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Screening and Genotypic Characterization of Lactic Cultures Isolated from Fermented *Idli* Batter

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present study was conducted during 2021-22 at Dairy Science College, Bangalore, India to study the microflora present in fermented control *idli* batter and batter added with paneer whey and characterize them to use as solid-state fermentation cultures for idli batter fermentation. Eighteen isolates were screened for their phenotypic characteristics after enumeration of microflora in ingredients and fermented batter. The paneer whey was used at 70% of the water used as value addition and by-product utilization for enhancing the nutritional quality of idli. Further, these lactic cultures were screened for their activity for acid production and DMC in the whey-based medium. Titratable acidity ranged from 0.42 to 0.70% lactic acid with a highest of 0.70 for Lb1 and lowest of 0.42 for Leu6 and W4 isolates. DMC was highest at 8.01 log10 cells/ml for E1 isolate and lowest at

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7.41 log10 cells/ml for W4 with a range from 7.41 to 8.01 log10 cells/ml. The isolates with code numbers Lb1, Lb2, Leu2, and E1 showed higher titratable acidity and DMC. DNA extracted from the selected four isolates was subjected to PCR and PCR products were sequenced. Based on the results obtained species of isolates were identified as *Leuconostoc* sp. strain Leu2, *Enterococcus* sp. strain E1, *Lactobacillus brevis* strain Lb1, and *Lactobacillus casei* strain Lb2. The nucleotide sequences of 16S rRNA were submitted to GenBank of NCBI and obtained accession numbers as MW 386845.1, MW386871.1, MW480882.1, and MW485119.1 respectively. These isolates were further used as solid-state fermentation cultures for *idli* batter fermentation.

Keywords: Screening; lactic cultures; batter; sequencing; fermentation; genotypic characterization; lactic acid bacteria.

1. INTRODUCTION

The microorganisms are responsible for characteristic change during the fermentation of *idli* batter. There will be a sequential change in bacterial flora, and the main responsible bacteria for gas production is due to leavening action caused by the activity of heterofermentative lactic culture, *Leuconostoc mesenteroides* [1]. Soni and Sandhu [2] enumerated lactic acid bacteria in the fermented batter ranging from 10^6–10^9/g that included *Leuconostoc mesenteroides, Enterococcus faecalis, Lactobacillus fermentum,* and *Pediococcus cerevisiae* essential for leavening of batter and acid production in idli.

The predominant yeasts identified belonged to the species of genera namely *Candida*, *Saccharomyces*, *Trichosporon,* and *Torulopsis*. *Saccharomyces* sp. were predominantly present and were identified at 0, 8, 16, and 24 h of fermentation. *Candida* sp. was identified at 0 and 8 h, *Trichosporon* sp., at 8 h, and *Torulopsis* sp., at 16 h of fermentation [3]. Fresh idli batter samples from ten households were analyzed during various fermentation time intervals and isolated 300 pure colonies and characterized by morphological and biochemical methods. Out of the 300 colonies isolated, 40 strains were characterized and identified as *Leuconostoc* spp, *Weissella* spp, *Pediococcus* spp, *Lactococcus* spp, and *Bacillus* spp. [4].

The study was conducted to isolate and identify the lactic isolates from fermented idli batters. Selected colonies on selective media plates used for viable count were isolated and maintained as pure cultures. All the 18 isolates were screened by preliminary and biochemical tests and identified seven isolates as *Leuconostoc* sp., four isolates as *Pediococcus pentosaceus*, One isolate as *Enterococcus* sp., Four isolates as *Weissella confusa* and two isolates as *Lactobacillus* sp [5].

Shukla and Dubey [6] replaced water with whey for soaking the rice and black gram, which enhanced the mean sensory scores of the 9-point hedonic scale to 8-8.5 compared to the control of 6.0 -7.9. There was an increase of protein by 0.67%, fat by 0.04%, carbohydrate by 1.28%, and a noticeable increase in calcium content approximately by five folds, i.e. 32.78 mg in control and 161.84 mg in whey-based idli batter. In one more study, concentrated paneer whey (15% TS) was used in the complete replacement of water for batter production, which improved the nutritional quality of idli and dosa as well as the effective utilization of whey by reducing the burden on effluent treatment. The idli made with whey concentrate had higher ash content (1.28%) and acidity (3.81 ml of 0.1 NaOH per g of sample), with a higher degree of hardness (21166.07 g) [7].

