

EFFECTIVE IDENTIFICATION OF *LACTOBACILLUS PARACASEI* SSP. *PARACASEI-1* BY 16S-23S rRNA INTERGENIC SPACER REGION SEQUENCING

Tariful Islam^{*1,2}, Md. Iqbal Hassan Khan², Kazi Farhana Afroz¹, Mohammed Zakiur Rahman², Md. Shawkat Hossain², Md. Emdadul Islam¹, Md. Morsaline Billah¹, Kazi Didarul Islam¹

Address(es): Tariful Islam,

¹Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna-9208, Khulna, Bangladesh.

² Biotechnology Derived Product Facility, Incepta Pharmaceuticals Ltd, Savar, Dhaka-1341, Bangladesh.

*Corresponding author: tarif_87@yahoo.com

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ABSTRACT

Fermentation profiling is a common tool to identify *Lactobacillus paracasei* from other related members of *Lactobacillus* genus. Because of the proximate biochemical characteristics, identification based on the fermentation pattern within *L. casei* group (*L. casei*, *L. paracasei* and *L. rhamnosus*) has considerable limitation. On the other hand, members of *L. casei* group are genetically very closely related and sequencing of signature region is the most reliable and rapid method of differentiation. In this study, instead of sequencing entire 16S or 23S ribosomal RNA gene, we have sequenced 16s-23s rRNA intergenic spacer region and compared them across the members of *L. casei* group. We also compiled thirteen signature sequences within 16s-23s ISR rRNA gene of *L. casei* group members. Our isolated strain (*L. paracasei* ssp. *paracasei-1*) resembled 100% and 99% similarity when compared with 16s-23s rRNA intergenic spacer region of *L. paracasei* and *L. casei* respectively. Our study summarizes that sequencing of short 16s-23s rRNA intergenic spacer region carries great significance in identification of closely related probiotic bacteria such as the members of *L. casei* group. The findings of this research could be very much helpful for food and pharmaceutical industries who are dealing with probiotic bacteria.

Keywords: Identification, *L. paracasei* ssp. *paracasei-1*, 16s-23s rRNA, signature sequence, probiotic

INTRODUCTION

The genus *Lactobacillus* is subject to continual taxonomic changes. It is often thorny to use classical microbiological methods to identify lactic acid bacteria even to genus level because of their similar nutritional and growth requirements (Dubernet et al., 2002). Moreover, identifying a *Lactobacillus* isolate accurately to the species level requires about 17 phenotypic tests (Tannock et al., 1999). Even bigger challenge is to accurately identify the species of *L. casei* group based on phenotypic tests (Ward and Timmins, 1999). However, confirming the identification of diverse species of lactic acid bacteria is very important for quality control and quality assurance perspective when it is intended for human use (Tilsala et al., 1997). Very often identification of *Lactobacillus* spp. appears as a dilemma to the researchers. *L. casei* group is an ideal example which must overcome some unusual and difficult obstacles in order to pacify confusion in their identification, especially within the *L. casei* group members. The taxonomy of *L. casei* group has been in dispute over the transfer of some species and strains from *L. casei* to *L. paracasei* (Kisworo et al., 2008). *L. casei* group gone through several rearrangement notably from five member group to four members enlisted in *Bergey's Manual of Systematic Bacteriology* (*L. casei* ssp. *alactosus* was combined with *L. casei* ssp. *casei*) (Mori et al., 1997). In 1989 Collins et al. (1989) reclassified the subspecies of *L. casei* into *L. casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* on the basis of DNA-DNA relatedness data. Although Coudeyras et al. (2008) classified *L. casei* into four group members, however, we have considered *L. casei* with three group members including *L. casei*, *L. paracasei* and *L. rhamnosus* based on the latest phylogenetic structure revealed by Salvetti et al. (2012).

Considering these identification enigma, use of a diagnostic marker specific to the probiotic strain of interest carries significant importance. Moreover, the beneficial characteristics associated with the probiotic are strain specific (Flint et al., 2005). Therefore nucleotide base sequences of *Lactobacillus* 16S ribosomal DNA (rDNA) is an important reliable analysis which provides an accurate basis for identification (Tannock et al., 1999). Barry et al. (1991) have shown that the ribosomal intergenic regions are more variable between bacterial species compared to 16S and 23S rRNA genes. Tannock et al. (1999) documented several studies which have demonstrated that the DNA sequence between the 16S

and 23S genes of lactobacilli is hypervariable. This intergenic spacer region (ISR) is about 200 bases in length. The 16s-23s spacer sequences of lactobacilli are adequately species specific which can be used as a marker to identify *Lactobacillus* species. In fact, using primer that will amplify 16s-23s ISR region is a relatively simple and quick method through which lactobacilli can be identified without resorting to the use of species-specific PCR primers (Tannock et al., 1999). While drawing significant relationship within closely related bacterial species, sequencing comparisons are often applied. Mori et al. (1997) compared the 16S rRNA sequences of all strains of the *L. casei* subspecies. Chen et al. (2000) reported intergroup comparisons of the 23S-5S rRNA spacer genes from *Lactobacillus casei* related strains. In this study we have compared the sequences of 16s-23s ISR region of *L. casei* group along with the identification of *L. paracasei* ssp. *paracasei-1*.

