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Partial Analysis 16S rRNA Gene in Lactobacillus spp. from Natural Fermented Milk

Doğal Fermente Olan Sütteki Lactobacillus Spp.16S rRNA Geninin Kısmi Analizi

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ABSTRACT

Purpose: The aim of this research is to analysis *16S rRNA* gene sequence of *Lactobacillus* spp. from natural fermented milk.

Material and Methods: The methods that used in this study are total DNA isolation using alkaline lysis, PCR amplification carried out using of specific primers, and identification of amplified DNA using sequencing. The result of PCR process is a nuclotide with approximately 585 bp length which contained V1-V3 variable regions. *16S rRNA* gene sequence then compared and aligned with existing *16S rRNA* data sequence from GenBank.

Results: The conserved region of *16S rRNA* gene from nuclotide number 189-358 and 384-445 were 95,9% and 94,9% respectively, and the total conserved region of *16S rRNA* gene is 78.8%. From total 7 isolates that isolated from natural fermented milk , 2 isolates were identified as *L. plantarum* with similarity value 98.28% and 97.78%, the other 5 isolates were identified as *L. rhamnosus* (2 isolates) with similarity value 98.38% and 97.51%, *L. zeae* (2 isolates) with similarity value 97.36% and 98.38%, and *L. casei* (1 isolate) with similarity value 98.20%.

Conclusion: These results indicate the test isolates are members of the genus Lactobacillus, but classified in different species.

Key Words: Lactobacillus, natural fermentation milk, 16S rRNA gene, partial analysis.

ÖZET

Amaç: Bu çalışmanın amacı doğal olarak fermente olan sütten Lactobacillus spp. türüne ait 16S rRNA ların gen sekansı yapmaktır.

Materyal ve Metod: Kullanılan metodlar; alkali lizis yöntemiyle total DNA izolasyonu, spesifik primerlerle PCR amplifikasyonu ve amplifiye edilmiş DNA ların sekans analizlerinin yapılması şeklindedir. Elde edilen PCR ürünleri yaklaşık 585 bp uzunluğunda olup V1-V3 değişken bölgelerini içermektedir. Sekanslanan 16s rRNA daha sonra gen bankasıyla karşılaştırıldı.

Bulgular: 16s rRNA geninin korunmuş bölgeleri; 189-358 ve 384-445 sırasıyla %95,9 ile %94,9 oranında korunurken, 16s rRNA geni totalde %78.8 oranında korunmuştur. Doğal sütün fermentasyonundan izole edilen bu 7 izolattan 2 tanesi L.Plantarum(benzerlik oranları %98.28 ve %97.78 diğer 5 izolattan 2'si L. Rhamnosus (benzerlik oranları (98.38 ve %97.51), diğer 2'si L. zeae (benzerlik oranları %97.36 ve % ve %98.38), biri ise L. case (benzerlik oranı%98.20) şeklinde tanımlanmıştır.

Sonuç: Çalışmanın sonuçları test edilen izolatın Laktobailus genusuna ait olduğunu, fakat farklı türde sınıflandırıldığını göstermiştir.
Anahtar Kelimeler: laktobacillus, doğal fermente süt, 16s rRNA, parsiyal analiz.

INTRODUCTION

Lactobacillus are members of the lactic acid bacteria, whose primary fermentation end product is lactic acid. Lactobacillus are nutritionally fastidious, and are associated with a large variety of plants and animals. Lactobacillus are used extensively for fermentation of plant material, dairy products and meat[1]. Generally Lactobacillus 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[2.]

Some Lactobacillus species have been attributed with probiotic properties, implying living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent nutrition[3]. This has added further incentive to detailed microbiological, biochemical and genomic studies of Lactobacillus[4].

Taxonomic analysis has already led to a recognition of the unusual diversity of the genus Lactobacillus[5] and one objective of this study was to extend the *16S rRNA* phylogeny of the Lactobacillus[6] and investigate its correlation with genome-based comparison. Lactobacillus group-specific primers were used to amplify the V1 to V3 regions of the *16S rRNA* gene.

