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# Isolation and Characterization of Lactobacillus Species From Local Indonesian Cow's Milk

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**Abstract**

**Probiotics are a group of beneficial microorganisms that can improve the health of their host. *Lactobacillus* species are lactic acid bacteria that have great potential as probiotics, in which cow's milk is a great source of lactic acid bacteria. In this study, *Lactobacillus* spp. were isolated from fresh local Indonesian cow's milk. The methods used to characterize the *Lactobacillus* spp. were (i) biochemical tests including catalase, hemolytic and sugar fermentation tests; (ii) tolerance tests against salt (NaCl 2%, 4% and 6%), low pH (2, 3, 4, 5, 6 and 7) and temperature (7°C, 37°C and 45°C); and (iii) 16S rRNA sequencing. The isolation yielded 14 isolates matching the criteria of *Lactobacillus* spp. colony and cell (i.e., Gram positive rods that did not produce endospores and did not have a waxy layer covering its cell wall). Based on subsequent biochemical tests, 5 isolates were suspected as potential probiotic *Lactobacillus* spp. The 16S rRNA sequencing revealed that the isolate L was *Limosilactobacillus fermentum*. In conclusion, this study demonstrated that local cow's milk could be used to isolate *Lactobacillus* spp.**

**Keywords: Biochemical identification, Lactic acid bacteria, *Limosilactobacillus fermentum*, Probiotics, 16S rRNA sequencing.**

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## Introduction

Humans have had a long history of consuming probiotic bacteria within fermented food and beverages, such as kimchi, sauerkraut, as well as dairy products, such as yogurt and kefir. Probiotics are microorganisms that could have a positive impact on their host if consumed in adequate amounts (Hill *et al*., 2014). Probiotics are measured in colony forming units (CFU), of which many products deliver in the range of 1-10 billion CFU/dose. However, the general optimum dose of probiotics is not available because the optimum dose varies greatly, depending on the strain and product (World Gastroenterology Organisation, 2017). Most probiotics available in today's market are members of a group called lactic acid bacteria, which, as the name suggests, are bacteria that able to ferment lactose and produce lactic acid, thus affecting a products texture, taste and aroma (Mithun *et al*., 2015).

Cow's milk is abundant in minerals, vitamins and micronutrients that are beneficial to human health. In addition, it is a rich source of lactic acid bacteria. Lactic acid bacteria found in cow's milk are in the genus of *Lactobacillus*, which is commonly used in the dairy industry as starter cultures to produce cheese and yogurt. *Lactobacillus* spp. from cows' milk can aid in maintaining the health of the human body, most notably in the wellbeing of the digestive system (Wu *et al*., 2022). *Lactobacillus* spp. can balance the microbiome of the digestive system and prevent dysbiosis, which has been linked to digestive system's dysfunction and inflammatory bowel diseases, such as Crohn's disease (Troche *et al*., 2020). *Lactobacillus* spp. can inhibit the growth of pathogenic bacteria within the gastrointestinal tract as well (Halder *et al*., 2017; Nemska *et al*., 2019).

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Due to the many health benefits, it is of interest to isolate *Lactobacillus* spp. from cow's milk. Hereby, this study was performed to isolate *Lactobacillus* spp. in fresh local Indonesian cow's milk, obtained from the Zhafira Farm, Ciracas, Jakarta.

## Materials and Methods

### Materials

Peptone saline water (0.1%) was made by dissolving 1 gram of peptone and 8.5 grams of NaCl in 1,000 mL and was used to dilute cow's milk. De Man, Rogosa, and Sharpe/MRS (Liofilchem, Italy) broth was used to test bacterial tolerance against different concentration of NaCl as well as various pH or temperature. The MRS agar (i.e., a mixture of MRS broth and 1.5% of bacteriological agar (Merck, USA) was used to support the growth of lactic acid bacteria derived from diluted cow's milk. The blood agar was made by mixing the blood agar base (Liofilchem, Italy) with 5% of sheep erythrocytes and was used to perform hemolytic test. Phenol red carbohydrate broth was made by mixing peptone, beef extract, NaCl, 0.25% of phenol red, distilled water as well as individual sugar (glucose, fructose, mannose, galactose, lactose, maltose, sucrose, mannitol or sorbitol) and was used to perform sugar fermentation test.