Hoque et al. (2010) reported the genus of a bacterial isolates as *Lactobacillus* spp. in their study. Based on classical microbiology and biochemical reaction, Islam et al. (2012) identified the same isolated bacteria reported by Hoque et al. (2010) as *L. paracasei* ssp. *paracasei-1*. Following that, Honi et al. (2013) also bolstered the identification, reported by Islam et al. (2012), as *L. paracasei* ssp. *paracasei-1* on the basis of PCR amplification of 16s-23s ISR region. However, none of these reports include sequencing of the ISR region of *L. paracasei* ssp. *paracasei-1*. *L. paracasei* and other members of *L. casei* group share extremely high similarities within the 16s-23s ISR region. So there is every possibility of getting a false positive result from PCR amplification based identification while identifying *L. paracasei* without the sequencing of 16s-23s ISR region. Sequencing of the 16s-23s ISR region of previously identified *L. paracasei* ssp. *paracasei-1* (Islam et al., 2012; Honi et al., 2013) and comparing the sequenced results across the already reported 16s-23s ISR region sequences of *L. casei* group members, in order to determine signature sequences, are the main focus of this research.

MATERIAL AND METHODS

Collection of test organism

The test organism (*L. paracasei* ssp. *paracasei*-1, isolated from regional yogurt) (Islam et al., 2012; Honi et al. 2013) was collected from the stock culture maintained by the Biochemistry and Molecular biology Laboratory of Biotechnology & Genetic Engineering Discipline, Khulna University.

Subculture of the bacteria

The test organism was grown in MRS broth, added with 0.05% cysteine, (HiMedia/India) and MRS agar (HiMedia/India) plated in an incubator (Shellab/USA) at 37°C for 16 hrs. pH (Mettler Toledo/Switzerland) of the media was adjusted to 6.5 (Islam et al., 2012; Honi et al., 2013).

Morphological, physiological and biochemical identification

Identification of *Lactobacillus* spp. was verified via gram staining, cell morphology, endospore assay, catalase test, motility test, colony morphology (Hoque et al., 2010; Islam et al., 2012; Honi et al., 2013).

Extraction of chromosomal DNA

Chromosomal DNA was extracted from overnight MRS culture, stored at 37°C, using bacterial DNA preparation kit (Jena bioscience/Germany) according to the manufacturer's instructions. Quantity of DNA extracts was checked with a spectrophotometer (Shimadzu: UV-1800 /Japan) (Skelin et al., 2012).

Quantification of genomic DNA of *Lactobacillus* spp.

Isolation of genomic DNA was confirmed by using horizontal gel electrophoresis system (BioRad: MINI SUB CELL /USA) and gel documentation system (CB Scientific: ALPHAIMAGER HP /USA). In this case 2% agarose was used. Then quantification of DNA was done by using spectrophotometer. Absorption reading was taken at 260, 280 and 320 nm. Purity, concentration and total yield of DNA were calculated from these readings which are detailed in Table 1.

Table 1 Concentration and purity of extracted DNA

Sample	A260	A280	A320	Concentration ¹ , µg/ml	Purity ²	Purity ³	DNA Yield ⁴ , µg
A	0.412	0.24	0.008	505	1.71	1.74	1010
B	0.424	0.24	0.012	515	1.76	1.80	1030
C	0.448	0.28	0.02	535	1.60	1.64	1070

1 = [(A260-A320) × Dilution Factor (= 25) × 50 µg/ml pure ds DNA]

2 = (A260÷A280)

3 = [(A260-A320)÷(A280-A320)]

4 = [(DNA Concentration in µg/ml × Total Sample volume (=2 ml)]

A260 = Absorption at 260 nm, A280 = Absorption at 280 nm, A320 = Absorption at 320 nm

PCR amplification

To determine the identification up to species level of *L. paracasei* isolates, species-specific PCR assay was carried out with *Lactobacillus paracasei* species-specific primer set, supplied by Bioneer Corp., USA. Primer set Lp1F- (5'-GGGGATCACCTCAAGCACCT -3') and Lp1R-(5'-GCGTCAGCGTTATGCGATGC -3') along with Applied Biosystem's thermal cycler (VERITY/USA) were used (Honi et al., 2013). Amplification of DNA from the 16s-23s ISR rRNA gene was carried out using promega PCR kit (Promega Corporation/USA) and PCR operating protocol described by Honi et

al. (2013). Table 2 gives information on primer details and Table 3 explains the PCR sample loading detail. Amplification products were separated on a 2% agarose gel using horizontal gel Electrophoresis System (BioRad: MINI SUB CELL /USA), stained with 2.5% ethidium bromide, visualized and documented using a Gel Documentation System (CB Scientific: ALPHAIMAGER HP /USA) (Honi et al., 2013). The amplified PCR products were purified using conventional ethanol based purification method and manufacturer's instruction of BigDye XTerminator Purification kit (AB Science, USA).

