10 min at 10,000 rpm, the cells were harvested and twice cleaned with sterile 0.1% peptone. Cells were suspended in 5 mL of sterile 0.1% peptone (Sisco Pvt. Ltd., India), which was mixed with 45 mL of sterile 2% (w/v) sodium alginate solution (Himedia, India). The suspension was injected with a disposable needle (0.70×38 mm) into a sterile 0.1 M CaCl₂ solution. The macro beads were immersed in a calcium chloride solution for 30 min before being washed with a 0.1% peptone solution and stored at 4°C until tested.

Determination of encapsulated cell number

Freshly prepared 1g of alginate microbeads were used to determine the encapsulated cell number. Encapsulated bacteria were broken down in a 2% (w/v) sterile sodium citrate solution at pH 6.0. The breakdown process was carried out by using laboratory sterile homogenizer. Then dilutions of the breakdown samples were prepared, dilution factors ranged from 10^{-1} up to 10^{-5} . Each dilution was plated onto MRS agar plates in 100 μ L aliquots for counting. The plates were then kept in anaerobic conditions for 24h at 37°C and the number of recovered colonies was counted.

Determination of encapsulation efficiency

The encapsulation efficiency was calculated using the following Equation:

$$EY = (N / N_0) * 100$$

N_0 represents the log number of viable cells in CFU/g of a wall material on a dry basis, while N represents the log number of viable cells in CFU/g of macrocapsules (Rajam and Anandharamakrishnan 2015)

Statistical analysis

Minitab statistical software was used to perform a variance analysis of the general linear model using independently triplicate data, using a 5% significance level. Tukey's simultaneous test was used to compare means at $P < 0.05$ level.

RESULTS AND DISCUSSION

Biochemical characterization of the isolate- Gram staining, catalase and oxidase test

Gram positive feature is a common characteristic of LAB. The Gram staining test indicated that the isolates belong to the group of Gram-positive bacteria. Gram staining helps to differentiate bacteria based on the characteristics of their cell walls. The identified cells exhibited a rod shaped morphology.

The catalase test finds out whether bacteria contain the catalase enzyme or not. When H_2O_2 is dripped onto the isolate, the absence of bubbles signifies a negative test outcome. This demonstrates that the catalase enzyme is absent from bacteria. Catalase enzyme converts hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O + O_2$) giving bubbles. The oxidase test is a biochemical process designed to detect the existence of cytochrome oxidase, an enzyme also known as indophenol oxidase when cytochrome C oxidase is present in bacteria, they become dark purple or blue in color. The isolates in our study showed negative for oxidase production, indicating that the isolated bacteria do not contain oxidase enzyme.

Out of stained 10 isolates, six isolates 101, 102, 104, 107, 109, and 110 stained in purple. In Gram staining purple color resemble for gram-positive bacteria while four colonies 103, 105, 106, and 108 stained in pink which represents the gram-negative bacteria. Gram differences and both Catalase and Oxidase tests results are shown in Table 1. All the strains did not show air bubbles in catalase test and not stained in purple color in oxidase test.

Table 1
Catalase and Oxidase test results of isolates of lactic acid bacteria

Isolate codeGram stain Catalase & Oxides		
101	gram-positive	Negative
102	gram-positive	Negative
103	gram negatives	Negative
104	gram-positive	Negative
105	gram negatives	Negative
106	gram negatives	Negative
107	gram-positive	Negative
108	gram negatives	Negative
109	gram-positive	Negative
110	gram-positive	Negative

Evaluation of probiotic properties- Acid Tolerance, Bile Tolerance, Antimicrobial activity and Thermo-tolerant capacity

Acid, like Hydrochloric acid (HCl) in the human stomach, is a strong oxidant; according to Gómez Zavaglia et al. (2002) the ability to withstand the extreme acidity of our stomach is a crucial requirement for an effective probiotic. To consider good probiotic sources, a minimum pH of 3.0 resistances should be exhibited (Fernández et al. 2003). The pH of 3.0 and 3h incubation period were chosen as the threshold parameters to assess acid resistance in this *in vitro* study to mimic residence time in the stomach (Prasad et al. 1998). Further, findings from Liong and Shah (2005) also claimed that a probiotic culture's ability to withstand acid is measured by its resistance at pH 3. The present study showed that isolated strains from carrot and Gotukola (101, 107, and 110) showed growth after 3h of incubation on MRSA showing relatively resistant to acid pH 3. However, three isolates, 102, 104, and 109 did not grow after 3 h of incubation, indicating that pH 3 killed while count remains relatively stable at pH 7. Since each isolate differs in its capacity for survival in the acidic pH conditions of the stomach, they all have differing viability. According to Azat et al. (2016), Following incubation at pH 3.0, the population density

of LAB having potential probiotics features must be greater than 10^6 CFU/g, which is consistent with the identified strains 101, 107, and 110.

