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Diversity of Lactic Acid Bacteria Isolated from Indonesian Traditional Fermented Foods

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The diversity of lactic acid bacteria was evaluated from Indonesian fermented foods such as dadih (buffalo fermented milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous rice). Lactic acid bacteria were enumerated using selective media and characterized based on a genotypic methods such as Repetitive bacterial DNA element (rep- PCR) and RAPD-PCR, as well as 16S rRNA gene sequencing of representative strains. Fourty-six colonies had successfully been isolated from Indonesian fermented foods. The great majority of these colonies originated from dadih (43.48%), tempoyak (39.13%), bekasam (13.04%), and tape (4,3%). The 46 isolates were characterized based on a genotypic methods such as RAPD and rep-PCR as well as 16S rRNA gene sequencing of representative strains. The rep-PCR result yielded seven clusters (I-VII) at a similarity level of 75-88% RAPD-PCR used LB2 primer, M13 primer and primer A, B, C. The RAPD result using LB2 primer yielded eight clusters (I-VIII) at a similarity level of 82-91%. Identification using 16S rRNA showed that the majority strains were closed to *Lactobacillus plantarum, Lactobacillus fermentum*, and *Pediococcus pentosaceus* strains.

Key words: 16S rRNA, Indonesian fermented foods, RAPD, rep-PCR

Keragaman bakteri asam laktat telah di evaluasi dari pangan fermentasi tradisional Indonesia seperti dadih (susu kerbau fermentasi), tempoyak (durian fermentasi), bekasam (daging fermentasi) dan tempe ketan. Bakteri asam laktat diseleksi dengan menggunakan media selektif dan dikarakterisasi secara genotip menggunakan rep-PCR dan RAPD PCR serta gen 16SrRNA. Sebanyak 46 koloni bakteri asam laktat diisolasi dari pangan fermentasi dengan komposisi dadih (43,48%), tempoyak (39,13%), bekasam (13,04%) dan tape (4,3%). Karakterisasi secara genotip dari 46 isolat dengan rep-PCR menghasilkan 7 kelompok dengan kesamaan 75-88%, sedangkan RAPD-PCR dengan primer LB2, M13, dan primer A,B,C terdapat 8 kelompok dengan kesamaan 82-91%. Identifikasi menggunakan 16S rRNA menunjukan bahwa isolate-isolat tersebut termasuk kedalam strain *Lactobacillus plantarum, Lactobacillus fermentum*, and *Pediococcus pentosaceus* strains.

Kata kunci : 16S rRNA, pangan fermentasi Indonesia, RAPD, rep-PCR

Lactic acid bacteria (LAB) are of considerable economic significance because of their widespread use in industrial food fermentation processes (Sudhamani *et al*. 2007). Certain LAB are also used as probiotics added to confer health benefits to consumers or to improve animal production. The lactic acid bacteria species are economically very important to the food fermentation industry (Korhonen 2010). Indonesian fermented foods, such as dadih (buffalo fermented milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous rice), have been consumed for centuries, but there is little investigation has been conducted to asses the diversity of LAB in Indonesian fermented foods.

LAB have complex nutritional requirements because of their limited biosynthetic capabilities. Most LAB strains must obtain essential components, such as carbohydrates, amino acids, peptides, fatty acid esters and vitamins, from their habitats. Indonesia fermented foods should be a suitable environment for LAB, since it contains plenty of protein and sugar units from the decomposed vegetables. Moreover, because the environment in Indonesia fermented foods differs from that in other fermented materials, it should be possible to collect LAB strains with unique characteristics, unlike those of the strains found in ordinary fermented materials such as fermented milk or vegetables.

Molecular approaches for LAB systematic studies include pulse field gel electrophoresis (PFGE) (Ventura and Zink 2002), random amplified polymorphic DNA (RAPD) analysis (Franciosi *et a*l. 2009; Chao *et al*. 2013;), PCR-denaturing gradient gel electrophoresis PCR-DGGE (Ercolini *et al*. 2001; Liu *et al.* 2012), PCR-RFLP (Yu *et al*. 2011) and DNA

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sequencing (Liu *et al.* 2012; Sulistiani *et al*. 2014), which have been extensively applied for the intraspecific identification and for genotyping LAB isolated from several fermented foods as well as from human gastrointestinal tract (Mc Cartney 2002).

For LAB, the RAPD is a method of choice for molecular typing. The profiles obtained can be stored in a computerized database, thus allowing rapid identification of unknown isolates (Berthier and Ehrlich 1999; Corroler *et al.* 1998). Alternatively, PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with the following characteristics: (i) a high discriminatory power, (ii) low cost, (iii) suitable for a high-throughput of strains, and (iv) considered to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria (Gever *et al*. 2001; Adimpong *et al*. 2012). Our aim is to investigate the diversity of the predominant LAB present in fermented foods using RAPD, rep PCR, and 16S ribosomal RNA sequences.