The study was conducted to characterize the bacteriocinogenic lactobacilli from fermented idli batter which could find application in bio-preservation and biomedicine. Isolates of 8 numbers out of 22 were characterized based on the various classical phenotypic, physiological, and biochemical tests including various carbohydrate utilization profiles. All isolates were homofermentative, catalase, and gelatin negative. Molecular characterization was performed by Random Amplification of Polymorphic DNA (RAPD), 16S rRNA analysis, Amplified rDNA restriction analysis (16S ARDRA), and Multiplex PCR for species identification. RAPD was carried out using the primer R2 5'-GGCGACCACTAG 3' and M13 5' GAGGGTGGCGGTTCT-3'. 16S rRNA analysis showed 99 to 100% homology towards *Lactobacillus plantarum*. Among the five clusters obtained in RAPD, three clusters were clearly identified as *Lactobacillus plantarum* ssp. *plantarum*, *Lactobacillus pentosus*, and *Lactobacillus plantarum* ssp. *argentoratensis* [8].

Saravanan [8] carried out an enumeration of the bacterial diversity of idli batter during fermentation and to characterize the potential functional properties of selected isolates. A total of 47 isolates were selected randomly, 16S rRNA was amplified and the sequences were analyzed by BLAST to identify up to strain level and the sequences were submitted to NCBI Gene Bank. The 47 isolates represented 10 genera and 15 species. Majorly, Bacillus spp., Weissella spp. *Leuconostocs* spp., *Pediococcus* spp., *Lactococus spp., Micrococcus* spp., *Enterobacter* spp., *Chryseobacterium* spp and *Acinetobacter* spp were identified. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There was a total of 624 positions in the final dataset and phylogenetic analyses were conducted in MEGA 4.

The study was carried out to isolate and identify lactic acid bacteria from fermented red and white fermented rice (Bathalagoda) variety in Sri Lanka. The potential probiotic lactic cultures were studied for phenotypic characteristics, biochemical characteristics using API 50CH kits, and five isolates were identified based on 16S rDNA gene sequencing as *Lactobacillus curvatus* GRLb1, *Latilactobacillus graminis* GRLb 8; *Limosilactobacillus fermentum* GRLb17; *Weissella confuse* GRLb4; and *Pediococcus pentosaceus* GRLc1 [9].

In the present study, lactic cultures were isolated from fermented idli batter using a plating method with selective growth media. Selected colonies were further purified by streaking, and subjected to screening for various phenotypic characteristics [10]. These isolates were screened for titratable acidity and cell count in whey medium. Further shortlisted four isolates with good biomass and acid development were taken for DNA extraction. Extracted DNA was subjected to PCR reaction, and obtained PCR products were sequenced in the external lab. The sequence data was analyzed by using BLAST from the NCBI website. Further, the identified cultures are used as inoculum in the form of SSF cultures for idli batter fermentation.

2. MATERIALS AND METHODS

Typical colonies from viable count plates were studied for their colony characteristics as well as cell morphology. A total of 18 isolates were selected based on colony morphology, isolates were obtained both from control and as well as whey-based idli batter. They were transferred to yeast glucose broth and incubated at 30ºC for 24 h. Further, they were streaked thrice on poured plates of Yeast Glucose Agar (YGA) and purified isolates were maintained in YGA stabs as stock and yeast glucose broth as working cultures. The phenotypic characterization of isolates was carried out by using preliminary tests, viz. Gram staining, catalase test, CO_2 from glucose, growth in litmus milk, and specific tests like dextran production on sucrose agar for sugar fermentation [11].

2.1 Screening of Isolates for Cell Count and Acid Production

After phenotypic characterization, lactic isolates were subjected to the growth study in a sterile whey medium. Young isolates (24 h) were inoculated individually in a 20 ml sterile whey medium at a 1% rate and incubated at 30℃ for 24 h. After 24 h, the whey medium was analyzed for Direct Microscopic count (DMC) [11] and counts expressed as $\log_{10}$ cells/ml and titratable acidity [11] were analyzed and expressed as % lactic acid. The highest values were compared with rest values, and screened, and selected the top four isolates.

