Genomic-Based Restriction Enzyme Selection for Specific Detection of *Lactobacillus rhamnosus and Lactobacillus plantarum* strain by 16S rDNA PCR-RFLP

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Abstract

Probiotics are discovered from the various natural sources from time to time as the functional food. Lactobacillus (LAB) is found because the most preferred genera during this direction with its utility within the dairy and allied sciences. The two isolates of *L. plantarum* and *L. rhamnosus* were isolated from the sheep milk. These strains are identified by Genomic-Based Restriction Enzyme Selection by digestion of *Hind III, EcoR1, BAM H e*nzymes to get a restriction profile using PCR technique. The PCR-RFLP method by using *Hind III, EcoR1, BAM H1* enzymes assist to establish the good correlation between the two Lactobacillus strains.

Keywords: *L. plantarum*, *L. rhamnosus*, PCR technique, Sheep milk.

1. Introduction

The current science of the nutraceutical world deals with the investigation of the new sorts of functional foods as an isolate for the betterment of the mankind [1]. The utilization of the microbes as functional food has explored its use in life science [2,3]. Probiotics are discovered from the various natural sources from time to time because the functional food lactobacillus (LAB) is found as the most preferred genera during this direction with its utility within the dairy and allied sciences [4–7]. The foremost difficult task to use these microbes because the functional food is studying the expansion parameters along side the genomic analysis [8]. The current study deals with the genomic analysis of microbial samples obtained from sheep milk and comparing the results with the existing culture of Lactobacillus.

2. Material and method

Milk of different animals were used to isolate the lactic acid bacteria. Frozen isolates were revived from the glycerol stock, thawed and re-inoculated into freshly prepared sterile MRS lactobacilli broth and incubated at 37 °C for 24 h [9]. After confirmation of purity, 10 µl of active broth culture was reinoculated into 10 ml sterile MRS broth and incubated at 37 °C for 10 h. Two-milliliter aliquots of active log phase cultures from this broth were then used to isolate genomic DNA from the processed sample (MRS lactobacilli broth). The bacteria were collected by centrifugation at 5200 rpm for 5 minutes in a refrigerated centrifuge. The supernatant was decanted from the medium along with washing of pellet using 2 ml of NaCl thrice with EDTA (25 mM NaCl, 4 mM EDTA, at pH 8.0). Later, 100 ul freshly prepared lysozyme solution was mixed (concentration 10 mg/ml in NaCl-EDTA) and incubated at 37 °C for 60 min. 4 ul of RNase-A solution (10 mg/ml, working concentration 100 µg/ml) was used to remove the RNA before incubation. The final volume was made up to 500 microliters by addition of NaCl-EDTA, 12 ul of proteinase solution (17 mg/ml) and 50 ul of a 10% SDS solution. The contents were completely mixed and incubated at 50 °C for 55 min. After incubation, an equal volume of phenol saturated with Tris (pH 8.0) was mixed and centrifuged at 11,000 rpm at 21 °C for 600 s. The upper aqueous phase was carefully removed free from proteins and cellular residues. This phase was repeated once with a new aliquot of the phenol-chloroform mixture (1: 1) with a collection of the supernatant. The DNA in the supernatant was precipitated using 0.7 volumes of isopropanol along with 0.3 M sodium acetate (pH 5.5). The obtained DNA was pelletized by centrifugation at 9,000 rpm at 4 °C for 6 min. The obtained DNA in precipitated form was collected and dried by washing with a 71% ethanol solution. Furthermore, the granules formed were dissolved in 50 ul of Tris-EDTA (10: 1, pH 8) and stored at -18 ° C. In about three isolates were tested by using a commercial kit with the protocol suggested by the manufacturer. The DNA was stored frozen at -20 °C until use [10].

A) Amplification of 16 S rDNA region of LAB

2 µl of genomic DNA was mixed with 48 µl of PCR mixture as per the instruction mentioned in the manufacturer user kit [11]. 50 µl of the final reaction mixture was taken to the PCR steps (Bio-Rad T 100 PCR, USA). The amplification was carried by using the EGE1 forward and EGE2 reverse primer of 16S rDNA region from the isolates. The forward primer is complementary to the 5'end of 16S rDNA and the reverse primer is complementary to the 3' end of the 16S rDNA region.