### Collection of cow's milk samples

The cow's milk was obtained from Holstein Friesian dairy cow raised at the Zhafira Farm at Jalan Raya Poncol, Ciracas, East Jakarta. While the fat content of Holstein Friesian cow's milk varies between 3.71% and 4.02%, its protein content varies between 2.94% and 3.08% (Sudrajat *et al*., 2021). The cow's milk samples were freshly collected and stored in sterile falcon tubes. The samples were subsequently transported to the lab in a cool box with ice packs and were tested on the same day.

### Isolation and purification of *Lactobacillus* species

The cow's milk samples were diluted at $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ in the 0.1% sterilized peptone saline water. The 50 µL of diluted samples were spread onto the MRS agar and incubated in a microaerophilic environment at 37°C for 48 hours. Purification of the colonies were performed using the 3-way streak method on a new MRS agar. The samples were incubated in a microaerophilic environment at 37°C for 48 hours. This step was performed multiple times until a single colony was obtained (El Kahlout *et al*., 2018).

### Bacteria morphological identification

Colony morphological identification was conducted by observing the shape, margin, color, elevation and size of the colonies, whilst the cell morphology's identification was done through Gram staining, endospore staining and acid-fast staining (Herbel *et al*., 2013).

### Biochemical tests

Catalase activity test was done using 3% hydrogen peroxide (Ogodo *et al*., 2022). Sugar fermentation test was performed by using glucose, fructose, mannose, galactose, lactose, maltose, sucrose, mannitol or sorbitol individually. Phenol red was used to indicate the pH for it was red at a pH of 7.4 and changed to yellow at a pH of <6.5. The bacteria's capability to produce gas as a fermentation product was detected using a Durham tube. While a lactose medium inoculated with *Escherichia coli* was used as a positive control, an uninoculated medium was used as a negative control.

### Resistance tests against various conditions

The NaCl resistance test was done by incubating 1% isolate sample in MRS with varying concentrations of NaCl (2%, 4% and 6%) at 37°C for 18 hours. The low pH resistance test was done by exposing 1% isolate sample in MRS with varying pH (2, 3, 4, 5, 6 and 7) at 37°C for 18 hours. Temperature resistance test was done by incubating 1% isolate sample in MRS at varying temperature (7°C, 37°C and 45°C). Once the incubation period in microaerophilic environment was concluded, the bacteria concentration was calculated by measuring the turbidity with a spectrophotometer at a wavelength of 600 nm (Anindita *et al*., 2017).

### Molecular identification using the 16S rRNA sequencing

Analysis through the 16S rRNA sequencing was done on one isolate by PT. Genetika Science Indonesia (Tangerang, Banten). Briefly, the genomic DNA was extracted with Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The polymerase chain reaction was performed with (2x) My Taq HS Red Mix (Bioline, BIO-25048) using universal primers 27F and 1492R, which spans nearly full-length of 16S rRNA gene, with an expected amplicon of ~1,400 base pairs. The 16S rRNA sequencing for isolate L was performed with ABI PRISM 3730xI Genetic Analyzer (Applied Biosystems, USA) using BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The partial sequence of 16S rRNA was subsequently processed with Sequence Scanner Software v.2 (Applied Biosystems, USA) and BioEdit (Ibis Therapeutics, USA).

The partial 16S rRNA sequence of isolate L was compared to reference RNA sequences (refseq rna) of NCBI GenBank database by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). In addition, the 16S rRNA sequence of isolate L was also compared against reference RNA sequences of *Limosilactobacillus fermentum* (taxid: 1613), *Lactobacillus delbrueckii* subspecies *lactis* (taxid: 29397) and *Lactobacillus acidophilus* (taxid: 1579). The phylogenetic tree was created based on partial 16S rRNA sequences of isolate L and various *Lactobacillus* spp. by using the MEGA software version 11 (https://www.megasoftware.net/). Briefly, the sequences were aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE). The phylogenetic tree was predicted using Maximum Likelihood (ML) statistical method, implemented using the Generalized Time Reversible (GTR) substitution model, and mutation rates and pattern were estimated using Gamma distribution with Invariant site (G+I), with discrete gamma categories was set to 5. The tree topology was searched using the Subtree-Pruning-Regrafting Extensive branch swap filter was set to none and statistical significance was measured from 500 replications.