MATERIALS AND METHODS

Source and Maintenance of Culture. Samples of LAB were isolated independently from dadih (buffalo fermented milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous). Samples were serially diluted in saline solution and plated onto MRS agar (Oxoid, England). The plates were incubated at 37 °C for 2 d . A total of 120 samples of LAB isolated independently from dadih (Fermented from fresh raw buffalo milk in bamboo tubes capped with banana leaves), tempoyak (Durian (*Duria zibethinus*) meat was mixed with small amount of salt (2.5%) and placed in a sealed container. Fermentation takes about 7 d), bekasam (Meat is mixed with 10-20% salt (w/v) and grind roasted rice, then fermented (in sealed container) for 14 d), and tape ketan (Glutinous rice is sleamed followed by inoculation with ragi tape, then fermentated about 1-2 d. This product is acid-alcoholic in taste) were used in this study. All LAB were maintained by subculturing in de Man Rogosa and Sharpe (MRS) broth (Oxoid, England) supplemented with 0.02% (w/v) sodium azide, using 1% inoculum and overnight of incubation at 37 °C; between transfer cultures were stored at 4 °C.

DNA Extraction. LAB isolates were cultured in MRS broth (pH 7.0) for 1 d. Bacterial cells were collected by centrifugation at 6000 rpm for 10 min. The genomic DNA was extracted as previously described, with modification (Zhu *et al.* 1993). The pellet was resuspended with TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA), 40 µL of lysozyme (60 mg mL^{-1}). Incubated at 37 ºC for 60 min and 200 µL 10% sodium dodecyl sulfate, 100 µL 5 M NaCL, 80 µL 10% CTAB was added. Warmed at 68 °C for 30 min and added an equal amount of chloroform. Centrifugation was conducted at 13000 rpm for 10 min. The supernatant was harvested and an equal amount of ethanol was added. The mixture was shaken again and then centrifuged at 13,000 rpm for 10 min. After being air-dried, the DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the concentration was adjusted to 10 µg mL^{-1} RNAse were stored at -20 °C until use.

RAPD-PCR and rep-PCR Genomic Finger-Printing. For both RAPD-PCR and rep-PCR finger-printing, total genomic DNA from all isolates, as well as various reference strains, was isolated according to the methods of Zhu *et al*. (1993) with modification. RAPD-PCR reactions were done for each strain, each employing a different primer. The primers used were primer M13 (50-GAG GGT GGC GGT TCT-30) (Huey and Hall 1989)[2] Lb2 (5'-GGT GAC GC-3') (Ben Omar *et al*. 2000), Primer A (5' CCG CAG CCA A 3'), Primer B (5'AACGCG CAA C 3'), and Primer C (5' GCGGAAATA G 3') (Chao *et al*. 2008).

RAPD was performed using methods and amplification conditions as described by Chao *et al*. (2008). It was performed in 20 µL of a mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 1.6 µM of primer, 200 µM of each dNTP, 0.96 U Taq polymerase and 50 ng of genomic DNA from the LAB isolates. The cycling program consisted of 1 cycle of 94 °C for 2 min; 6 cycles of 94 °C for 30 s, 36 °C for 1 min, and 72 °C for 90 s; 30 cycles of 94 °C for 20 s, 36 °C for 30 s, and 72 °C for 90 s; and finally 1 cycle of 72 °C for 3 min. Rep-PCR was performed using the primer GTG5 (5'-GTG GTG GTG GTG GTG-3') and methods as previously described by Gevers *et al*. (2001). The cycling program consisted of 1 cycle of 95 °C for 7 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 65 °C for 8 min; and finally 1 cycle of 65 °C for 16 min. PCR products were separated by electrophoresis on 1.8% (w/v) agarose gel using 1 x TBE buffer. The gels were stained in ethidium bromide solution and photographed on a UV transilluminator.

The RAPD and the rep-PCR fingerprints obtained with both primers were analysed separately as a single data set by calculating the average matrix from the two separate similarity matrices for primer fingerprint sets

to obtain a single dendrogram. It was coded in binary form 1 or 0, respectively. Statistical analysis software NTSYSpc 2.11p (Exeter Software, Setauket, USA) was used for clustering.

PCR Amplification for 16S rRNA. For 16S rRNA sequencing, primers 8F (5'-AGA GTT TGA TCA TGG CTC AG-3'; positions 8 to 27 bp) and 15R (5'-AAGGAG GTG ATC CAA CCG CA-3'; positions 1541 to 1522 bp) were used to amplify the full length of bacterial 16S rRNA fragment (Cho *et al*. 2008). Each 25 µL PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 400 nM of each primer, 1 U of Taq polymerase, and 10 ng of the DNA template. The PCR conditions were 96 °C for 5 min; 35 cycles consisting of 96 °C for 1 min, 55 °C for 3 min, and 72 °C for 1 min; and 72 °C for 7 min. The PCR products were subjected to gel electrophoresis in 1% agarose gel, followed by ethidium bromide staining.