Table 2 Primers used for PCR amplification of 16s-23s intergenic spacer region

Primer	Sequences (5' > 3')	Length	Start	Stop	Tm	GC%
Lp1F	GGGGATCACCTCAAGCACCT	22	63	84	64.0	63.64
Lp1R	GCGTCAGCGTTATGCGATGC	21	208	188	64.3	61.90

Table 3 PCR reaction mixture

Sl No.	Reagents	PCR Master Mix (µl)	Forward Primer ¹ (µl)	Reverse Primer ¹ (µl)	DNA Template ² (µl)	DNA Template ³ (µl)	Nuclease Free Water (µl)	Total Vol. (µl)
	Sample							
1	Sample 1	12.5	0.5	0.5	3	0	8.5	25
2	Sample 2	12.5	0.5	0.5	3	0	8.5	25
3	Sample 3	12.5	0.5	0.5	3	0	8.5	25
4	Sample 4	12.5	0.5	0.5	0	3	8.5	25
5	Sample 5	12.5	0.5	0.5	0	3	8.5	25
6	Sample 6	12.5	0.5	0.5	3	0	8.5	25
7	Sample 7	12.5	0.5	0.5	3	0	8.5	25
8	Sample 8	12.5	0.5	0.5	3	0	8.5	25
9	Sample 9	12.5	0.5	0.5	0	3	8.5	25
10	Sample10	12.5	0.5	0.5	0	3	8.5	25

1= Stock Concentration (100 nano mole)

2= Sample-B of Table 1

3= Sample-C of Table 1

16s-23s ISR DNA sequence

The PCR products of the amplification were used for re-identification at the species level and to recheck the primer specificity for *Lactobacillus paracasei* ssp. *paracasei*-1 as reported by **Honi et al. (2013)**. For DNA sequencing we have used sequencing kit (AB science, USA). An in-house developed protocol was used in determining the sequencing. Briefly, in each cycle, during initial denaturation the temperature was increased to 96°C for 1 minute, followed by 96°C and 10 seconds denaturation cycle. Then temperature decreased to 50°C for 5 seconds to anneal the primer to the template, and finally increased to 60°C for 4 minutes during elongation step involving enzymatic synthesis. A total of 25 cycles were completed. Finally the reaction was kept at 4°C. The PCR product

was purified using ethanol precipitation method and purification kit. **Table 4** contains the cycle sequencing sample loading detail which was performed in a DNA analyzer unit of Applied Biosystem science (Applied Biosystem: 3500 GENETIC ANALYZER /USA). The obtained sequences were aligned and compared with the *L. casei* group member's 16s-23s rRNA ISR sequences (accession number U32964.1, U32966.1 and AF121200.1) stored at GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA). BLAST and ClustalW2 algorithm were used in this comparison. To test the reliability of the cycle sequencing method, we have sequenced pGEM vector (AB Science, USA) using the same sequencing protocol described above.

Table 4 Cycle sequencing sample preparation:

SI No.	Reagents	Ready Reaction Mix (μl)	Dilution Buffer (μl)	DNA Template ¹ , μl	Forward Primer ² , μl	Reverse Primer ² , μl	Nuclease Free Water (μl)	Total Vol. (μl)
	Sample							
1	Sample 1	1.0	1.5	1.5 ^a	2.0 ^b	0	4.0	10
2	Sample 2	1.0	1.5	1.5	2.0	0	4.0	10
3	Sample 3	1.0	1.5	1.5	0	2.0	4.0	10

1= Sample B of Table 1, 2= Concentration is 100 nano mole, a= pGEM control, b= Forward primer for pGEM

Comparison of 16s-23s ISR of *L. casei* group

Tilsala et al. (1997) determined the 16s-23s rRNA sequence of *L. paracasei* (accession number U32964.1) and *L. Rhamnosus* (accession number U32966.1). **Tannock et al. (1999)** determined the 16s-23s rRNA sequence of *L. casei* ATCC 334 (accession number AF121200.1). We tried to identify the signature sequences of ISR region within the *L. casei* group. The homology between *L. paracasei* ssp. *paracasei*-1 and *L. casei* group members were determined in order to differentiate *L. paracasei* ssp. *paracasei*-1 from *L. casei* group members based on 16s-23s ISR rRNA sequencing. Finally we compared 16s-23s ISR rRNA gene sequence of *L. paracasei* ssp. *paracasei*-1 with the 16s-23s ISR rRNA regions of *L. casei* and *L. paracasei* to confirm the identity based on sequencing similarity pattern. All the FASTA sequences were obtained from National Center for Biotechnology Information (NCBI).

Three types of alignment were performed using Clustal W2:

1. Alignment of 16s-23s ISR sequence of *L. casei* group (*L. paracasei*, GenBank U32964.1, *L. rhamnosus* (U 32966.1) and *L. casei*, (AF121200.1))
2. Alignment of 16s-23s *L. paracasei* (U32964.1) and *L. casei* (AF121200.1)
3. Alignment of 16s-23s *L. paracasei* (U32964.1), *L. casei* (AF121200.1) and *L. paracasei* ssp. *paracasei*-1

RESULTS

Morphological, physiological and biochemical characterization

The isolate showed their colonies morphology small, circular, white-creamy in colors. Microscopically they were gram-positive rod shaped, non-motile, catalase negative and non spore forming. Their colony and microbiological characteristics have been summarized in **Table 5**. According to their characteristics, the isolates were identified as *Lactobacillus* spp.

Table 5 Different characteristics of isolated *Lactobacillus* spp.

Morphological, physiological and biochemical test	Findings
Gram staining	Positive
Cell morphology	Rod shaped
Endospore staining	Negative
Catalase test	Negative
Motility test	Negative
Colony morphology	Small round, creamy white

Extraction and quantification of genomic DNA from *Lactobacillus* spp.