## Results and Discussion

This study was performed to isolate *Lactobacillus* spp. in fresh milk obtained from Holstein Friesian dairy cow. Upon incubation of diluted cow's milk samples for 48 hours on the MRS agar plate, 30 colonies were chosen based on their initial appearance that matched the colony's characteristics of *Lactobacillus* spp. (i.e., round, white with an entire margin and raised/convex elevation; Figure 1) and subsequently purified via the 3-way streak on the MRS agar plate to obtain single colonies. According to Bergey's Manual and Brachter (2018), colonies of *Lactobacillus* spp. are round, 2-5 mm with an entire margin, convex or raised elevation, a smooth and glistening surface, as well as opaque in color. Upon purification, only 19 colonies, among the 30 chosen colonies, with size of 2-3 mm matched the colony's characteristics of *Lactobacillus* spp. The characteristics of those 19 candidates can be seen in Table 1. The cell morphological identification revealed that 14 of those 19 isolates were Gram positive bacteria with a rod morphology, endospore negative and acid-fast negative. This finding was consistent with the characteristics of *Lactobacillus* spp.

### Biochemical Tests

The results of biochemical tests are displayed in Table 2. The catalase testing revealed that all 14 isolates were catalase negative indicated by a lack of bubbles produced by the bacteria when exposed to hydrogen peroxide. *Lactobacillus* spp., as well as all other lactic acid bacteria, indeed do not produce any catalase enzyme due to its ability to survive without oxygen (Martinez *et al.*, 2014).

**Table 1**. Characteristic of isolated colonies

| Name | Shape | Margin | Color | Surface | Elevation |
|---|---|---|---|---|---|
| **A** | Round | Entire | White | Smooth & Glistening | Convex |
| **B** | Round | Entire | White | Smooth & Glistening | Convex |
| **C** | Round | Entire | White | Smooth & Glistening | Raised |
| **D** | Round | Entire | White | Smooth & Glistening | Raised |
| **E** | Round | Entire | White | Smooth & Glistening | Convex |
| **F** | Round | Entire | White | Smooth & Glistening | Raised |
| **G** | Round | Entire | White | Smooth & Glistening | Convex |
| **H** | Round | Entire | White | Smooth & Glistening | Convex |
| **I** | Round | Entire | White | Smooth & Glistening | Convex |
| **J** | Round | Entire | White | Smooth & Glistening | Raised |
| **K** | Round | Entire | White | Smooth & Glistening | Convex |
| **L** | Round | Entire | White | Smooth & Glistening | Convex |
| **M** | Round | Entire | White | Smooth & Glistening | Convex |
| **N** | Round | Entire | White | Smooth & Glistening | Convex |
| **O** | Round | Entire | White | Smooth & Glistening | Raised |
| **P** | Round | Entire | White | Smooth & Glistening | Convex |
| **Q** | Round | Entire | White | Smooth & Glistening | Raised |
| **R** | Round | Entire | White | Smooth & Glistening | Convex |
| **S** | Round | Entire | White | Smooth & Glistening | Convex |