2.2 Genotypic Characterization of the Selected Isolates

The selected four isolates Lb1, Lb2, Leu2, and E1 among 18 isolates that showed good biomass and acidity were further subcultured, in yeast glucose broth and taken for DNA extraction. The cultures were subjected to DNA extraction using a ready-to-use DNA extraction kit from Genei Laboratories, Bangalore. Further, this genomic DNA of isolates was subjected to PCR. The reaction mixture comprising 10X PCR buffer (containing $MgCl_2$), dNTPs, primers, and taq polymerase was prepared and distributed to reaction tubes according to the requirements. The final volume of the PCR mix was adjusted to 25 µl and the PCR tubes were transferred to a thermocycler (S-96 Satellite Gradient Thermal Cycler). The PCR Cycling steps comprised one cycle of initial denaturation (9 min at 94ºC), followed by 45 cycles each of denaturation (30 s at 94ºC), primer annealing (30 s at 50ºC), and extension (30 s at 72ºC) followed by a single cycle of final extension of 7 min at 72ºC. The reaction was terminated by cooling the contents to 4ºC. After the run was over, the amplified PCR

products were kept at -20 °C after dissolving in 20 µl of TE buffer until further use [12].

2.3 Sequencing and Analysis Using Blast

The PCR products obtained were sent to an external laboratory, Theracues Innovations Pvt. Ltd, Bangalore for sequencing. After getting sequence data from the external lab, analysis was performed using the Basic Local Alignment Search Tool (BLAST) from the NCBI (https:/www.ncbi.nlm.nih.gov.>BLAST) website. Based on the results obtained, species of isolates were identified. The nucleotide sequences of 16s rRNA were deposited in the GenBank of NCBI and obtained an accession number.

3. RESULTS AND DISCUSSION

3.1 Screening of Isolates for Cell Count and Acid Production

After phenotypic characterization, the selected 18 isolates were screened for their activity and growth in whey medium. They were inoculated at a 1% rate in the sterile whey medium. They were incubated at 30℃/ 24 h in a candle jar. Their activity was determined by titratable acidity and Direct Microscopic Count (DMC). The titratable acidity (% LA) was 0.58, 0.63, 0.56, 0.46, 0.54 and 0.42; DMC (log_{10} cells/ml) was 7.43, 7.90, 7.61, 7.82, 7.72, 7.87 and 7.60 for isolates Leu1, Leu2, Leu3, Leu4, Leu5, Leu6, and Leu7, respectively. Isolates P1, P2, P3, and P4 showed titratable acidity of 0.59, 0.59, 0.59, and 0.55% lactic acid, whereas DMC (log_{10} cells/ml) was 7.67, 7.79, 7.87 and 7.83, respectively. The isolate E1 exhibited titratable acidity of 0.59% LA and DMC (log_{10} cells/ml) of 8.01. The titratable acidity was 0.46, 0.44, 0.46, and 0.42% lactic acid, whereas DMC (log_{10} cells/ml) was 7.74, 7.41, 7.56, and 7.65 for isolates W1, W2, W3, and W4, respectively. Lactobacillus isolates Lb1, and Lb2 showed titratable acidity of 0.70, 0.60, and DMC of 7.91 and 7.98 log_{10} cells/ml, respectively (Table 1 and Fig. 1).

Table 1. Screening of batter isolates for titratable acidity and cell count in whey medium

Sl. No	Name of the isolate	Isolate code	Titratable Acidity (% LA)	DMC (log_{10} cells/ml)
1	*Leuconostoc* sp.	Leu1	0.58^{cd}	7.43^{f}
2	*Leuconostoc* sp.	Leu2	0.63^{b}	7.90^{ab}
3	*Leuconostoc* sp.	Leu3	0.56^{de}	7.61^{def}
4	*Leuconostoc* sp.	Leu4	0.46^{g}	7.82^{abcd}
5	*Leuconostoc* sp.	Leu5	0.54^{e}	7.72^{bcde}
6	*Leuconostoc* sp.	Leu6	0.42^{h}	7.87^{abc}
7	*Leuconostoc* sp.	Leu7	0.49^{f}	7.60^{def}
8	*Pediococcus pentosaceus*	P1	0.59^{c}	7.67^{bcde}
9	*Pediococcus pentosaceus*	P2	0.59^{c}	7.79^{abcde}
10	*Pediococcus pentosaceus*	P3	0.59^{c}	7.87^{abc}
11	*Pediococcus pentosaceus*	P4	0.55^{de}	7.83^{abcd}
12	*Enterococcus* sp.	E1	0.59^{c}	8.01^{a}
13	*Weissella confusa*	W1	0.46^{g}	7.74^{bcde}
14	*Weissella confusa*	W2	0.44^{gh}	7.41^{f}
15	*Weissella confusa*	W3	0.46^{g}	7.56^{ef}
16	*Weissella confusa*	W4	0.42^{h}	7.65^{cdef}
17	*Lactobacillus brevis*	Lb1	0.70^{a}	7.91^{ab}
18	*Lactobacillus casei*	Lb2	0.60^{c}	7.98^{a}
	CD (*P*=.05)		0.02	0.28