Forward Primer: EGE1: 5'-AGGAGTTTATCCTGGCTCAG-3'

Reverse Primer: EGE2: 5'CTTACGGCACCTTGTTACGA-3'

The PCR Conditions:

Step 1: 94°C for 360 s

Step 2: 94°C for 60 s (denaturation)

Step 3: 56°C for 60 s (annealing)

Step 4: 72°C for 60 s (elongation)

Step 5: 72°C for 600 s

Table 1. PCR mixture composition

Sr. no	Media ingredient	Formula (µl)
1	Mg-freeTaq DNA polymerase buffer	5
2	MgCl2 (25Mm)	3
3	Sterile deionized water	33
4	Oligo forward 10 picomole/µl	1
5	Oligo reverse 10 picomole/µl	1
6	dNTP (2 mM each)	5
7	DNA	2
Total		50

B) Separation of amplified PCR products

a) Preparation of agarose gel

0.8% prepared agarose gel was dissolved in boiling 100 ml TAE buffer. The gel was cooled at 45 °C with the addition of 15 µl ethidium bromide solution (9.9 µg/ml) was added. The agarose gel was placed into the gel casting stand along with the combs. The combs were removed after getting the rigid gel and used for loading the DNA samples [12].

b) Loading of Agarose Gel

2 µl loading dye was added to 5 µl of PCR products which was introduced into wells. The first well was loaded with the DNA size-marker (1 kb, Fermentas) to observe the amplification range.

c) Electrophoresis of the products

The electrophoreses of the PCR products were carried out at 79 mA for 40 min. The visualization of the PCR products was carried out by a gel documentation system (Vilber-Lormat). The amplication of the DNA fragments observed in the range 1500-2000 bp shows the proper output of the amplification.

d) Purification of PCR products

The restriction enzymes were used for PCR products prior to digestion.
The purification procedure carried out were as follows:

• 50 µl 1XTE buffer was used to PCR product to make up the volume to 100 µl
• Addition and mixing of 200 µl chloroform solution was carried out in duplicate
• The formed solution was centrifuged at 5.000 rpm for 11 min
• The upper aqueous phase was mixed with the 0.l volume of 3 M sodium acetate (pH 5.2) solution
• Addition and mixing of 200 µl 99% ethanol solution was carried out in duplicate along with the centrifugation at 8.000 rpm for 10 min
• The supernatant was discarded and the formed DNA pellets were added with 500 µl of 70% ethanol
• The formed solution was centrifuged at 5.000 rpm for 11 min
• The formed pellet were dried at 37 °C for 10 min
• The formed DNA is dissolved into 50µl 1X TE solution
• DNA was stored as a solution at -20 °C.

e) DNA sequencing

The two isolates were sent out for 16S rRNA gene molecular identification, by illumination Nextseq platform, Netherlands. The results obtained after sequencing were then BLAST using the BLASTn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and identification of individual isolates were done. To elucidate the phylogenetic relation of our isolated *Lactobacillus*, we retrieved the 16S rRNA sequences of other *Lactobacillus* species from GenBank nucleotide database and the phylogenetic tree was constructed using the Maximum likelihood method.

C) Restriction fragment length polymorphism (RFLP)

11 µl of purified amplification PCR products were used for each of the restriction enzyme digestion. Three different enzymes *Bam HI*, *Hind* III, and *EcoR*I are used for enzyme digestion study. 50 µl final reaction volumes were used for digestion; for *Bam HI* at 65 °C, for *Hind* III and *EcoR*I, it was 37 °C. All of the reactions were performed overnight and additionally *Bam HI* restriction reactions were overlaid with mineral oil to avoid evaporation[13].