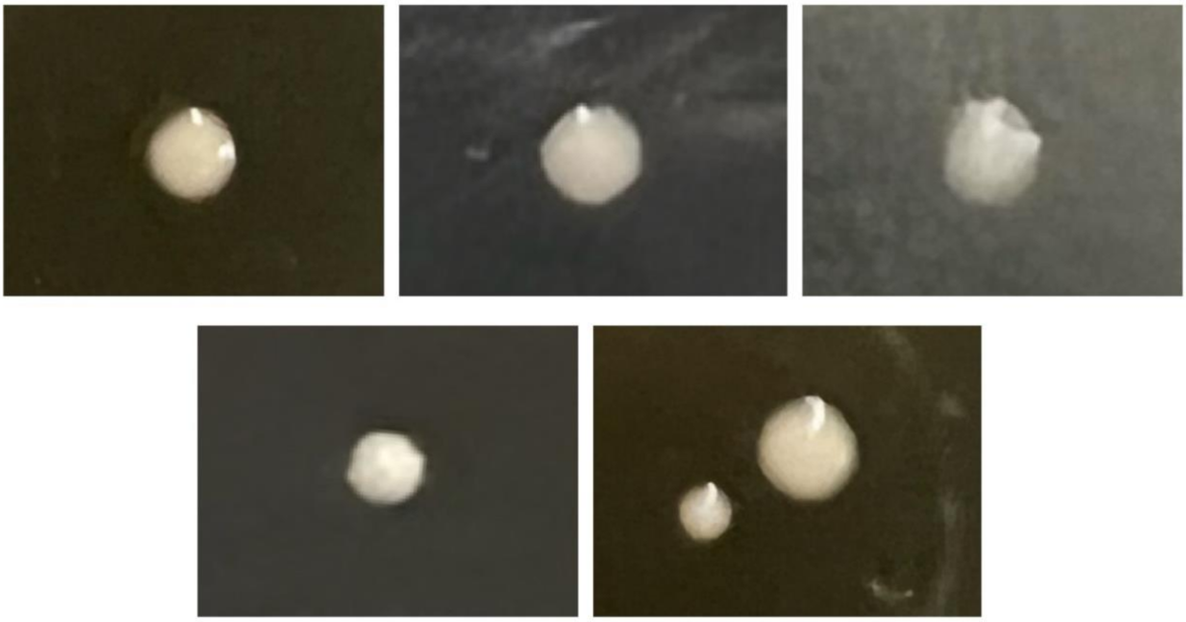

**Figure 1**. A representative figure of various colonies exhibiting colony’s characteristics of *Lactobacillus* spp. Of note, 19 colonies demonstrated characteristics of *Lactobacillus* spp. in this study.

Hemolytic testing revealed that 4 of the 14 isolates had alpha hemolytic characteristics. This result was in line with the findings of Goldstein *et al*., (2015), revealing that *Lactobacillus* spp. were alpha hemolytic bacteria. Isolate N, P, R and M did not indicate any hemolytic activity, thus was categorized as gamma hemolytic. In contrast, isolate L displayed alpha hemolytic capability as indicated by the green zone formation around the colonies. Of note, Owusu-Kwarteng *et al*. (2015) as well as García *et al*. (2017) had suggested that *L. fermentum* as alpha-hemolytic bacterium, hence the isolate L was likely to be *L. fermentum*.

Sugar fermentation test was done by using individual sugar, including monosaccharides (glucose, fructose, mannose and galactose), disaccharides (lactose, maltose and sucrose) and sugar-alcohols (mannitol and sorbitol). If the bacteria were able to ferment the sugar, the pH of the broth will decrease and the color will change due to the production of lactic acid (Borriss, 2020). Results of the sugar fermentation tests are displayed in Table 2. According to Ludwig *et al*. (2015), *Lactobacillus* spp. fermented a variety of sugars, including amygdalin, cellobiose, galactose, lactose, maltose, mannitol, mannose, melibiose, raffinose, salicin, sucrose, trehalose, arabinose, esculin and xylose. In addition, Rizkinata *et al*. (2018) had also isolated *Lactobacillus delbrueckii* from local cow's milk, in which this isolate had exerted the ability to ferment glucose, fructose, lactose, maltose, sucrose, mannitol and sorbitol. The results were compared to the Bergey's Manual of Systematic Bacteriology and were predicted to be *Lactobacillus* spp. Most notable of the isolates were N, P and R (suspected to be *L. acidophilus* or *L. delbrueckii* subspecies *lactis*), M (suspected to be *L. delbrueckii* subspecies *indicus*) and L (suspected to be *L. delbrueckii* subspecies *lactis* or *L. fermentum*). These five isolates were selected for subsequent testing.

## Tolerance Tests

Sodium chloride is an inhibitory substance that could prevent the growth of some bacteria. Table 3 showed that the isolates isolated from local cow's milk could withstand 2% and 4% of NaCl with strong (OD > 0.5) to moderate (OD > 0.3) tolerance. All isolates showed weak tolerance to 6% of NaCl with an OD of 0.1 – 0.2. These results were in line with the Bergey's Manual as well as with the findings of Rizkinata *et al*. (2018) and Sharafi *et al*. (2015), which reported that *L. delbrueckii*, *L. fermentum* and *L. acidophilus* were able to grow in medium with NaCl of 1 – 4%, but experienced weak to no growth at the concentration of NaCl above 5%.