Note:

- *The values were average of three trials*
- *CD – Critical difference*
- *Inoculum level was 1% with incubation anaerobically in a candle jar at 30ºC/24 h in whey medium*
- *Highest value was compared with other values*
- *Same superscripts in the column indicate non-significance while different superscripts indicate significant difference*

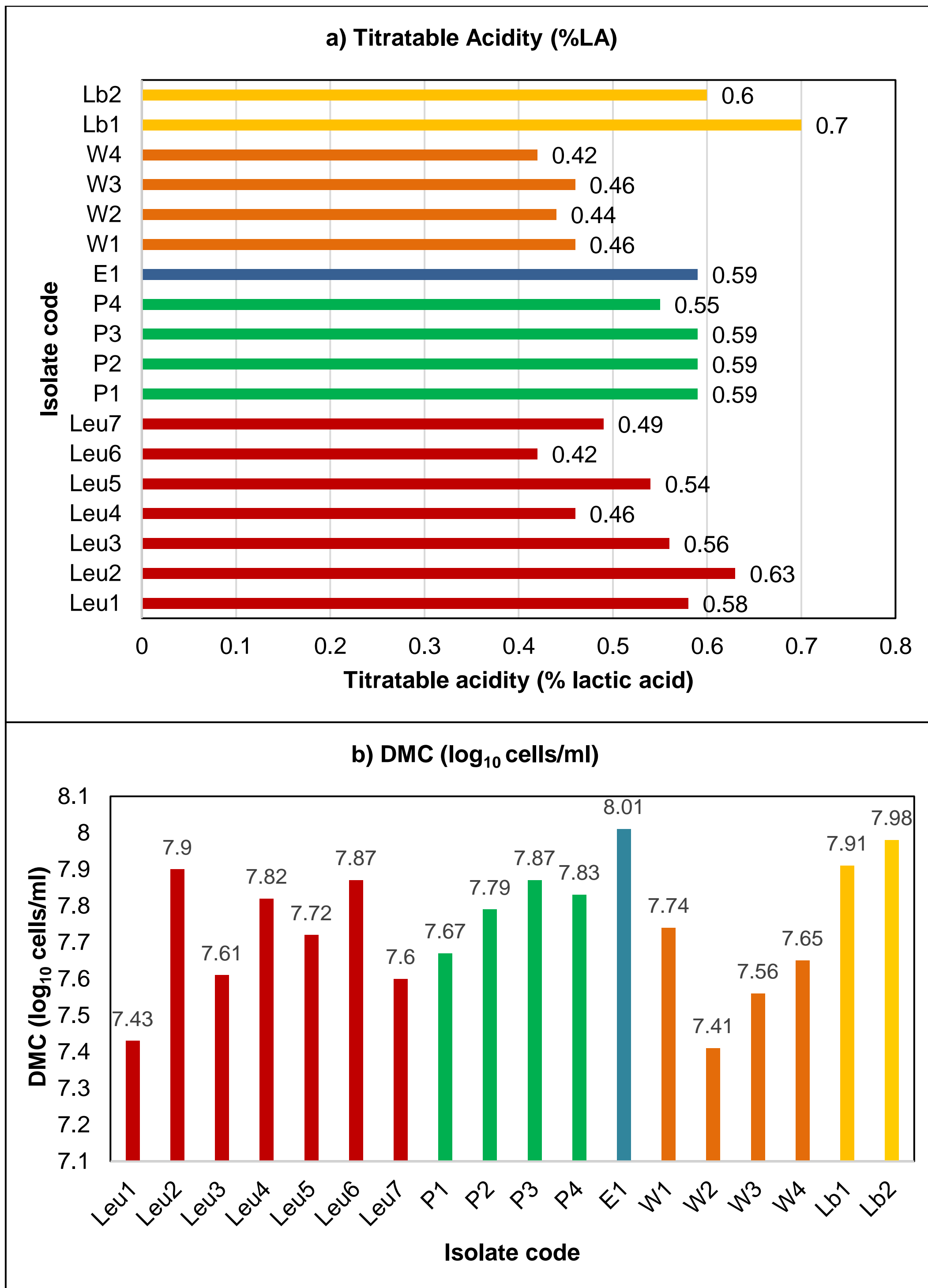

Fig. 1. Titratable acidity and DMC of lactic isolates in sterile whey medium

Titratable acidity ranged from 0.42 to 0.70% lactic acid and DMC from 7.41 to 8.01 log_{10} cells/ml. The highest titratable acidity was found with Lb1 of 0.70% LA, lowest was 0.42 by Leu6 and W4. The DMC was highest for E1, 8.01 log_{10} cells/ml, and lowest for Leu1 of 7.43 log_{10} cells/ml. There was a significant difference in the values from one isolate to another with few exceptions (P=.05). From the above results, it was found that the isolates with code numbers Lb1, Lb2, Leu2, and E1 showed higher titratable acidity and DMC and were selected for further study as solid-state fermentation cultures.

Table 2. Genotypic identity of selected lactic isolates obtained from idli batter

Sl. No	Isolate code	Source	Name of isolate	Accession number
1	Leu2	Control batter	*Leuconostoc sp.*	MW386845.1
2	E1	(100% water)	*Enterococcus sp*	MW386871.1
3	Lb1	Whey based batter	*Lactobacillus brevis*	MW480882.1
4	Lb2	(70% paneer whey)	*Lactobacillus casei*	MW485119.1

3.2 Genotypic Characterization of Selected Isolates

Based on the results, isolates were correctly identified as *Leuconostoc* sp. strain Leu2, *Enterococcus* sp. strain E1, *Lactobacillus brevis* strain Lb1, and *Lactobacillus casei* strain Lb2. The nucleotide sequences of 16s rRNA were submitted in GenBank of NCBI and obtained accession numbers for Leu2, E1, Lb1, and Lb2 as MW386845.1, MW386871.1, MW480882.1 and MW485119.1 respectively (MW in accession number indicates direct submission to GenBank) (Figs. 2-5 (Appendix), Table 2). Similar studies were conducted on antibacterial activity and probiotic properties of LAB isolates from idli batter purchased in West Bengal. The