The survivability of different LAB strains to acidic conditions pH 3 and 7 is presented in the Table 2. There was a significant different resistant on pH variations. Out of three LAB isolated strains, 101 showed the significant highest resistance at low at pH 3.0 after 3 h of incubation compared to stains 107 and 110. The strain 101 showed significantly highest resistance ($p < 0.05$) 0.52 log CFU/mL reduction at pH 3 compared to counts at pH 7 for 3 h. However, strains 102, 104, and 109 did not viable under highly acidic condition pH 3.

Table 2
Bacterial survival at the end of 3h of acid tolerance test

pH value	Total plate counts (log CFU/mL)					
	101	102	104	107	109	110
7	9.58 ± 0.06 ^{a,x}			6.63 ± 0.05 ^{z*}	6.17 ± 0.23 ^z	8.96 ± 0.05 ^{b,y}
3	9.06 ± 0.06 ^{a,x}	ND	ND	ND	6.59 ± 1.12 ^{c,z}	8.18 ± 0.03 ^y
						8.82 ± 0.05 ^{b,y}

One of the primary factors taken into consideration during the *in vitro* selection process to find probiotic bacteria is the microorganisms' capacity to live in bile salt conditions. The mean intestinal bile concentration is nearly 0.3% (w/v) with some variations, and four hour stay in the intestine (Prasad et al. 1998). The effect of the bile salt on the viability of the isolated strains is presented in Table 3. Tested strains 101 and 110 showed significant tolerance to bile. Isolated strain 101 did not show any significant ($p > 0.05$) loss of viability for 0.3% and 0.7% bile salt after 8h incubation period. However, strain 110 showed significant different viability loss at 0.7% bile compared to the control and 0.3% bile.

Table 3
Survival of isolated strains in MRS Broth supplemented with 0%, 0.3%, and 0.7% Bile salt

Bile salt (w/v)	Isolate code	
	Total plate counts (log ₁₀ CFU/ mL)	
	101	110
0%	8.39 ± 0.01 ^{a*}	8.12 ± 0.01 ^a
0.3%	8.36 ± 0.06 ^a	8.08 ± 0.02 ^a
0.7%	8.35 ± 0.01 ^a	7.91 ± 0.01 ^b
* Mean value with different superscripts in the same row differs significantly ($P < 0.05$)		

The control group in each trial had the highest growth rate at 0% bile. This validates the findings of Jensen et al. (2012), who found that some *Lactobacillus* species can withstand gastric juice without losing the viability. Similarly, Abriouel et al. (2012) found that, at 0.3% (w/v) bile salt, all LAB isolated from fermented olives could grow and survive. According to the results obtained from the Bile tolerance test, isolate 101 was selected to microencapsulation process.

Previous research examined the antibacterial efficacy of a few *Lactobacillus* strains against pathogenic bacteria, including *Staphylococcus aureus* ATCC 25212, *Escherichia coli* EC 108 LMHAESA, *Listeria innocua* LMHAE-LI 107, and *Escherichia coli* ATCC 25922. The DIZ of all LAB isolated strains tested against food borne bacteria is shown in Table 4. Isolate 101 showed a significantly ($p < 0.05$) higher DIZ 11.5 mm and 10.73 mm against *S. Typhimurium* and *S. aureus* ATCC 25925 respectively compared to other strains. None of the isolate strains showed any antimicrobial activity against *E. coli*. The Isolates 102 and 110 showed significantly ($p < 0.05$) lower inhibition (8.43 mm) against *S. Typhimurium* compared to strain 101. Two isolates, 101 and 110, were selected from the six examined isolates as they both showed higher inhibitory effects against *S. Typhimurium* and *S. aureus* irrespective of the gram positive or negative feature. Therefore, isolates 101 and 110 were selected for further testing to better characterize their probiotic properties.