DNA Sequencing and Phylogenetic Analysis. The DNA sequencing was performed in Macrogen, South Korea. Similarity searches with sequences were performed by online BLAST analysis in NCBI. For phylogenetic analysis, sequences were aligned by using the CLUSTAL X software (Thompson *et al*. 1997) and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987).

RESULTS

Isolation of Lactic Acid Bacteria. Mean total of bacteria concentrations enumerated on selective media (MRS) agar were varied ranging from 1.0×10^7 to 9.0×10^8 CFU/mL. The latter samples were collected from dadih (buffalo fermented milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous). Several colony morphologies could be observed on most of the agar plates. Colonies showing different characteristics (colour, shape, etc.) were collected (46 colonies) and plated again on the same agar medium for purification and preliminary identification.

RAPD and rep-PCR Genomic Finger Printing. A taxonomical approach was utilised in this investigation to identify the predominant LAB associated with Indonesia fermented foods. PCR-based identification techniques (RAPD-PCR and rep-PCR) and 16S rRNA gene sequencing of representative strains showed that the majority of predominant isolates from Indonesian tradisional food consisted of *Lactobacillus* and *Pediococcus*.

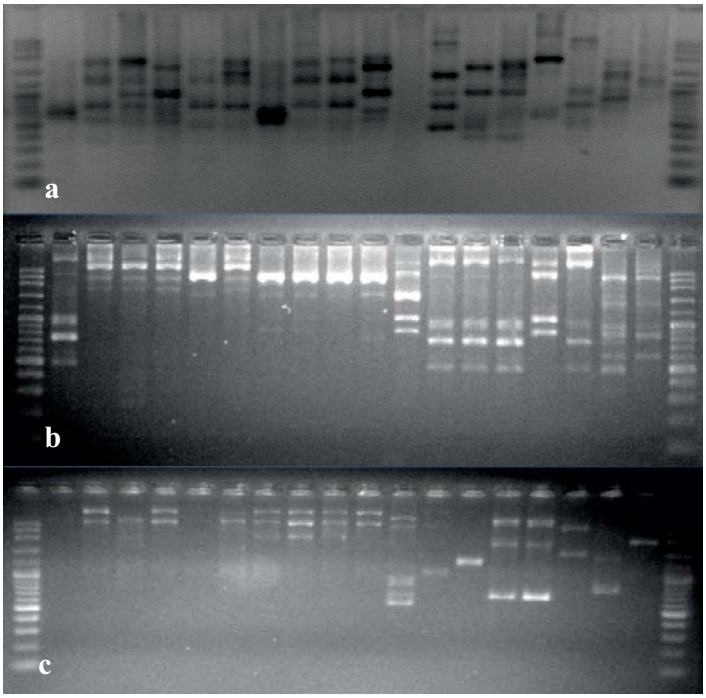

Fig 1 DNA Profiling of LAB using primer a) primer A b) primer $(GTG)_5$ and c) primer Lb_2 on 2% agarose initially tested for their ability to type a subset of 18 LAB. Lane 1: 100 bp DNA ladder; lane 2-11 LAB isolated from dadih; lane 12-17 LAB isolated from bekasam.

* primer A, the random primer with 50% to 70% G+C content were designated by Cho *et al*. 2008; $(GTG)_5$ primer, the single oligonucleotide primer with repetitive GTG (Gevers *et al*. 2000); primer Lb_2, RAPD-PCR fingerprinting (Ben Omar *et al*. 2000)

For the evaluation of the RAPD-PCR and rep-PCR fingerprinting technique, five single oligonucleotide primers for RAPD (M13, Lb2 and primer A, B, C) and single primer for rep PCR $(GTG)_5$ were initially tested for their ability to type a subset of 46 LAB.

The 46 strains were submitted to rep PCR analysis using (GTG)5 primer. The rep-PCR result yielded seven clusters (I-VII) at a similarity level of 75-88%. The majority of LAB could be classified into four major groups (called G1, GII, GIII and GVI) (Fig 2). Group 1 (GI) contained 12 isolates, majority of isolates isolated from dadih and group 2 (GII) contained 18 isolates, majotity of isolates were from tempoyak. The majority of isolates from bekasam clustered into group VI. Four groups were composed of only four isolates (GIII) or one isolate (GIV, GV, GVII)).