isolates were identified as gram-positive, non-spore-forming, non-motile rod-shaped, catalase-negative and oxidase-negative. The molecular identity validation was done by 16S rRNA gene sequencing and phylogenic analysis. the isolates were identified as *Lactobacillus pentosus* LMEM1001, *Lactobacillus plantarum* LMEM1002, *Lactobacillus* sp. LMEM1003 and *Lactobacillus* sp. LMEM1004, *Lactobacillus* sp. LMEM1005, *L. plantarum* LMEM1006, *Lactobacillus* sp. LMEM1007 and *Lactobacillus fermentum* LMEM1008 [13]. These isolates were used for further studies for employing them in solid-state fermentation cultures for *idli* batter fermentation.

4. CONCLUSION

After phenotypic characterization, the selected 18 isolates were screened for DMC and titratable acidity in whey medium. Titratable acidity ranged from 0.42 to 0.70% lactic acid with a highest of 0.70 for Lb1 and lowest of 0.42 for Leu6 and W4 isolates. DMC was the highest of 8.01 log10 cells/ml for E1 isolate and the lowest of 7.41 log10 cells/ml for W4, ranging from 7.41 to 8.01 log10 cells/ml. The isolates with code numbers Lb1, Lb2, Leu2, and E1 showed higher titratable acidity and DMC and hence they were selected for further study for solid-state fermentation on black gram dhal. Genomic DNA extracted from the selected four isolates was amplified through PCR and PCR products were outsourced for gene sequencing. After receiving the sequence data, analysis was done using the Basic Local Alignment Search Tool (BLAST) from the NCBI website. Based on the results obtained species of isolates were correctly identified as *Leuconostoc* sp. strain Leu2, *Enterococcus* sp. strain E1, *Lactobacillus brevis* strain Lb1, and *Lactobacillus casei* strain Lb2. The nucleotide sequences of 16S rRNA were submitted to GenBank of NCBI and obtained accession numbers as MW 386845.1, MW386871.1, MW480882.1, and MW485119.1 respectively. These identified cultures are grown on solid-state media and are used as inoculum for hastening the batter fermentation process compared to the natural process.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