MATERIALS and METHODS

Subject

Raw unpasteurized milk samples from cow and goat were collected from the local area of UPT Singosari in a sterile screw cap tubes, processed within 144 hours as product natural fermentation. Milk fermentation samples were serially diluted in peptone medium. Diluted samples were plated onto De Man Rogosa Sharpe (MRS) medium for Lactobacillus isolation and incubated at 37 ºC for 48 h. Well-isolated colonies were picked from each plate and transferred to MRS broth.

The sequence of *16S rRNA* gene is aligned with same gene of *L.acidophilus* 1001H (JQ031741.1), *L.bulgaricus* ATCC-11842T (FR683102.1), *L.rhamnosus* ATCC-7469T (FR653106.1), *L.zeae* JCM-11302 (AB289313.1), *L.rhamnosus* JCM 8849 (AB690234.1), *L.plantarum* JCM 1100 (AB239347.1).

Ethical Consideration

This study was approved by animal research ethics comitee, Brawijaya University, as a member of Research Ethics committee in Indonesia.

Procedure

Bacterial DNA from goat and cow natural fermentation milk samples was isolated according to alkaline lysis method of Villalobos et al[7]. Quality & quantity DNA were measured by using NanoDrop spectrophotometer and 1% agarose gel electrophoresis.

DNA isolate was amplified using Eub *l6S rRNA* 7f, Lact S-G-Lab-0677-r and GC Clamp primer[8]. PCR programs included 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. PCR products were measured qualitatively using 2% agarose gel electrophoresis.

The PCR product was purified using ethanol absolute and 3 M of sodium acetate. After that PCR product was sequenced using bigDye™ terminator cycling condition. Sequencing process was conducted in German.

Data Analysis

The sequences were compared with the sequences deposited in the GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/; 1). Data was analyzed using MEGA5 (Molecular Evolutionary Genetic Analysis version 5.0) software to constructed phylogeny tree using Maximum likelihood (ML) method, tree construction was bootstrapped 1000x and BioEdit software for conserved region analysis. The sequence data were alignment using Clustal X in MEGA5 software.