After performing gel electrophoresis (2% agarose, voltage: 85V) there was a distinctive band of genomic DNA (Figure 1). Quantification of DNA concentration carried out by spectrophotometer. The purity range of isolated DNA of the test organism was within 1.60 to 1.80 (**Table 1**)

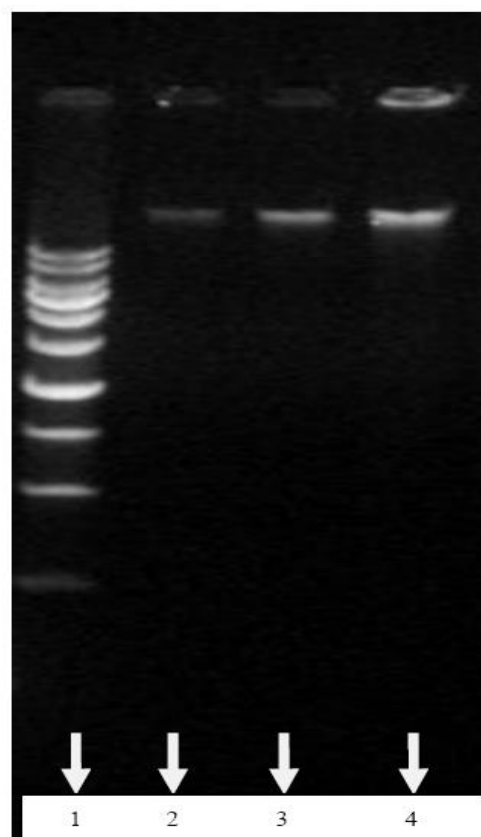


Figure 1 Isolated genomic DNA of *Lactobacillus* spp. (1= 1Kb ladder, 2= sample A, 3= sample B and 4= sample C of Table 1)

PCR amplification of the 16s-23s rRNA ISR from *Lactobacillus paracasei* DNA

One primer pair (Lp1F & Lp1R) was used for PCR amplification of spacer region of our *Lactobacillus* spp. PCR product was obtained with the primer pair (Figure 2). The amplified product with the same primer pair has been reported to be 146 bp long (**Honi et al., 2013**).

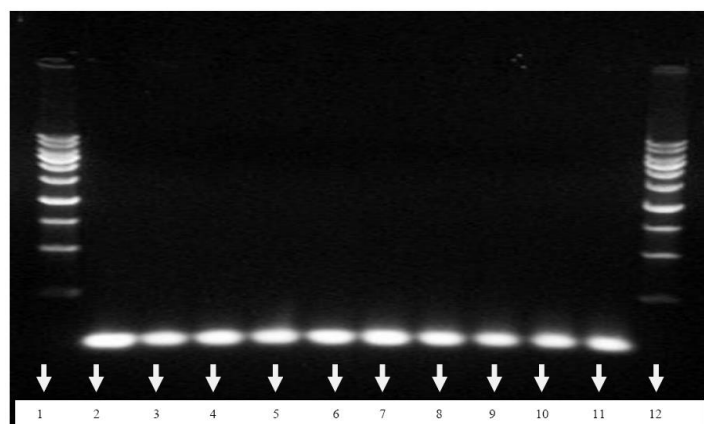


Figure 2 Gel run of PCR amplified sample

Lane 1: 1Kb Ladder	Lane 2: Sample-1	Lane 3: Sample-2	Lane 4: Sample-3	Lane 5: Sample-4	Lane 6: Sample-5
Lane 7: Sample-6	Lane 8: Sample-7	Lane 9: Sample-8	Lane 10: Sample-9	Lane 11: Sample-10	Lane 12: 1Kb Ladder

16s-23s ISR rRNA gene sequencing result

Obtained forward sequence using forward primer Lp1F

>

TTGAAAACCTGGATATCATTGTATTAATTTGTTTAAATTGCCGAGAACACAGCGTATTGTATGAGTTTCTGAA
AAAGAAATTCGCATCGCATAACCGTCAGCGAA

Query sequence (obtained forward sequence) :

1 TTGAAAACCTG GATATCATTG TATTAATTTG TTTAAATTCG CGAGAACACA GCGTATTGT
61 ATGAGTTTCT GAAAAAGAAA TTCGCATCGC ATAAACCGCTG ACGCAA

Subject sequence (*Lactobacillus paracasei paracasei*, 16s-23s rRNA spacer region, U32964.1)

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCTCAAGCA CCTARCGGG TCGGACTTTG TTCCTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTAAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTGCGA TCGCATAACC GCTGACGCAA GTCAGTACAG G

Sequence ID: lcl|49579|Length: 221|Number of Matches: 1
Score Expect Identities Gaps
196 bits (106) 1e-55 106/106 (100%) 0/106 (0%)

Query 1 TTGAAAACCTGGATATCATTGTATTAATTTGTTTAAATTGCCGAGAACACAGCGTATTGT 60
|||||
Sbjct 105 TTGAAAACCTGGATATCATTGTATTAATTTGTTTAAATTGCCGAGAACACAGCGTATTGT 164

Query 61 ATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACCGCTGACGCAA 106
|||||
Sbjct 165 ATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACCGCTGACGCAA 210

Among 221 bases of reference sequence 106 bases perfectly matched with the results of sample's forward sequence.