Table 2. RFLP reaction mixture composition

Sr. no	Media ingredient	Formula (µl)
1	Restriction enzyme buffer	5
2	Sterile deionized water	34.5
3	Restriction enzyme (5U)	0.5
4	DNA	10
	Total	50

a) Electrophoresis of restriction fragments

1.6% agarose gel was used for separation of restricted fragments.

b) Preparation of agarose gel

2.4 g agarose gel was dissolved in boiling 150 ml TAE buffer. The gel was cooled at 45 °C with the addition of 22 µl ethidium bromide solution (9.9 µg/ml) was added. The agarose gel was placed into the gel casting stand along with the combs. The combs were removed after getting the rigid gel and used for loading the DNA samples

c) Loading of agarose gel

In electrophoresis tank, the solidified agarose gel was placed with the pouring of 1.5 X TAE buffer containing 300 µl of ethidium bromide. 2 µl of gel loading dye was added to 11 µl solution of digestion products. The loading of the samples was carried out from the second well on the gel. 2 µl (500 ng) of DNA was loaded to the first well on the gel with the molecular weight marker from 100 bp to 1kb.

e) Electrophoresis of the products

The electrophoreses of the samples were carried out at 60 mA for 29 min and at 80 mA for 4 h. The visualization of the amplified products was carried out by a gel documentation system (Vilber-Lormat).

f) Interpretation of results

The images obtained by gel documentation were modified in Adobe Photoshop 7.0. using BIO-ID.++software (Vilbeer-L'ourmat). The strains similarities were observed automatically by using the formula of Jaccard. The unweighted_pair_group method was used for strain clustering studies using arithmetic averages and UPGMA, BIO-ID++ techniques. 10% of homology were prepared for the homology coefficient studies.

3. Result and Discussion

Evaluation of amplification and digestion regions of 16S r DNA region of LAB

Genomic DNAs of the isolates are visualized by agarose gel electrophoresis under UV-light (Fig. 1). Then they are taken to the PCR step.

