

GENETIC DIVERSITY OF *Lactobacillus* spp. OF NATURAL ETHAWAH GOAT MILK-FERMENTED WAS DETERMINED BY USING 16SRDNA WITH DDGE ANALYSIS

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ABSTRACT

The aim of this research is to characterize of *Lactobacillus* spp. from natural Ethawah goat milk-fermented were using 16SrDNA by denaturation gradient gel electrophoresis (DGGE) analysis. Goat and bovine (as negative control) milk naturally fermented among 24hour until 6 days. Morphological and biochemistry of bacteria were characterized by standard methods. The total DNA of bacteria were isolated using alkali lysis, PCR amplification was carried out using 3 pairs of specific primers, DNA-amplified using DGGE and then to determine the relationship among Lactobacteria using NTSys package software V2.0. Phenotypical and biochemical study showed that the 11 strains are belonging to genus *Lactobacillus*. The dendrogram results show all of isolates had similarity characters with genus *Lactobacillus* around 56-76%. According to morphological and DDGE profiles, we were identified that bacteria isolate of goat milk-fermented are K1A, and K3A are *L. casei*, and bacteria isolate K3B is *L. plantarum*.

Key words: Ethawah goat, *Lactobacillus*, natural milk-fermented

INTRODUCTION

Fermented milk products have been thoroughly characterized for its beneficial effect and unique flavor. Lactobacilli generally dominate the non-starter lactic acid bacteria (NSLAB) population in milk. Heterogeneity of NSLAB strains with unique and diverse properties represent a key factor for improving authenticity of traditional milk, when compared to commercially available starter strains (Abdullah & Osman, 2010).

Lactobacillus is organism plays a major role in making the intestine a home for beneficial organism and a barrier for pathogens. It is largely found as the dominant species in the last step of natural food raw- material fermentation, including a variety of vegetables, meat and milk (Daeschel et al., 1987).

Molecular-based methods and specific primers and probes that target genes coding for the 16S rDNA have allowed precise description and identification of *Lactobacillus* strains that dominate the fermentation process and ripening of raw milk (Sweringen et al., 2001). *Lactobacillus* group-specific primers were used to amplify the V1 to V3 regions of the 16S rDNA gene. Our study will identify the bacteria growth on goat and bovine milk naturally fermented by using morphological characteristics and molecular profile on 16SrDNA by DDGE analysis.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

Raw unpasteurized milk samples of bovine were collected from the local area of Coimbatore during lactation period under aseptic conditions in a sterile screw cap tubes, processed within three hours and used for further studies. Milk samples were serially diluted in peptone medium and incubated at 23°C for 30 min before plating by which 50% of recovery of Lactic acid bacteria (LAB) was increased. Diluted samples were plated onto De Man Rogosa Sharpe (MRS) medium for *Lactobacillus* isolation and incubated at 37°C for 48-72 h. Well-isolated colonies with typical characteristics namely pure white, small (2 - 3 mm diameter) with entire margins were picked from each plate and transferred to MRS broth (Bovine and Steel, 1993).

Identification of lactic acid bacteria (LAB)

Identification of the *Lactobacillus* was performed according to their morphological, cultural, physiological and biochemical characteristics (Kandler and Weiss, 1986; Sharpe et al., 1979): Gram reaction, production of catalase, carbohydrate fermentation patterns, growth at 15°C and 45°C in the lactobacilli de Man Rogosa and Sharpe (MRS) broth. Purified cultures were maintained at -20°C in MRS broth with 10% glycerol and enriched in MRS broth by incubating at 37°C for 24 h. the positive control used *L. casei* and *L. plantarum* (Kusmiyati et al., 2014).

DNA Isolation of Bacteria

Bacterial DNA from goat and bovine natural fermentation milk samples was isolated according to alkaline lysis method of Villalobos et al (2004). Quality & quantity DNA were measured by using NanoDrop spectrophotometer and 1% agarose gel electrophoresis.