Obtained reverse sequence using reverse primer Lp1 R:

>

ATACAAATACGCTGTGTCTCGGCAATTTAAACAATTAATACATGATATCCAGTTTCAAAGAACAAGT
CGCACCCGYTAGG

Blasting was performed in following way

Query sequence (obtained reverse sequence):

1 ATACAAATAC GCTGTGTCT CGGCAATTTAAACAATTAATACAATGATATCCAGTTTTC
61 AAAGAACAAGTTCGACCCGYTAGG

Subject sequence (*Lactobacillus paracasei paracasei*, 16s-23s rRNA Spacer Region, U32964.1):

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCTCAAGCA CCTARCGGG TCGGACTTTG TTCCTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTAAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTGCGA TCGCATAACC GCTGACGCAA GTCAGTACAG G

Sequence ID: lcl|19777|Length: 221|Number of Matches: 1
Score Expect Identities Gaps
154 bits (83) 6e-43 85/85 (100%) 0/85 (0%)

Query 1 ATACAAATACGCTGTGTCTCGGCAATTTAAACAATTAATACATGATATCCAGTTTTC 60
|||||
Sbjct 166 ATACAAATACGCTGTGTCTCGGCAATTTAAACAATTAATACATGATATCCAGTTTTC 107

Query 61 AAAGAACAAGTTCGACCCGYTAGG 85
|||||
Sbjct 106 AAAGAACAAGTTCGACCCGYTAGG 82

Among 221 bases of reference sequence 85 bases perfectly matched, shown in bold, with the results of sample's reverse sequence. Sequence given below, shown within box, shows the forward primer position within the reference sequence. Good signal sequence was obtained after 20 initial bases which continued up to 210 no. base.

Forward

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCTCAAGCA CCTARCGGG TCGGACTTTG TTCCTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTAAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTGCGA TCGCATAACC GCTGACGCAA GTCAGTACAG G

The following sequence, shown within box, shows the reverse primer position within the reference sequence. Good signal sequence was obtained after 21 bases which continued up to 82 no. base.

Reverse

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCTCAAGCA CCTARCGGG TCGGACTTTG TTCCTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTAAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTGCGA TCGCATAACC GCTGACGCAA GTCAGTACAG G

After combining both the results it was found that almost 148 bases, shown within box, were completely matched with the reference sequence.

Combined result:

1 CTAAGGAAAC AGACTGAAAGTCTGACGGAAACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCTCAAGCA CCTARCGGG TCGGACTTTG TTCCTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTAAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTGCGA TCGCATAACC GCTGACGCAA GTCAGTACAG G

Sequences of the 16s-23s ISR rRNA gene of isolated *L. paracasei* ssp. *paracasei-1*

From the combined cycle sequencing result we did determine the intended region designed for our primer. Sequence of *L. paracasei* ssp. *paracasei-1* showed 100% similarity (comparison - 3) with the 16s-23s ISR sequence of *L. paracasei* (accession number U32964.1).

Comparison of 16s-23s ISR rRNA gene of *L. casei* group and *L. paracasei* ssp. *paracasei-1*

The 16s-23s ISR rRNA gene sequences of the *L. casei* group species obtained from the data bank (accession number U32964.1, U32966.1 & AF121200.1) and shown in comparison 1. While comparing *L. casei* and *L. paracasei* sequences we saw that *L. paracasei* possess four extra bases at position no 1, 2, 220 & 221 (comparison 2). Apart from that, we observed a single base difference at 86 no. position. Then we blasted ISR sequence of *L. paracasei* ssp. *paracasei-1* with *L. paracasei* and *L. casei* in order to confirm the identification. From these blasts (comparisons 1 – 3, Table 6 & 7) we became 100% ascertain that our isolate was *L. paracasei* ssp. *paracasei-1*.

Table 6 Signature sequences between the members of *L. casei* group and *L. paracasei* ssp. *paracasei*-1

Nucleotide position ¹	1	2	69	86	126	127	131	175	177	179	180	221	222
<i>L. rhamnosus</i> (U32966.1)	C	T	T	G	T	G	A	A	T	A	T	G	G
<i>L. paracasei</i> (U32964.1)	C	T	C	R	A	T	T	G	A	-	A	G	G
<i>L. casei</i> (AF121200.1)	-	-	C	A	A	T	T	G	A	-	A	-	-
<i>L. paracasei</i> ssp. <i>paracasei</i> -1			C	R	A	T	T	G	A	-	A		

1= From the 5' end of the 16s-23s ISR rRNA sequence of *L. rhamnosus***Table 7** Comparison of 16s-23s ISR rRNA region of isolated *L. paracasei* ssp. *paracasei*-1 with *L. casei* group members

Strain	Homology with the sequence of <i>L. paracasei</i> - ssp. <i>paracasei</i> -1	Sequencing start-end position ¹	Position no. where difference were found ¹
<i>L. paracasei</i> (U32964.1)	100%	63-210	None
<i>L. casei</i> (AF121200.1)	99%	63-210	86
<i>L. rhamnosus</i> (U32966.1)	93%	63-210	69, 86, 126, 127, 131, 175, 177, 178, 179, 180,

1= Numbering position with respect to *L. rhamnosus***Comparison 1** CLUSTAL 2.1 multiple sequence alignment no. 01 (*L. rhamnosus* (U32966.1), *L. paracasei* (U32964.1) and *L. casei* (AF121200.1))