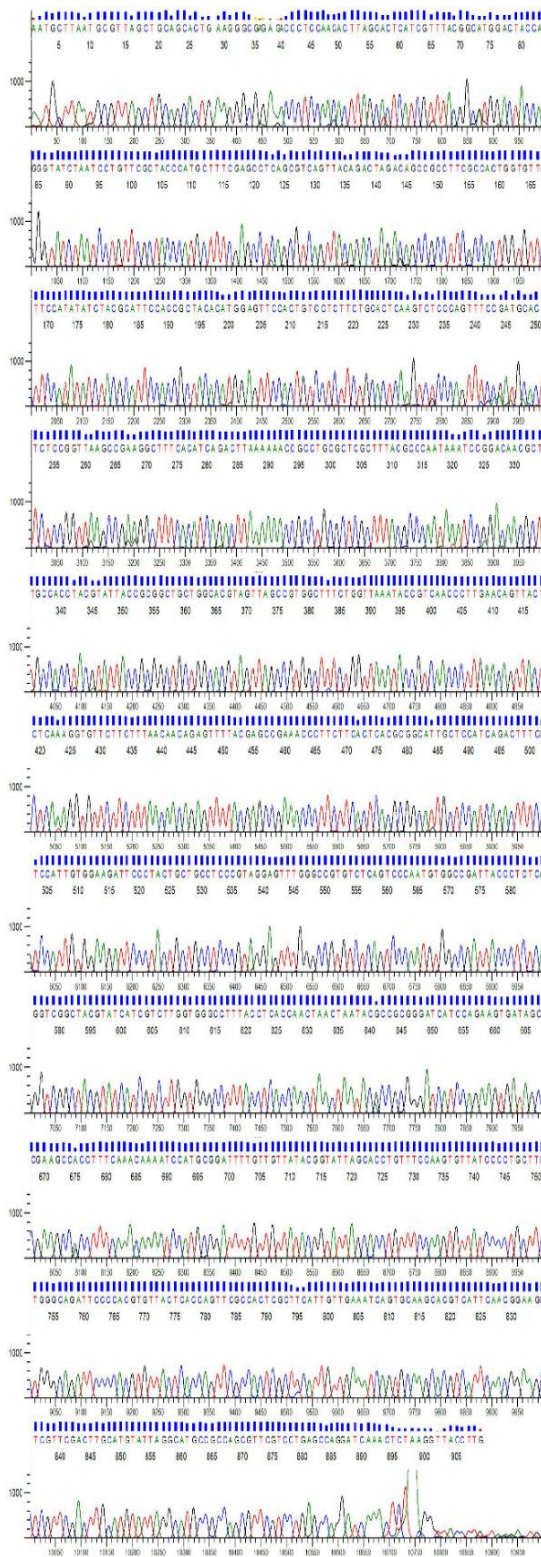

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Fig. 2. 16s rRNA sequence *Leuconostoc* sp. Leu2 with Accession number – MW 386845.1