DNA amplification and Sequencing

Polymerase chain reaction (PCR) analyses were carried out with paired primer Eub 16S rDNA 7-f1,7-f2, 7-f3, and Lact S-G-Lab-0677-r. The PCR reaction was prepared with mixture of 5 µL PCR master mix 2x solutions, 1 µL of each primer, 2 µL ddH₂O, and 1 µL DNA (100-250 ng/µL). DNA was amplified using Master Cycler Gradient Eppendorf. The PCR program started with hot start 94°C for 3 min, denaturation 94°C for 30 s, annealing 52°C for 30 s, extension 68°C 1.5 min (30 cycles) and final extension 68 °C 7 min (Randazzo et al., 2002).

DGGE (Denaturing Gradient Gel Electrophoresis)

DGGE analysis of the PCR products was performed with a DCode universal mutation detection system (Bio-Rad, Hercules, USA). The 6% (w/V) polyacrylamide gel (acrylamide/bis-acrylamide ratio, 37.5 : 1) was made with a denaturing gradient range between of 30% - 60% (A 100% denaturants corresponding to 7 mol/L urea and 40% formamide). The electrophoresis was performed at a constant voltage of 100 V for 16 h at 60°C in 1xTAE buffer (40mmol/L Tris base, 20mmol/L Acetic acid, 1mmol/L pH 8.0 EDTA). After electrophoresis, gels were stained with ethidium bromide (0.5 mg/L) for 30 min, destained with TAE, and photographed under UV light.

Data Analyses

Morphological similarities measured by similarity index, and were used to construct a dendrogram using UPGMA (Un-weight pair group method with arithmetic average). All the statistical analyses were carried out using the NTSYSpc 2.1 version (Exeter Software, Setauket, NY).

RESULTS

The morphological analysis was determined by 18 characters as well (data not shown). All of the characters were conferred into character states. Character states data were converted to binary digits that formed the source of data for phylogenetic analysis. The phylogenetic tree based on UPGMA analysis of the phenotypic data comprising 18 character states indicated a similarity among the 11 bacteria isolate and 2 reference isolate at the 54 % level (Figure 1).

Nevertheless, it was interesting to note that there were two sister-clade (automorphy 1 and 2) on sub-clade A (apomorphy A) that suggested of *Lactobacillus* family. The first sister-clade consisted of LA, S1A, K1A, and K3A with 76 % degree of similarity. The second sister-clade consisted of LB, K3B, S2A, K1B, and K2A; five of them had 77 % degree of similarity. Sub-clade B (apomorphy B) consisted of S1B, S3B, K2B, and S3A most likely the genus *Lactobacillus* because had 54 % degree of similarity with sub-clade A.

T K3B have a single band. Position band of bacteria isolates S1A, K1A, K3A, and K3B equal to reference isolate *L. casei* while isolate K3B equal to *L. plantarum*. Bacteria isolate S1A, K1A, and K3A are *L. casei*, while bacteria isolate K3B is *L. plantarum*. Species determination of bacteria isolate is caused by similarity of band position of migration with reference isolate. Isolates S1B, S2A, S3A, S3B, K1B, K2A, and K2B are cannot be identified because the position of migration is not same migration with reference isolates and used reference isolate are limited. Those seven isolate are high potentially included as genus *Lactobacillus* sp. especially based on their morphological characteristic.

DISCUSSIONS

Similarity value bacteria isolates and reference isolates in the similarity value of the specified to classify an isolates to a species. Isolates can be classified into a specific species if the similarity value of 80-85% (OECD, 1997). Both sub-clade had the same characters, and called sinapomorphy characters. Sinapomorphy characters for both sub-clades were gram-positive, rod, negative endospore, catalase and oxidase. Morphological data had limitations; however, the morphological analysis was very useful for field analysis. The morphological analysis established in bacteria phylogeny.