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gi|1854588|gb|U32966.1|LRU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAACCTGCACACACGAAACTTT 50
gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|          --AAGGAAACAGACTGAAAGTCTGACGGAACCTGCACACACGAAACTTT 48
*****

gi|1854588|gb|U32966.1|LRU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCCTACGGGTGCGACTTTG 100
gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCCTACGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|          GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCCTACGGGTGCGACTTTG 98
*****

gi|1854588|gb|U32966.1|LRU3296      TTCTTTGAAAACTGGATATCATTGTGTAATGTTTTAAATTGCCGAGAA 150
gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|          TTCTTTGAAAACTGGATATCATTGTATTAATGTTTTAAATTGCCGAGAA 148
*****

gi|1854588|gb|U32966.1|LRU3296      CACAGCGTATTGTATGAGTTTCTGAAATAGAAATTCGCATCGCATAAC 200
gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTGTATGAGTTTCTGAAA--AAGAAATTCGCATCGCATAAC 199
gi|5902977|gb|AF121200.1|          CACAGCGTATTGTATGAGTTTCTGAAA--AAGAAATTCGCATCGCATAAC 197
*****

gi|1854588|gb|U32966.1|LRU3296      CGCTGACGCAAGTCAGTACAGG 222
gi|1854587|gb|U32964.1|LPU3296      CGCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|          CGCTGACGCAAGTCAGTACA-- 217
*****