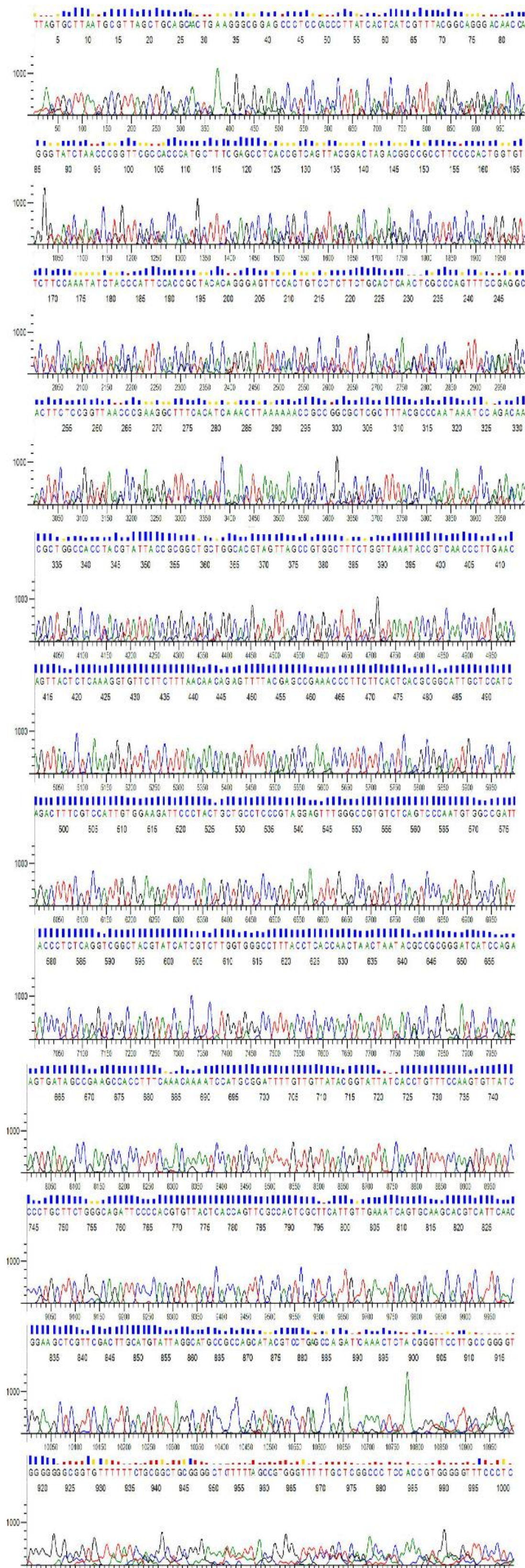

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Fig. 3. 16s rRNA sequence of Enterococcus sp. E1 with Accession number – MW386871.1