According to Bergey's Manual and Abdel-Daim *et al*. (2013), *Lactobacillus* spp. were able to withstand a low pH of 2 – 4. Studies done by Liu *et al*. (2022) as well as Jose *et al*. (2015) stated that *Lactobacillus* spp. could tolerate and grew at pH of 3, but their viability decreased at pH of 2. In this study, the isolates were inoculated to MRS broth with pH ranging from 2 – 7. The results supported the aforementioned findings, in which while the isolate N, M and L experienced very weak growth, the isolate R and P showed no growth at the low end of the pH range.

According to Bergey's Manual, lactic acid bacteria are categorized as mesophilic bacteria. Furthermore, *L. delbrueckii*, as well as *L. fermentum* and *L. acidophilus*, were able to grow at temperatures up to 45°C, but could not grow at a temperature of 15°C. According to Śliżewska & Chlebicz-Wójcik (2020), the optimum temperature for the growth of lactic acid bacteria ranged between 30 – 45°C. In this study, the isolates were incubated at temperatures of 7°C, 37°C and 45°C for 18 hours. As can be seen in Table 3, all isolates grew well at 37°C and at 45°C, but did not grow when incubated at 7°C.

**Table 2**. Biochemical tests on the selected fourteen isolates

| **Test \ Isolate** | | **B** | **D** | **E** | **G** | **J** | **K** | **L** | **M** | **N** | **O** | **P** | **Q** | **R** | **S** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Catalase** | | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Hemolytic** | | γ | γ | α | α | γ | α | α | γ | Γ | γ | γ | γ | γ | γ |
| **Sugar Fermentation** | **Glucose** | + | + | + | + | + | + | + | + | + | + | + | - | + | + |
| | **Mannitol** | +<br>gas | + | + | +<br>gas | - | + | + | - | - | - | - | + | - | - |
| | **Lactose** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | **Sucrose** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | **Mannose** | + | - | - | - | - | + | + | + | + | - | + | + | + | - |
| | **Galactose** | + | - | + | + | - | + | + | - | + | + | + | - | - | + |
| | **Sorbitol** | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | **Fructose** | + | + | + | +<br>gas | + | + | + | + | + | + | + | +<br>gas | + | +<br>gas |
| | **Maltose** | + | - | + | + | + | - | + | - | + | - | + | + | + | + |

Note: (-): negative; (+): positive; (+) gas: positive and produced gas; (γ): gamma hemolytic/no hemolysis; (α): alpha hemolytic/partial hemolysis. Each biochemical test was conducted once.

**Table 3**. Tolerance tests on the selected five isolates

| **Isolate** | **NaCl** | | | | **pH** | | | | | | **Temperature** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0%** | **2%** | **4%** | **6%** | **2** | **3** | **4** | **5** | **6** | **7** | **7°C** | **37°C** | **45°C** |
| **R** | +++ | +++ | +++ | + | - | - | ++ | +++ | +++ | +++ | - | +++ | +++ |
| **P** | +++ | +++ | +++ | + | - | - | ++ | ++ | +++ | +++ | - | +++ | +++ |
| **N** | +++ | +++ | +++ | + | + | + | + | ++ | +++ | +++ | - | +++ | +++ |
| **M** | +++ | +++ | ++ | + | + | ++ | + | ++ | ++ | +++ | - | +++ | +++ |
| **L** | ++ | ++ | ++ | + | + | + | ++ | ++ | +++ | ++ | - | +++ | +++ |

Note: (+++): tolerant, OD>0.5; (++): moderate, OD of 0.3-0.5; (+): weak, OD of 0.1-0.3; (-): negative, OD <0.1. Each tolerance test was conducted once.

## Molecular Identification

The 16S rRNA sequencing was conducted on one isolate to confirm its identity. As an arbitrary choice, isolate L was selected due to its similarity to either *L. delbrueckii* subspecies *lactis* or *L. fermentum*, in which both species were known to exert probiotic effect. The 16S rRNA sequencing revealed that isolate L was indeed *Limosilactobacillus fermentum*, previously known as *Lactobacillus fermentum* (Table 4, Table 5 and Figure 2).