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Comparison 2 CLUSTAL 2.1 multiple sequence alignment no. 03 (*L. paracasei* (U32964.1) and *L. casei* (AF121200.1))

```

gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|          --AAGGAAACAGACTGAAAGTCTGACGGAACCTGCACACACGAAACTTT 48
*****

gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCCTACGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|          GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCCTACGGGTGCGACTTTG 98
*****

gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|          TTCTTTGAAAACTGGATATCATTGTATTAATGTTTTAAATTGCCGAGAA 148
*****

gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTGTATGAGTTTCTGAAAAGAAATTCGCATCGCATAACC 200
gi|5902977|gb|AF121200.1|          CACAGCGTATTGTATGAGTTTCTGAAAAGAAATTCGCATCGCATAACC 198
*****

gi|1854587|gb|U32964.1|LPU3296      GCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|          GCTGACGCAAGTCAGTACA-- 217
*****

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Comparison 3 CLUSTAL 2.1 multiple sequence alignment (*L. paracasei* (U32964.1), *L. casei* (AF121200.1) and *L. paracasei* ssp. *paracasei*-1 (Our isolate))

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gi|1854587|gb|U32964.1|LFU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAACTTT 50
gi|5902977|gb|AF121200.1|          --AAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAACTTT 48
gi|KU|gb|AFKU|                      -----

gi|1854587|gb|U32964.1|LFU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCCTACCGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|          GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCCTACCGGGTGCGACTTTG 98
gi|KU|gb|AFKU|                      -----GGGGATCACCCTCAAGCACCCCTACCGGGTGCGACTTTG 38
                                   *****

gi|1854587|gb|U32964.1|LFU3296      TTCTTTGAAACTGGATATCATTGTATTAATTGTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|          TTCTTTGAAACTGGATATCATTGTATTAATTGTTTAAATTGCCGAGAA 148
gi|KU|gb|AFKU|                      TTCTTTGAAACTGGATATCATTGTATTAATTGTTTAAATTGCCGAGAA 88
                                   *****

gi|1854587|gb|U32964.1|LFU3296      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 200
gi|5902977|gb|AF121200.1|          CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 198
gi|KU|gb|AFKU|                      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 138
                                   *****

gi|1854587|gb|U32964.1|LFU3296      GCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|          GCTGACGCAAGTCAGTACA-- 217
gi|KU|gb|AFKU|                      GCTGACGCAA----- 148
                                   *****

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DISCUSSION**Morphological, physiological and biochemical identification of collected test organism**

The isolated bacteria were grown on MRS selective media. Addition of 0.05% cysteine in MRS media increased the specificity for isolation of *Lactobacillus* spp. (Hoque et al., 2010; Hartemink et al., 1997). Small, rounded, creamy, white colony and rod-shaped cells further support our claim on morphological background. The isolate was found to be gram positive and at the same time, endospore, catalase and motility negative, summing it up to be a *Lactobacillus* sp. These characteristics can be correlated with Hoque et al. (2010), Islam et al. (2012) and Honi et al. (2013).

Extraction of chromosomal DNA

In gel electrophoresis, extracted DNA appeared as a distinct band denoting the manufacturer's isolation protocol was mild to generate enough DNA yield. Spectrophotometer revealed good quality DNA ratio was isolated with very good yield (1010-1070 µg/ml). Quantification of DNA concentration was done at 260 nm, 280 nm and 320nm.

PCR amplification of 16s-23s rRNA gene ISR of *Lactobacillus paracasei* ssp. *paracasei*-1

In this study, in order to sequence the 16s-23s ISR rRNA gene, we have used primer pairs Lp1F and Lp1R (Honi et al., 2013). The primer pairs specifically anneal and amplify 16s-23s ISR rRNA gene when correct genomic DNA was present during the thermal cycling reaction. The PCR condition was same as described by Honi et al. (2013). PCR amplification with the primer pairs followed by electrophoresis produced bands. Honi et al. (2013) reported an outcome of 146 bp band, using the same primer pairs Lp1F and Lp1R, which was in exact concordance to *L. paracasei* ssp.

Sequences of the 16s-23s ISR rRNA gene of *L. paracasei* ssp. *paracasei*-1

The internal spacer region of 16s-23s rRNA gene sequence of *Lactobacillus* genus is 221 bp long (Tilsala et al., 1997). Sequence signals of pGEM vector confirmed the reliability of our hardware and the protocol used in the sequencing purpose. We have obtained 148 nucleotides sequences of the 16s-23s ISR rRNA gene homologous to position no. 63 to 210 of the *L. paracasei* ssp. *paracasei*-1 using PCR DNA-sequencing method. There is scope, for the future, to improve the primer design so that it can bind more upstream position of the 16s-23s ISR region of *L. paracasei* ssp. *paracasei*-1.

Comparison of 16s-23s ISR rRNA gene of *L. casei* group

There have been lots of controversies over the identification of *L. casei* group (*L. casei*, *L. paracasei* and *L. rhamnosus*) (Ward et al., 1999). A few species of *Lactobacillus*, including species of *L. casei* group, may be recovered from a variety of diverse habitats because of their ecological adaptability. It is empirical that bacterial genome adaptation and evolution occur via three major processes:

1) decay of genes that no longer offer a fitness benefit 2) gaining of exogenous genes or gene clusters by bacteria through horizontal gene transfer (HGT) and 3) modification of existing genes by mutation with vertical inheritance (Broadben et al., 2012). In our previous experiment (Honi et al., 2013) we supported that the bacteria reported by Hoque et al. (2010) and Islam et al. (2012) was *Lactobacillus paracasei* ssp. *paracasei*-1. We obtained PCR amplification of 16s-23s ISR rRNA gene based on which we reported the isolate as *Lactobacillus paracasei* ssp. *paracasei*-1. Because of the taxonomic dispute and close genetic similarities, however, in this study we extrapolate that the isolate could be one of the members of *L. casei* group rather than *L. paracasei*. We wanted to confirm the identification based on nucleotide sequencing, more specifically by sequencing of 16s-23s ISR rRNA gene, which provides an accurate basis for identification (Tannock et al., 1999). In doing so, we had to compile the sequence signature of 16s-23s ISR rRNA genes of *L. casei* group obtained from the NCBI data bank (comparison 1). By comparing 16s-23s ISR rRNA gene (217 to 222 bp) across the three members of *L. casei* group we have identified thirteen signature sequence locations. Mori et al. (1997) also reported thirteen signature sequences in *L. casei* group members by comparing, however, 1.5 Kbp long 16rDNAs of *L. casei* group. The high frequency of variability between 16s-23s ISR rRNA gene could have evolved because of the higher sequence and fragment length polymorphism (Felis et al., 2001). From the determined signature sequences between these three species of *L. casei* group, we were confirmed that our isolated stain (*L. paracasei* ssp. *paracasei*-1) was *L. paracasei*.