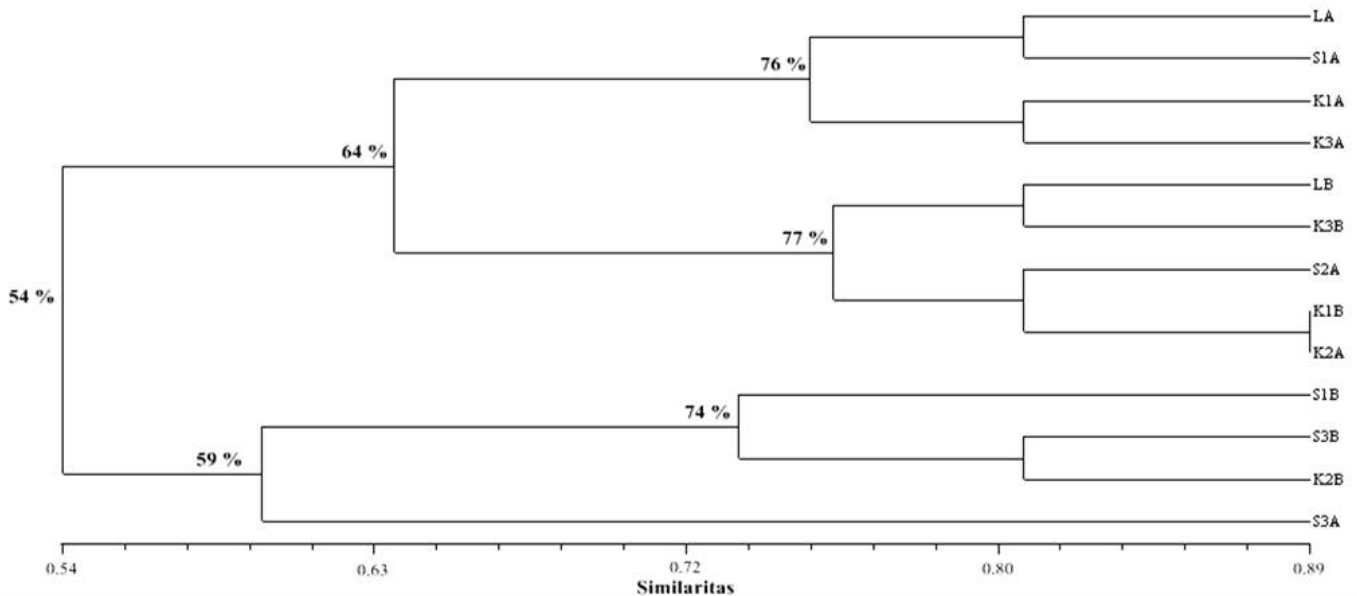


Figure 1. A dendrogram of phenotypical characteristics in variant of *Lactobacillus* spp. (LA: *L. casei*; LB: *L. plantarum*; S1A, S1B, S2A, S3A, S3B: bacteria isolate from bovine milk; K1A, K1B, K2A, K2B, K3A, K3B: bacteria isolate from goat milk). Dendrogram was carried out using NTSyspc software with UPGMA algorithm.

PCR Product bacteria isolates S1A, S1B, S2A, S3A, S3B, K1A, K1B, K2A, K2B, K3A, and K3B shows that one bands size ± 450 bp with same position of migration (Figure 2). Band of reference isolate *L. casei* (LA) and *L. plantarum* (LB) on PCR-DGGE are used to marker identification bacteria isolates because commonly used in fermented milk (Figure 3). The bands as result of PCR-DGGE are different, band of reference isolate LB is above LA.

Band of PCR-DGGE indicated bacteria isolates S1A, S1B, S2A, S3A, S3B, K1A, K1B, K2A, K2B, K3A, and

Clustal-X alignment program showed amplification of 16S rDNA gene size 463 bp, so there is a difference between PCR products and alignment. Band from PCR products differentiate isolates so that research was conducted PCR-DGGE. This difference is caused by the amount of GC base of *L. plantarum* is more than *L. casei*. DNA sample which has high contained of GC is harder to migrate, so it will move slower than DNA sample with low GC. DNA double string is harder to be separated because GC has three hydrogen bonds (Chen et al., 2008). Primary our study was used partially