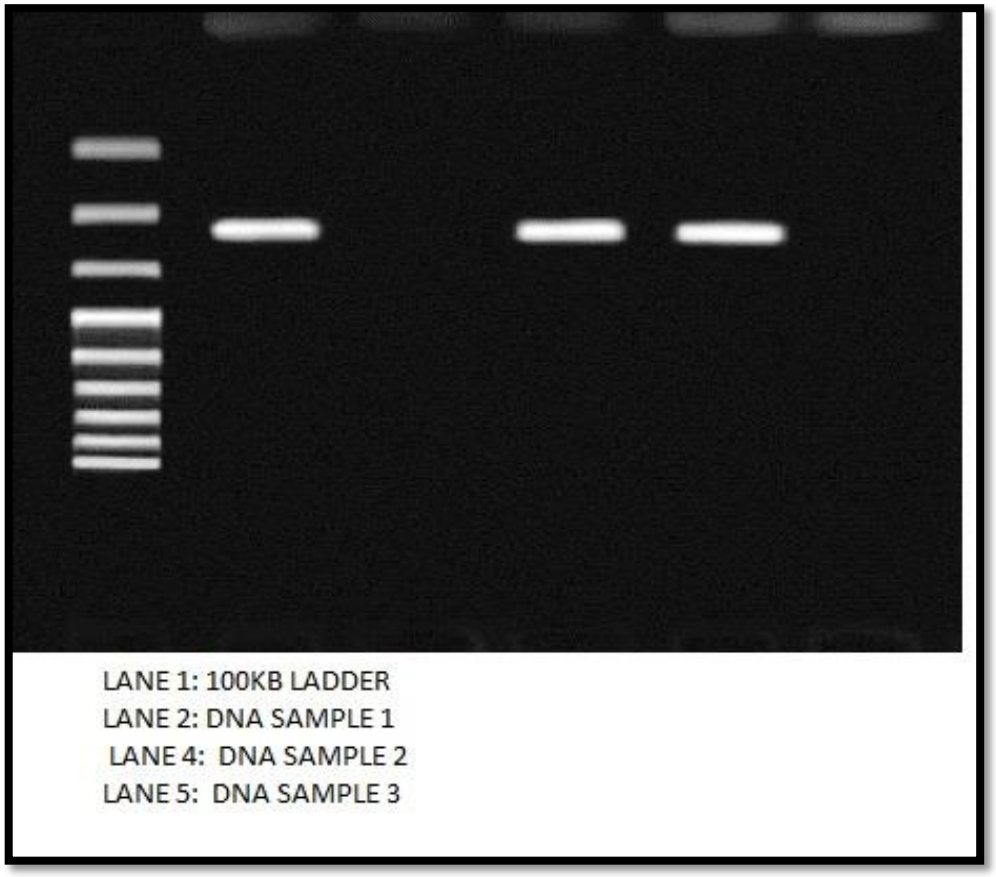

Fig. 1. DNA isolation Lactobacillus with the reference strain

b) Amplification of 16S rDNA region

After DNA isolation the 16S rDNA region is amplified by PCR protocol [14,15]. Then 50 µl of PCR products are visualized by agarose gel electrophoresis under UV-light. The length of the amplification products varied from 1850 to 2000 bp (Fig 2).