Between *L. paracasei* (U32964.1) and *L. casei* (AF121200.1) we have observed five signature sequence for 16s-23s ISR sequence, based on *L. paracasei* numbering, chronologically which were base position no. 1, 2, 86, 220, and 221 (comparison 2). The base pair location 86, based on *L. paracasei* numbering, appeared to be a critical signature sequence for separating *L. casei* and *L. paracasei*. It suggests that in order to differentiate *L. paracasei* from *L. casei*, based on 16s-23s ISR rRNA gene sequencing, we have to confirm the base pair of location no. 86. When we blasted the sequence of 16s-23s ISR rRNA gene of *L. paracasei* ssp. *paracasei*-1 with strain U32964.1 (*L. paracasei*) and AF121200.1 (*L. casei*) we have found only difference at position no 86 (comparison 3). In *L. paracasei* ssp. *paracasei*-1 sequence it was R (A or G) which matched with *L. paracasei* strain. The result of comparison-3 (*L. paracasei* ssp. *paracasei*-1, *L. casei* and *L. paracasei*) reveals that our isolate was *L. paracasei* ssp. *paracasei*-1.

CONCLUSION

Our study shows that comparison of the percentages of similarity between 16s-23s ISR rRNA gene sequences of lactobacilli provides a practical method of strain identification. The small 16s-23s ISR rRNA gene spacer sequences of lactobacilli are around 200 bp in length. These relatively short sequences can be easily sequenced on both polynucleotide strands and provide reliable information for comparative bacterial identification research. Moreover, sequencing of the spacer region restrains the advantage in distinguishing *Lactobacillus* spp from the members of *L. casei* group which cannot be accomplished by comparison of 16S V2-V3 region sequences. The findings of this research is a very strong bolstering evidence for the research works of Islam et al. (2012) and Honi et al. (2013) who also identified the bacteria, in different approaches though, as *L. paracasei*

ssp. *paracasei*-1. We suggest that while differentiating between *L. caesi* and *L. paracasei*, the base position no. 86, based on *L. paracasei* numbering, can solve the identification dilemma. Purine (R) base at position no. 86 confirms the strain as *L. paracasei*. Similarly, it can be suggested that other closely related bacterial group, as like *L. casei* group, can be separated from the group members by sequencing 16s-23s ISR rRNA gene because of the higher sequence and fragment length polymorphism. The series of our research activities (Hoque et al., 2010; Islam et al., 2012 and Honi et al., 2013), including this one, can be utilized in screening potential probiotic bacteria for food and pharmaceuticals application.

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REFERENCE

- Barry, T., Collieran G., Glennon M., Dunican L.K., Cannon F. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria, PCR Methods Appl, 1, 1, 51-56. <http://dx.doi.org/10.1101/gr.1.1.51>
- Broadbent J.R., Neeno-Eckwall E.C., Stahl B., Tandee K., Cai H., Morovic W., et al. 2012. Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation, BMC Genomics, 13, 533. <http://dx.doi.org/10.1186/1471-2164-13-533>
- Chen H., Lim C.K., Lee Y.K., Chan Y.N. 2000. Comparative analysis of the genes encoding 23S-5S rRNA intergenic spacer regions of *Lactobacillus casei*-related strains, Int J Syst Evol Microbiol, 50, 471-478. <http://dx.doi.org/10.1099/00207713-50-2-471>
- Collins M.D., Phillips B.A., Zanoni P. 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov., Int J Syst Bacteriol, 39, 105-108. <http://dx.doi.org/10.1099/00207713-39-2-105>
- Coudeyras S., Marchandin H., Fajon C., Forestier C. 2008. Taxonomic and strain-specific identification of the probiotic strain *Lactobacillus rhamnosus* 35 within the *Lactobacillus casei* group, Appl Environ Microbiol, 74, 9, 2679-2689. <http://dx.doi.org/10.1128/AEM.02286-07>
- Dubernet S., Desmasures N., Guéguen M. 2002. A PCR-based method for identification of *lactobacilli* at the genus level, FEMS Microbiol Lett, 214, 271-275. <http://dx.doi.org/10.1111/j.1574-6968.2002.tb11358.x>
- Felis G.E., Dellaglio F., Mizzi L., Torriani S. 2001. Comparative sequence analysis of a *recA* gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group, Int J Syst Evol Microbiol, 51, 2113-2117. <http://dx.doi.org/10.1099/00207713-51-6-2113>
- Flint J.F., Angert E.R. 2005. Development of a strain-specific assay for detection of viable *Lactobacillus* sp. HOFG1 after application to cattle feed, J Microbiol Methods, 61, 2, 235-243. <http://dx.doi.org/10.1016/j.mimet.2004.12.002>
- Hartemink R., Domenech V.R., Rombouts F.M. 1997. LAMVAB - A new selective medium for the isolation of lactobacilli from faeces, J. Microbiol. Meth., 29, 77-84. [http://dx.doi.org/10.1016/S0167-7012\(97\)00025-0](http://dx.doi.org/10.1016/S0167-7012(97)00025-0)
- Honi U., Sabrin F., Islam T., Islam M.E., Billah M.M., Islam K.D. 2013. Enzymatic activity and antibiotic resistance profile of *Lactobacillus paracasei* ssp. *paracasei*-1 isolated from regional yogurts of Bangladesh, J Microbiol Biotechnol Food Sci, 3, 3, 235-239
- Hoque M.Z., Akter F., Hossain K.M., Rahman M.S.M., Billah M.M., Islam K.M.D. 2010. Isolation, identification and analysis of probiotic properties of *Lactobacillus* Spp. from Selective regional yoghurts. World J Dairy Food Sci, 5, 1, 39-46
- Islam T., Sabrin F., Islam M.E., Billah M.M., Islam K.M.D. 2012. Analysis of antimicrobial activity of *Lactobacillus paracasei* ssp. *paracasei*-1 isolated from regional yogurt, Int Res J Appl Life Sci, 1, 4, 66-72
- Kisworo D., Elegado F.B., Barraquio V.L. 2008. Phenotypic and genotypic characterization of probiotic bacteria isolated from probiotic dairy products in the philippines, Phil J Sci, 137, 1, 77-83
- Mori K., Yamazaki K., Ishiyama T., Katsumata M., Kobayash K., Wiwai Y., et al. 1997. Comparative sequence analyses of the genes coding for 16s rRNA of *Lactobacillus casei*-related taxa, Int J Syst Bacteriol, 47, 1, 54-57. <http://dx.doi.org/10.1099/00207713-47-1-54>
- Salveti E., Torriani S., Felis G.E. 2012. The Genus *Lactobacillus*: A Taxonomic Update, Probiotics & Antimicro. Prot., 4, 217-226. <http://dx.doi.org/10.1007/s12602-012-9117-8>
- Skelin A., Fuka M.M., Majhenic A.C., Redzepovic S., Samarzija D. and Matijasic B.B. 2012. Phenotypic and genotypic characterization of indigenous *Lactobacillus* community from traditional istrian ewe's cheese, Food Technol. Biotechnol, 50, 3, 362-370
- Tannock G.W., Tilsala-Timisjarvi A., Rodtong S., Ng J., Munro K., Alatossava T. 1999. Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons, Appl Environ Microbiol, 65, 9, 4264-4267

- Tilsala-Timisjarvi A., Alatossava T. 1997. Development of oligonucleotide primers from the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR, Int J Food Microbiol, 35, 1, 49-56. [http://dx.doi.org/10.1016/S0168-1605\(97\)88066-X](http://dx.doi.org/10.1016/S0168-1605(97)88066-X)
- Ward L.J.H., Timmins M.J. 1999. Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction, The Soc Appl Microbiol, Lett Appl Microbiol, 29, 90-92. <http://dx.doi.org/10.1046/j.1365-2672.1999.00586.x>