Table 4
The antimicrobial activity of LAB isolates against some food-borne pathogenic bacteria as per the disc diffusion assay

Isolate code	Diameter of Inhibition Zone (mm)		
	<i>Escherichia coli</i>	<i>Salmonella Typhimurium</i>	<i>Staphylococcus aureus</i>
101	0	$11.5 \pm 1.32^{a*}$	10.73 ± 0.46^a
102	0	8.43 ± 0.01^b	0
104	0	0	0
107	0	0	0
109	0	0	0
110	0	8.40 ± 1.04^b	8.53 ± 1.50^b
* Means within a column with different superscripts are significantly different ($p < 0.05$).			

An essential prerequisite for bacterial development is temperature, and the range of temperatures that was chosen was meant to approximate the average body temperature of an individual. The LAB ability to survive within the chosen temperature range (37–70°C) may be an indication of their capacity to withstand the temperature of the human gut and food processing. The viability of probiotics throughout the storage is a crucial aspect in determining their effectiveness; hence this factor plays a major role in determining probiotic functionality. In this investigation, the isolated 101 and 110 showed abundant

survival (> 300 CFU/mL) after 60 min of heating at 70°C, demonstrating an inherent thermotolerance potential.

Surprisingly, there was no gas production observed in 101 isolate confirming another probiotic feature.

Molecular characterization of the isolate

L. rhamnosus related species was identified by 16S rRNA sequencing, which classified under the *Lacticaseibacillus* genus.

The sequencing of the strongest potential probiotic properties having LAB isolate 101 was compared with Gen Bank data using the BLAST program on the NCBI website (<http://www.ncbi.nlm.nih.gov>), showing a maximum similarity level of 93.42% but not 99% with the *Lacticaseibacillus rhamnosus* strain KX057502.1. The phylogenetic tree based on the 16S rRNA gene sequence is shown in Fig. 1. Further, 101 strain genome was compared with other related *Lacticaseibacillus rhamnosus* (OQ117234.1, OL434981.1, OM877488.1, KX057502.1, and MZ836778.1) and developed the phylogenetic tree to identify the related species. However, the 16S rRNA gene sequences obtained in this study exhibited to similarity to above reference species 91.30%, 91.48, 90.94%, 93.42% and 93.24% of sequences in NCBI. According to the Fig. 1, not even one species showed 99% similarity level to 101 isolated strain. Therefore, it can be concluded that 101 was confirmed as the new species and new addition to the NCBI database and deposited as *Lacticaseibacillus rhamnosus* OUSLNIMADU accession no PX050959.1.

Many food formulations, health foods, and functional foods use specific strains of the *L.rhamnosus* species as probiotics (Kalliomaki et al. 2001). One of the best-researched probiotic bacteria is *L. rhamnosus* strain GG (Gorbach–Goldin), which was first isolated from adult, healthy human feces samples (Moslem et al. 2017). Adhering to the intestinal epithelial cells, *L. rhamnosus* is able to survive and grow through the gastrointestinal tract. When contrasted with comparable strains like *L.johnsonii* LJ1 and *L. casei* Shirota, *L. rhamnosus* strain has demonstrated excellent mucus adhesion (De Champs et al. 2003).

Encapsulation of probiotic strain and the efficiency

The extrusion method was employed for the encapsulation process. This method involves forcing a liquid or semi-liquid mixture containing probiotic bacteria through a small opening or nozzle to form capsules. The size of the capsules is a crucial factor, particularly for oral delivery of probiotics. The identified *L. rhamnosus* strain OUSLNIMADU was encapsulated and formed Alginate macrobeads. The capsules average diameter ranged between 1 and 1.5 mm and showed 91% encapsulation efficiency.

The capsules developed in this study were in the range of 1-1.5 mm, which aligns with the extrusion method to encapsulate probiotic bacteria and achieved 1.2 mm capsules by Martin-Dejardin et al. (2013). This consistency in size is important for dosage and delivery purposes.

Surprisingly, Alginate beads displayed a high encapsulation yield (91%) compared *Bifidobacteria* encapsulated in whey protein which had an effectiveness of roughly 25.57% (Picot and Lacroix, 2004).

However, Tülek et al. (2025) reported that using *Ceratonia siliqua* L. fruit containing carob flour as a natural carrier material resulted in encapsulation efficiencies of $79.51\% \pm 0.36\%$ for *L. rhamnosus* and $74.98\% \pm 0.20\%$ for *L. paracasei*.