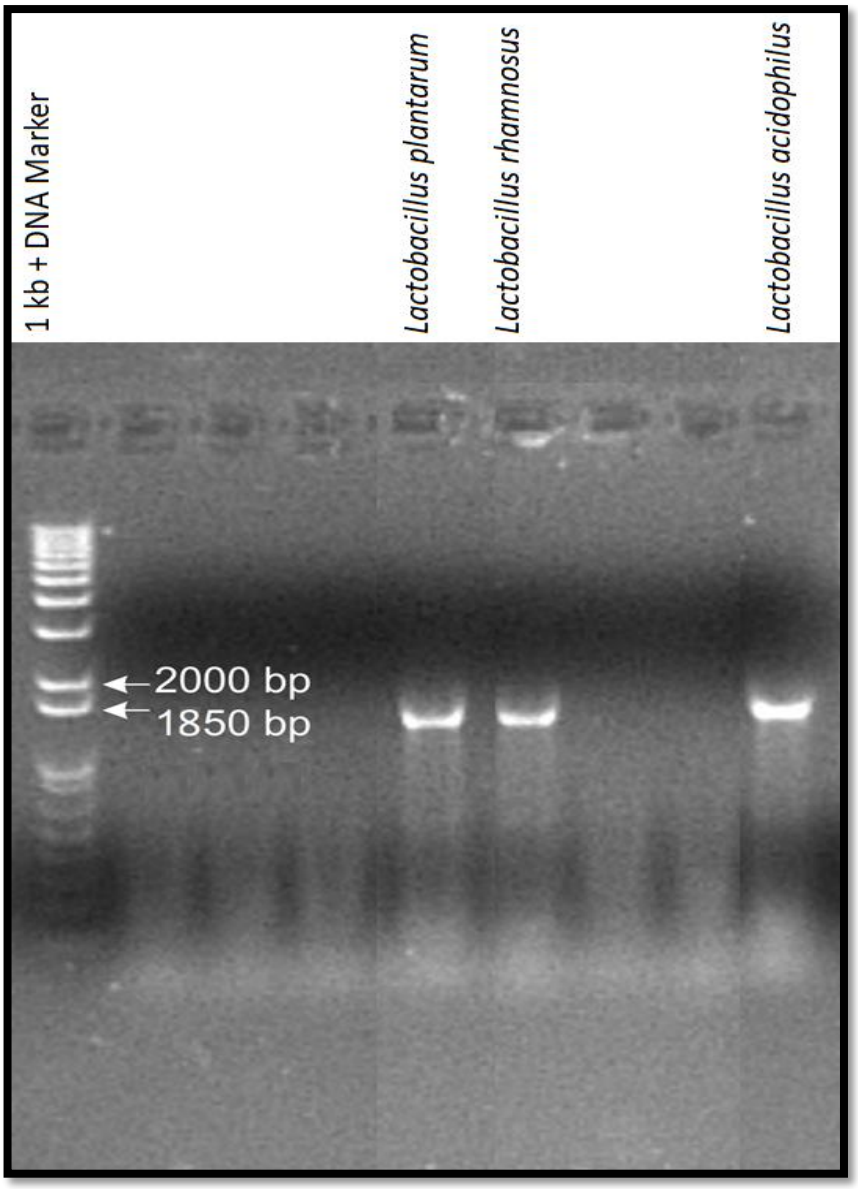

Fig 2. 16S Amplification products of isolates and reference strains

Identification of avian LAB isolates by 16S rRNA sequencing carried out by BLAST (Basic local alignment search tool) shows that sample A with 98% similarity with *Lactobacillus plantarum (Lp)*. Similarly, sample B shows a 96% similarity with *Lactobacillus rhamnosus (Lr)*. The correct identification of LAB with the accurate method and precision; having fast high discriminatory power is achieved by 16S rRNA gene sequencing [14].

Thus *L. plantarum* determined from (lane 5), *L. rhamnosus* (lane 6), and *L. acidophilus* (lane 9) used as the reference standard and are compared with 1kb DNA ladder gene ruler^{TM}.