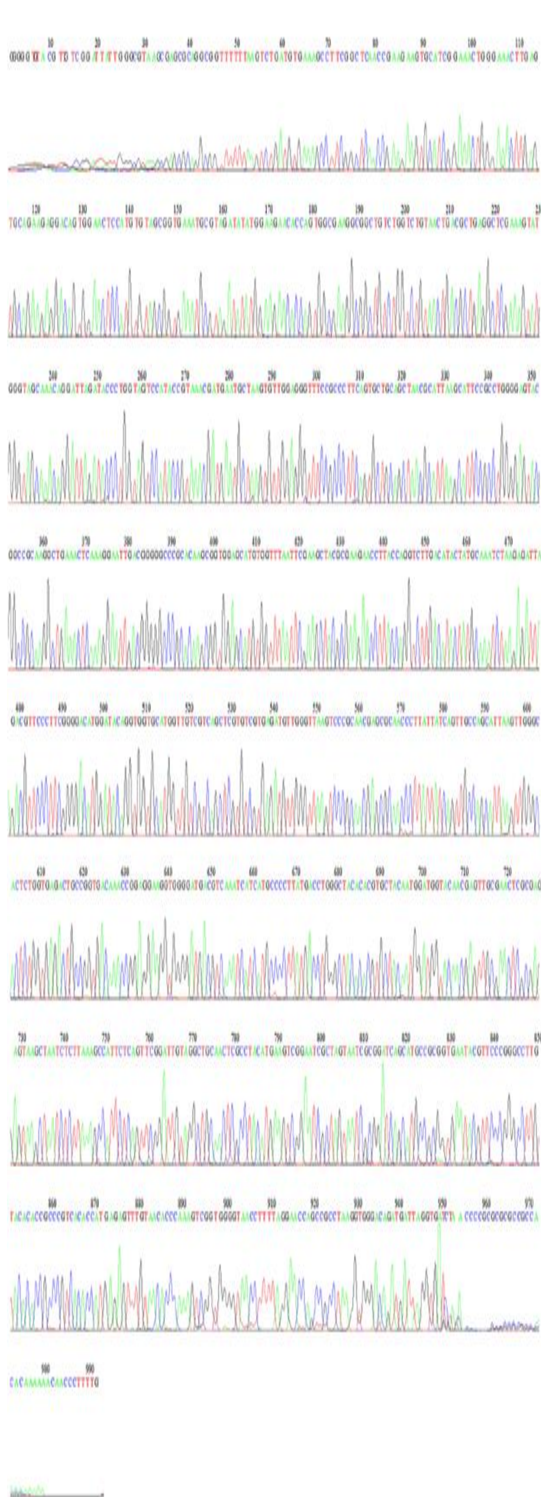

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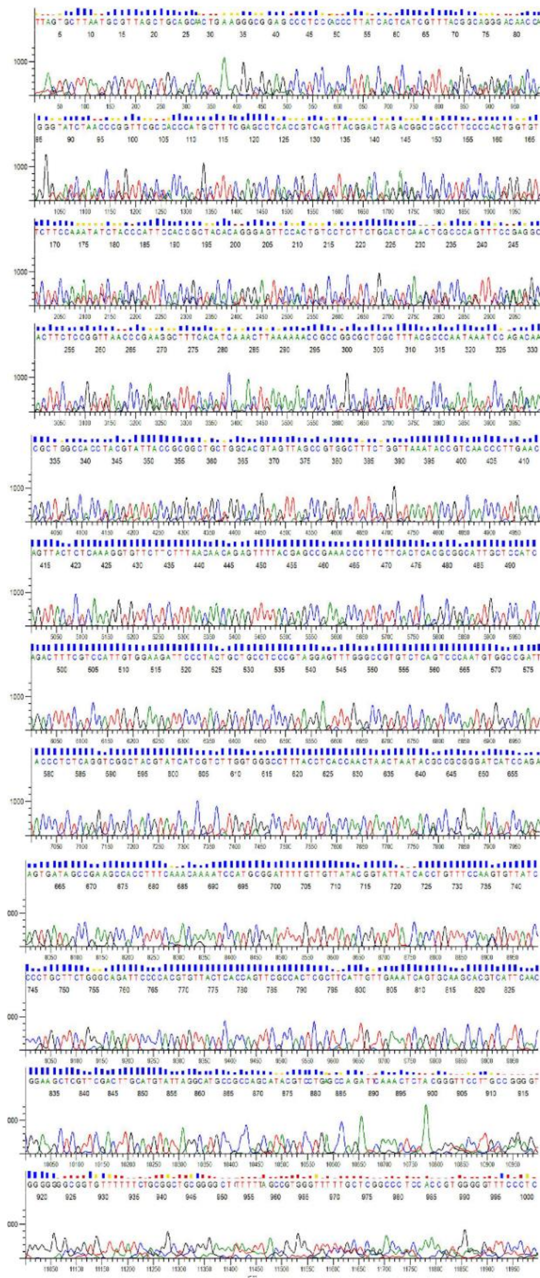
Fig. 4. 16s rRNA sequence of Lactobacillus brevis Lb1 with Accession number – MW480882.1



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GGTTCGCCACCCATGCTTTCGAGCCTCACCGT
CAGTTACGGACTAGACGGCCGCCTTCCCCACT
GGTGTTCTTCCAAATATCTACCCATTCCACCGC
TACACAGGGAGTTCCACTGTCCTCTTCTGCAC
TCAACTCGCCCAGTTTCCGAGGCACTTCTCCG
GTTAACCCGAAGGCTTTCACATCAAACTTAAAA
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CCAGACAACGCTGGCCACCTACGTATTACCGC
GGCTGCTGGCACGTAGTTAGCCGTGGCTTTCT
GGTTAAATACCGTCAACCCTTGAACAGTTACTC
TCAAAGGTGTTCTTCTTTAACAACAGAGTTTTA
CGAGCCGAAACCCTTCTTCACTCACGCGGCAT
TGCTCCATCAGACTTTCGTCCATTGTGGAAGAT
TCCCTACTGCTGCCTCCCGTAGGAGTTTGGGC
CGTGTCTCAGTCCCAATGTGGCCGATTACCCT
CTCAGGTCGGCTACGTATCATCGTCTTGGTGG
GCCTTTACCTCACCAACTAACTAATACGCCGCG
GGATCATCCAGAAGTGATAGCCGAAGCCACCT
TTCAAACAAAATCCATGCGGATTTTGTTGTTATA
CGGTATTATCACCTGTTTCCAAGTGTTATCCCC
TGCTTCTGGGCAGATTCCCCACGTGTTACTCA
CCAGTTCGCCACTCGCTTCATTGTTGAAATCAG
TGCAAGCACGTCATTCAACGGAAGCTCGTTCG
ACTTGCATGTATTAGGCATGCCGCCAGCATACG
TCCT

Fig. 5. 16s rRNA sequence of *Lactobacillus casei* Lb2 with Accession number – MW485119.1

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