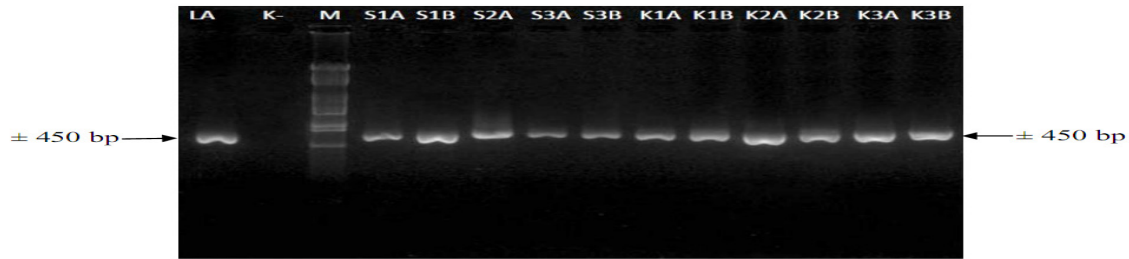


Figure 2. Polymerase Chain Reaction (PCR) visualization result of *Lactobacillus* sp. 16S rDNA gene amplified sequence from goat and bovine milk on 1,5% agarose gel.

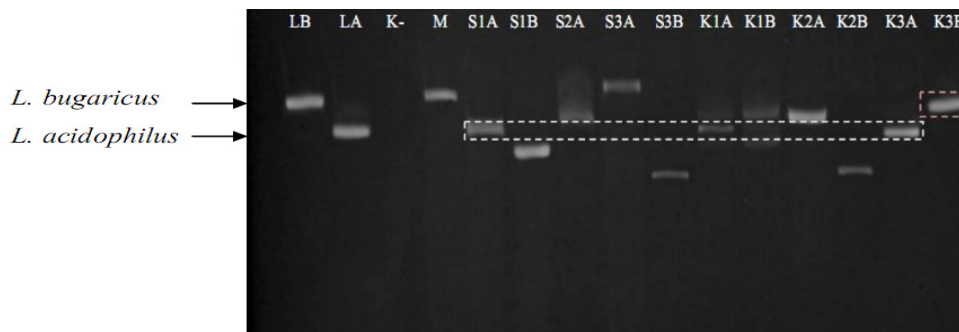


Figure 3. DGGE profiles of references isolate *Lactobacillus* (LA is *L. casei* and LB is *L. plantarum*) and bacteria isolate from bovine and goat milk with denaturing gradient from 30% - 60%.

bacteria with 1-600 bp which is nucleotide sequence on V1-V3 region of the 16S rDNA gene. Topology of the phylogenetic tree shows all test isolates is one clade genus *Lactobacillus* to vary on 97-99% similarity (Kusmiyati et al., 2014).

PCR-DGGE by using 16S rDNA gene sequence can be used as DNA barcoding. According to Walter et al, (2000) it is known that discriminative strength from PRC-DGGE technique can reach species level and easily distinguish *L. delbrueckii* species until the subspecies level (Chen et al., 2008). Chen et al, (2008) also stated that there is no difference observed on species identification based on sequencing result with PCR-DGGE product on bacteria and fungi determination. Temmerman, et al, (2004) reported, if different species are too many in DGGE gel pattern, and the amount of reference isolate are limited, then it will required advance analysis such as sequencing for identification.

PCR-DGGE result with two reference isolate indicates that *L. casei* bacteria are found in naturally fermented goat and bovine milk, while *L. plantarum* are only found in naturally fermented goat milk. Fermented milk product in the first 24 h is dominated by hetero fermentative BAL such as *L. fermentum* but after 24 h, fermented milk product is dominated by homo fermentative BAL (*L. plantarum*, *L. casei*, *L. delbrueckii* and *L. acidophilus*) (Ampe et al., 2001).

CONCLUSIONS

The most of bacterial colony growth on natural goat milk-fermented are the genus *Lactobacillus*. Bacteria isolate of goat milk-fermented are K1A, and K3A are *L. casei*, and bacteria isolate K3B is *L. plantarum*. Isolates other test species can not be known because of the limitations of reference isolates that require additional reference isolates and further analysis to determine the species.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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