c) Digestion of amplified 16S rDNA region by *Hind III, EcoR1, Bam H1*

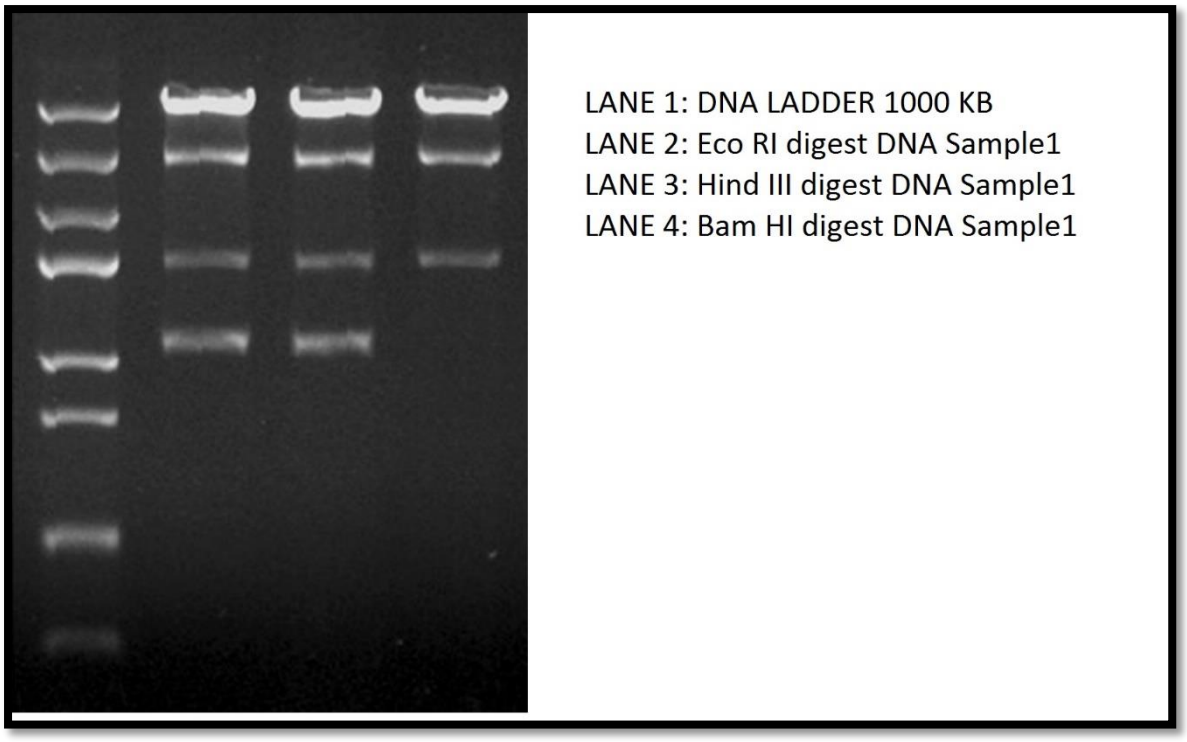

Fig 3. *Hind III, EcoR1, BAM H1* digests of *Lactobacillus plantarum*

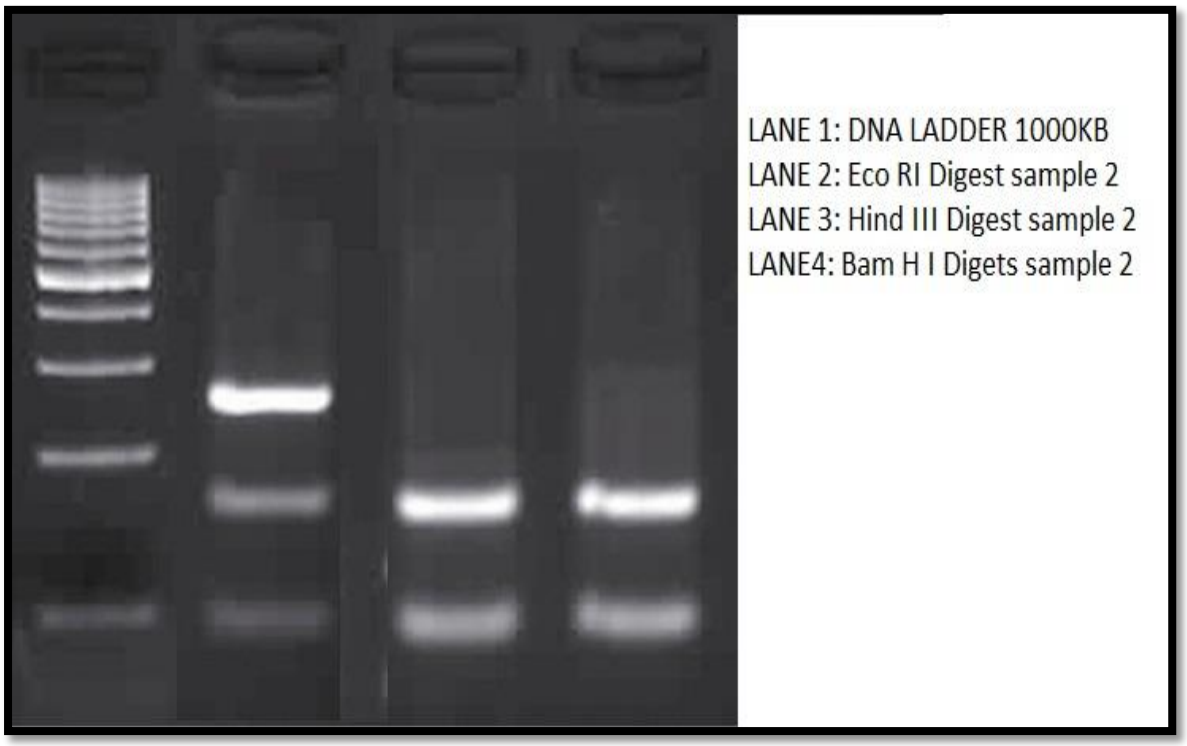

Fig 4. *Hind III, EcoR1, BAM H1*digests of *Lactobacillus rhamnosus*

The two isolates of *L. plantarum* (Fig 3) and *L. rhamnosus* (Fig 4) are digested by *Hind III, EcoR1, BAM H e*nzymes to get a restriction profile. It could be concluded that PCR-RFLP method by using *Hind III, EcoR1, BAM H1* enzymes revealed a good correlation between the two Lactobacillus strains. Because of the absence of some reference strains, isolates were identified by 16SDNA sequencing and BLAST in earlier studies [16].

4. Conclusion

The DNA of the strains were isolated and analysed for the 16 S rDNA investigation by taking standard reference strain of *L. acidophilus* (La). On the basis, amplification and digestion regions of 16S r DNA region of lactic acid bacteria these culture were identified as *L. plantarum* and *L. rhamnosus* and deposited the culture in NCIM, Pune as MCC 3595 and 3594 respectively.

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