*L. fermentum* is a lactic acid bacteria found in the digestive tract of mammals including humans. Commercially, *L. fermentum* has been used in the cheese-making industry as starter culture and in the making of sourdough bread. *L. fermentum* has been categorized as probiotics due to its ability to fight infection in the digestive tract, produce antioxidants (e.g., superoxide dismutase, glutathione peroxidase, catalase and nitric oxide), as well as exerts anti-inflammatory and immune-stimulatory properties (Sharma *et al*., 2014; Suo *et al*., 2016; Zhao *et al*., 2019). Studies had shown that the consumption of *L. fermentum* prevented infection of the respiratory and digestive tract among infants via the production of short-chain fatty acids that inhibited the growth of pathogens (Naghmouchi *et al*., 2020). Taken together, this study demonstrated an ability to isolate potential probiotics from local cow's milk.

**Table 4**. Top 10 BLAST results of 16S rRNA sequence of isolate L

| Description | Max Score | Total Score | Query Cover | E value | % Identity | Accession |
|---|---|---|---|---|---|---|
| *Limosilactobacillus fermentum* strain CIP 102980 16S ribosomal RNA, partial sequence | 2656 | 2656 | 100% | 0 | 100 | NR_104927.1 |
| *Limosilactobacillus fermentum* strain NBRC 15885 16S ribosomal RNA, partial sequence | 2652 | 2652 | 100% | 0 | 99.93 | NR_113335.1 |
| *Limosilactobacillus gorillae* strain KZ01 16S ribosomal RNA, partial sequence | 2501 | 2501 | 100% | 0 | 98.05 | NR_134066.1 |
| *Limosilactobacillus ingluviei* strain KR3 16S ribosomal RNA, partial sequence | 2324 | 2324 | 100% | 0 | 95.84 | NR_028810.1 |
| *Limosilactobacillus equigenerosi* strain NRIC 0697 16S ribosomal RNA, partial sequence | 2313 | 2313 | 100% | 0 | 95.7 | NR_041566.1 |
| *Limosilactobacillus gastricus* strain Kx156A7 16S ribosomal RNA, partial sequence | 2311 | 2311 | 100% | 0 | 95.69 | NR_029084.1 |
| *Limosilactobacillus fermentum* strain NCDO 1750 16S ribosomal RNA, partial sequence | 2276 | 2276 | 95% | 0 | 95.5 | NR_118978.1 |
| *Limosilactobacillus alvi* strain R54 16S ribosomal RNA, partial sequence | 2266 | 2266 | 100% | 0 | 95.15 | NR_118032.1 |
| *Limosilactobacillus mucosae* strain S32 16S ribosomal RNA, partial sequence | 2263 | 2263 | 100% | 0 | 95.08 | NR_024994.1 |
| *Limosilactobacillus balticus* strain BG-AF3-A 16S ribosomal RNA, partial sequence | 2174 | 2174 | 100% | 0 | 93.96 | NR_181344.1 |

Note: the partial sequence of isolate L was compared to reference RNA sequences (refseq_rna) of NCBI GenBank database by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

**Table 5**. Top 10 BLAST results of 16S rRNA sequence of isolate L against *Limosilactobacillus fermentum*, *Lactobacillus delbrueckii* subspecies *lactis* and *Lactobacillus acidophilus*