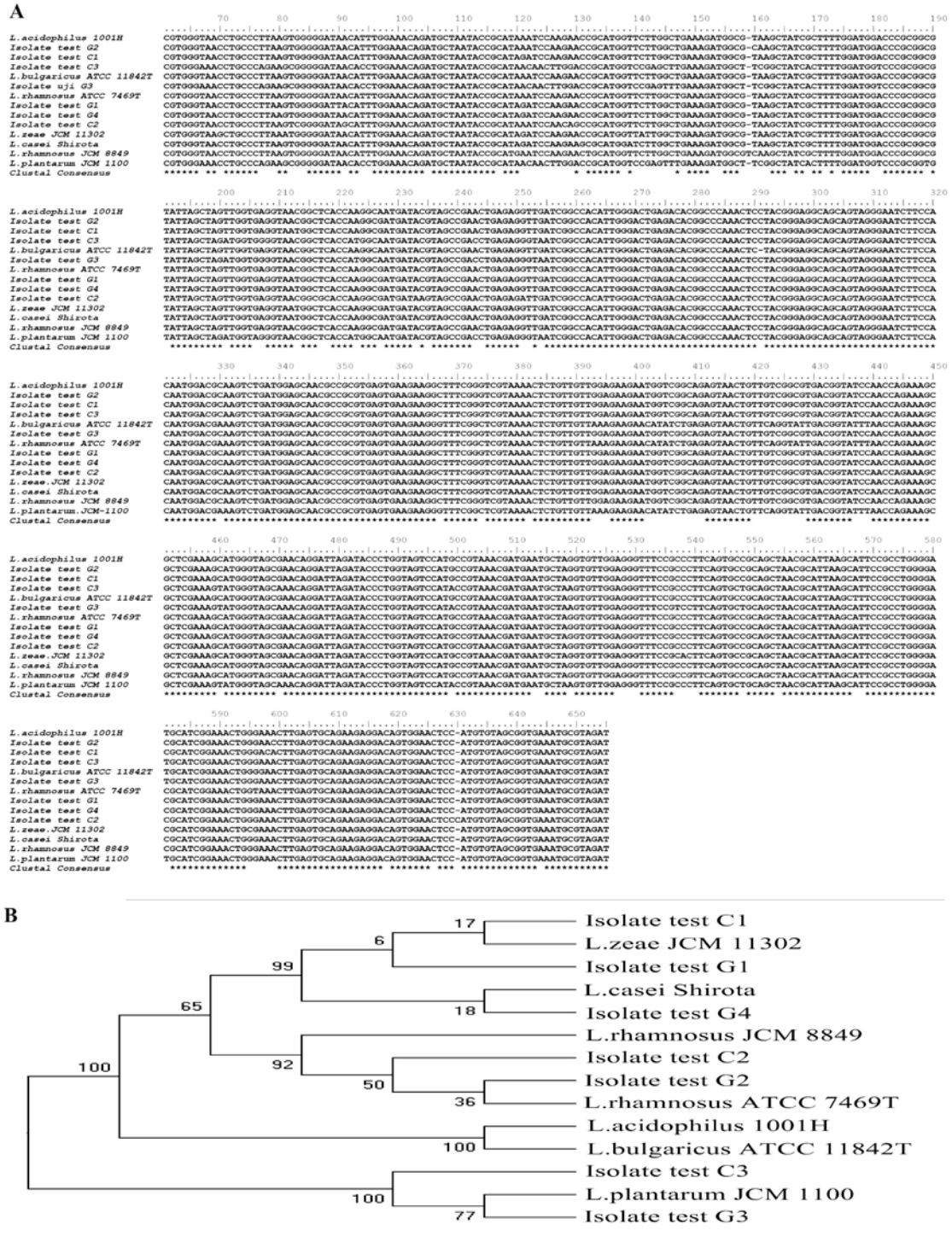
RESULTS

Nucleotide bases of *16S rRNA* gene were alignment with the program Clustal X. The Result of NCBI Genebank reference sequence alignment indicates that the *16S rRNA* gene has a 585 bp amplicon (Figure 9). Amplicons of *16S rRNA* gene have the conserved regions base of 189-358 bp at 95.9% and 384-445 bp at 94.9% with 78.8% of conserved sequences. Primary DNA can be used to identify to species level when similar areas have more than 70%[9]. The similarity regions, indicates a potential the area while the *Lactobacillus* group variability can be used to identify the species level. This indicated that the primary 7f and SG-Lab-0677 can only amplify V1 and V2 in the genus Lactobacillus.

Alignment of several genera of Lactic Acid Bacteria other than the genus *Lactobacillus* (*Pediococcus*, *Lactococcus*, *Eubacteriun*, and *Leuconostoc*) shows the position between forward and reverse did not never met (data not shown). This indicated that the primary 7f and SG-Lab-0677 can only amplify V1 - V3 in the genus *Lactobacillus*. The amplicons could differentiate between bacterial species *Lactobacillus* one another so often used for identification. Primary in this study were used to partially bacteria with 1-600 bp sequence of bases on V1-V3 region of the *16S rRNA* gene.

Topology of the phylogenetic tree shows all test isolates is one clade genus *Lactobacillus.* The Isolates G3 and C3 formed by one *sister-clade* with *L. plantarum,* while the isolates G2, C2, G1, C1 and G4 formed by one *sister-clade* with *L. rhamnosus*, *L. zeae*, and *L*. *casei*. *Sister-clade* generated by the seven isolates with the reference species is still in the same species but different subspecies.