Both the free and encapsulated forms of the non-dairy isolate *L. rhamnosus* OUSLNIMADU contributed to a probiotic drink with pleasant acidity after fermentation effectively in dairy matrices, supporting its potential use as a starter culture for dairy products (Dissanayake et al. 2026)

In this study, among the different strains isolated from vegetables, isolate 101 isolated from carrot was confirmed for a new strain belonging to *L. rhamnosus*. The new strain showed promising probiotic properties, such as acid and bile tolerance, antimicrobial susceptibility, heat tolerance, no gas production from the glucose with encapsulation efficiency. This strain possesses properties of potential probiotic and can consider a new addition to the probiotic strain collection.

Declarations

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Author contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ayesha Madushani and Judy Ranaweera. The first draft of the manuscript was written by Ayesha Madushani and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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Competing interests:

the authors declare no competing interests

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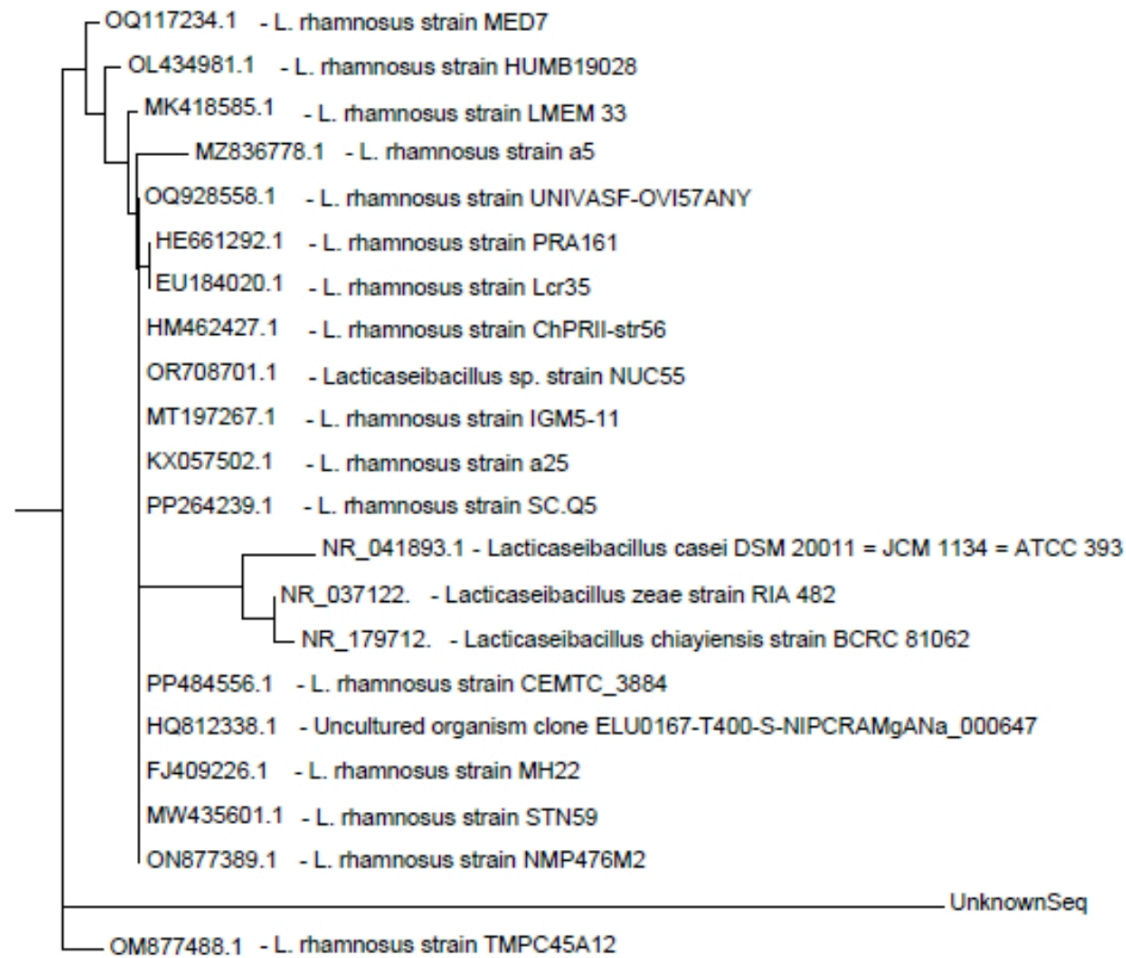
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Figures

Phylogenetic tree



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Figure 1

Phylogenetic tree for the related *L. rhamnosus* species