| **Description** | **Max Score** | **Total Score** | **Query Cover** | **E value** | **% Identity** | **Accession** |
|---|---|---|---|---|---|---|
| *Limosilactobacillus fermentum* strain CIP 102980 16S ribosomal RNA, partial sequence | 2656 | 2656 | 100% | 0 | 100 | NR_104927.1 |
| *Limosilactobacillus fermentum* strain NBRC 15885 16S ribosomal RNA, partial sequence | 2652 | 2652 | 100% | 0 | 99.93 | NR_113335.1 |
| *Limosilactobacillus fermentum* strain NCDO 1750 16S ribosomal RNA, partial sequence | 2276 | 2276 | 95% | 0 | 95.5 | NR_118978.1 |
| *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072 strain ATCC 12315 16S ribosomal RNA, partial sequence | 1755 | 1755 | 95% | 0 | 89.73 | NR_042728.1 |
| *Lactobacillus delbrueckii* subsp. *lactis* strain NBRC 102622 16S ribosomal RNA, partial sequence | 1744 | 1744 | 95% | 0 | 89.51 | NR_114168.1 |
| *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072 16S ribosomal RNA, partial sequence | 1744 | 1744 | 95% | 0 | 89.58 | NR_117076.1 |
| *Lactobacillus acidophilus* strain NBRC 13951 16S ribosomal RNA, partial sequence | 1742 | 1742 | 94% | 0 | 89.77 | NR_113638.1 |
| *Lactobacillus acidophilus* strain VPI 6032 16S ribosomal RNA, partial sequence | 1742 | 1742 | 94% | 0 | 89.77 | NR_117062.1 |
| *Lactobacillus acidophilus* strain JCM 1132 16S ribosomal RNA, partial sequence | 1742 | 1742 | 94% | 0 | 89.77 | NR_117812.1 |
| *Lactobacillus acidophilus* strain BCRC10695 16S ribosomal RNA, partial sequence | 1742 | 1742 | 94% | 0 | 89.77 | NR_043182.1 |

Note: the partial sequence of isolate L was compared to reference RNA sequences (refseq_rna) of *Limosilactobacillus fermentum* (taxid: 1613), *Lactobacillus delbrueckii* subspecies *lactis* (taxid: 29397) and *Lactobacillus acidophilus* (taxid: 1579) in the NCBI GenBank database by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

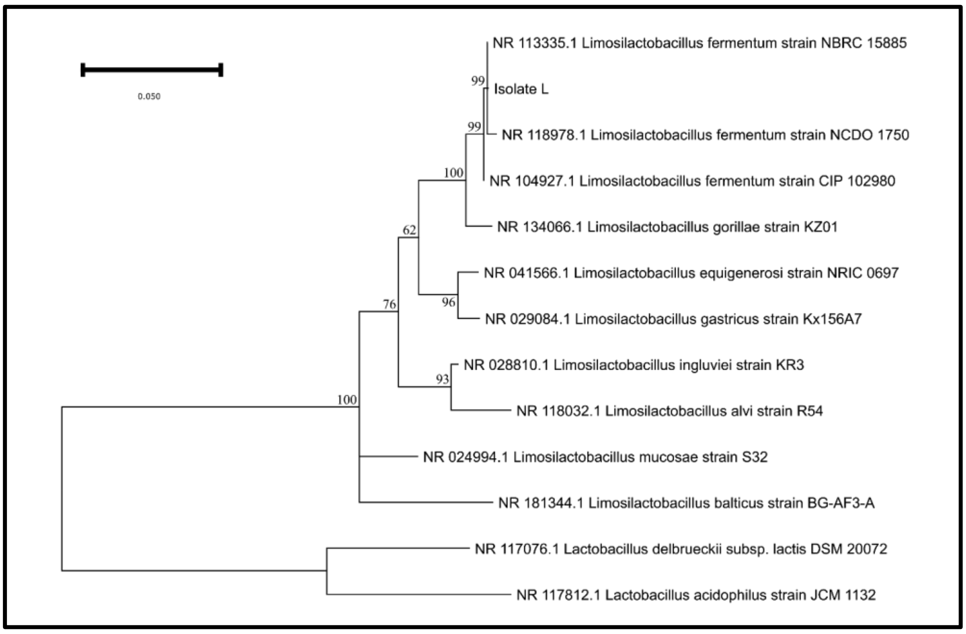

**Figure 2**. Phylogenetic tree of isolate L and various *Lactobacillus* spp. according to their partial 16S rRNA sequences. The phylogenetic tree was constructed with the MEGA software version 11 (https://www.megasoftware.net/) using the Maximum Likelihood statistical method tree. The numbers shown below and above the branch points denote the confidence levels of the relationship of the paired sequences determined by boot strap statistical analysis. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

## Conclusion

It can be concluded that local fresh milk samples, obtained from Holstein Friesian dairy cow, contained *Lactobacillus* spp. Five isolates were highlighted to be *Lactobacillus* spp. according to the colony & cell morphology's identification as well as biochemical tests. Finally, the isolate L was molecularly identified as *Limosilactobacillus fermentum* (previously known as *Lactobacillus fermentum*).

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