The Isolates G3 and C3 have a close kinship with *L. plantarum,* sequentially similarity value of 98.28% and 97.78%. The Isolates G2 and C2 have a close kinship with *L. rhamnosus,* sequentially similarity value of 98.38% and 97.51%. The Isolates G1 and C1 have a close kinship with *L.zeae* sequentially similarity value of JCM-11302 amounted to 97.36% and 98.38%. While isolate G4 have a close kinship with *L.casei* amounted to 98.20%. Isolates were found not include *L.acidophilus* and *L.bulgaricus* but produced fermented milk product similar to a product containing *L.acidophilus* and *L.bulgaricus*.



Kusmiyati et al., 2013

Table 1. Similarity value of test isolates and reference isolates

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L.acidophilus 1001H	---													
Isolate test G2	93.68	---												
Isolate test C1	94.69	95.43	---											
Isolate test C3	88.11	94.98	97.54	---										
L.bulgaricus ATCC 11842T	97.48	92.48	91.98	92.48	---									
Isolate test G3	89.83	95.37	93.88	96.43	91.49	---								
L.rhamnosus ATCC 7469T	93.28	93.78	93.69	94.97	92.07	96,77	---							
Isolate test G1	92,09	95.98	96.99	93.99	91.21	95.98	94,31	---						
Isolate test G4	93.46	93.48	94.56	95.69	90.99	96.65	94.90	94.48	---					
Isolate test C2	92.68	96.68	92.43	94.99	91.37	94.99	95.48	95.48	94.68	---				
L.zeae.JCM 11302	93.69	94.69	98.38	95.56	90.78	92.56	93.83	97.36	93.43	93.69	---			
L.casei Shirota	93.99	95.11	95.48	94.43	93.98	94.43	93.28	98.20	93.98	94.99	93.69	---		
L.rhamnosus JCM 8849	93.56	97.51	95.37	93.78	92.48	95.98	92,09	93.98	93.48	98.38	94.99	93.69	---	
L.plantarum JCM 1100	89.43	94.83	93.78	98.28	89.68	97.73	93.46	93.48	94.37	95.43	93.56	94.99	92.69	---

Figure 1. *16S rRNA* gene Lactobacillus in single colony from Lactobacillus by PCR with the primer 7f and the specific primer Lab-0677r (A) Alignment results from data sequencing Lactobacillus isolate and Lactobacillus from GeneBank. (B) Phylogenetic tree based upon the *Maximum Likelihood* method of partial *16S rRNA* gene sequences (60 to 655 bp)

DISCUSSION

16S rRNA gene consists of highly conserved regions and hyper variable region. Highly conserved region describe the level of phylogenetic relationships, whereas the hyper variable region shows the level of kinship[9,10]. *16S rRNA* gene contains nine hypervariabel regions (V1-V9) which sequence diversity among different bacteria. Region V1 is able to distinguish Pediococcus sp., V2 and V3 best distinguish all species of the genus except Enterobacteriaceae bacteria levels[11]. For identification purposes, the necessary amplification using PCR and sequence analysis of the bases at least 500 base pairs[12].

The presence of mutations or differences in the sequence of bases will serve as a determinant of bacterial identification at the genus and species level[13]. Primary SG-Lab-0677-r-f and primer 7 is special to the genus Lactobacillus group[8]. Specific groups of Lactobacillus primer SG-Lab-0677-r and-f 7 primer used to amplify the V1-V3 region of *16S rRNA* gene[14].

Isolates with *16S rRNA* gene similarity values of ≥ 99% can be are grouped into a single species[9]. Test isolates were found to have a value of ≤ 99% similarity with the reference isolates. These results indicate the test isolates are members of the genus Lactobacillus, but classified in different species. While according to the phylogenetic species concept stated that a species can be the same if the value of similarity more than 95% and declared a strain if the value is more than 99% similarity[15].

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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