The rep-PCR result confirmed the RAPD-PCR using LB2 primer, M13 primer and primer A, B, C. The RAPD using LB2 primer result yielded eight clusters (I-VIII) at a similarity level of 82-91%; M13 primer yielded six cluster at similarity level 67-78% and A, B, C primer yielded ten cluster at similarity level 77-85%. Majority of isolates emerged from RAPD analysis using LB2 and M13 as primer pair could be classified into four major groups (GI, GII, GIII and GII) (Fig 3, 4). RAPD analysis using A, B and C primers was done in separate reactions. For each strain, the three RAPD patterns were merged for computations. The majority of strains could be classified into three major groups (Fig 5).

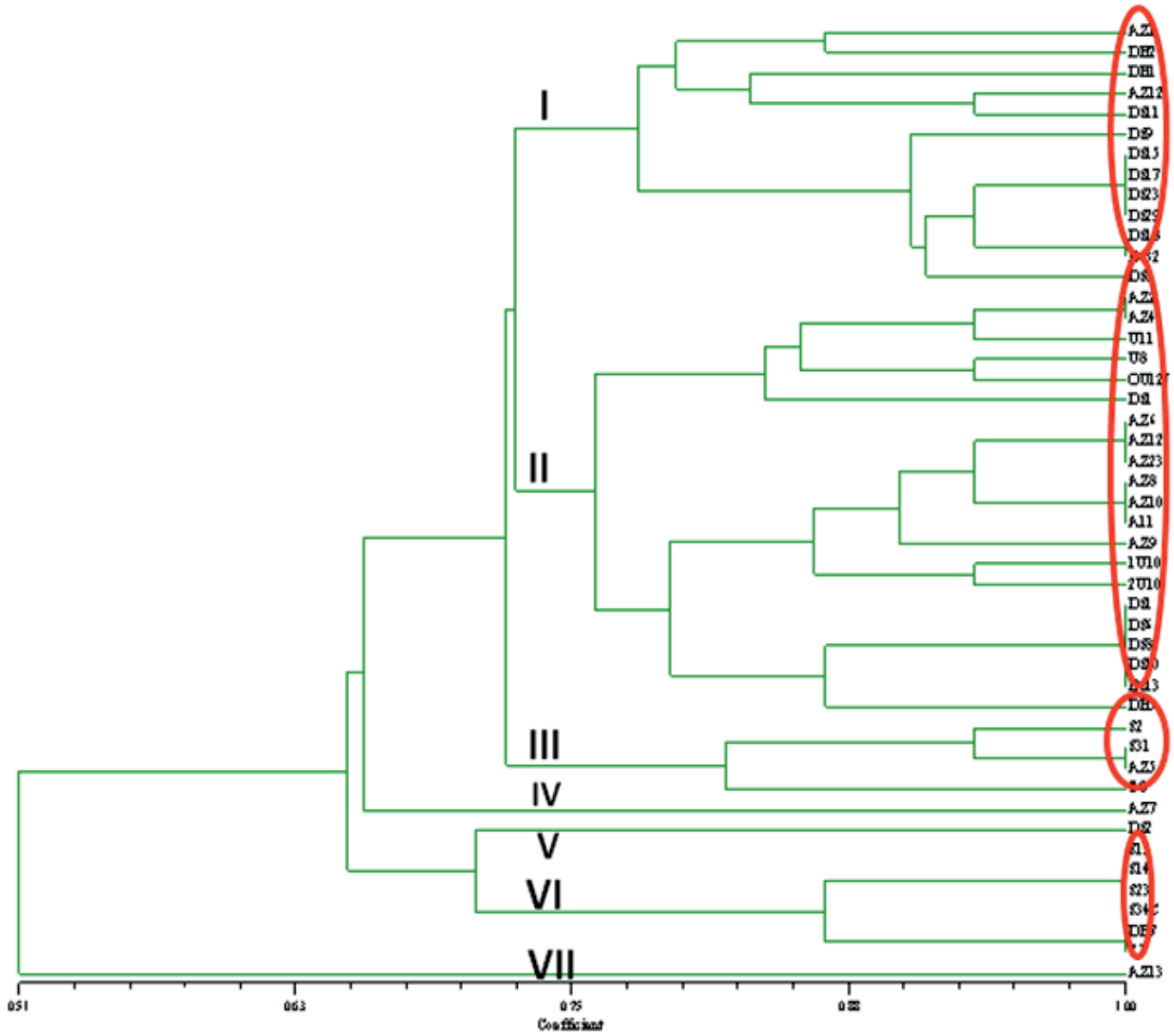

Fig 2 Dendrogram generated after cluster analysis of the digitized (GTG)5-PCR fingerprints of the LAB isolated from Indonesia traditional fermented foods. The majority of LAB classified into four major groups (called G1, GII, GIII and GVI). GI contained 12 isolates from dadih and GII contained 18 isolates from tempoyak. The isolates from bekasam clustered into group VI.

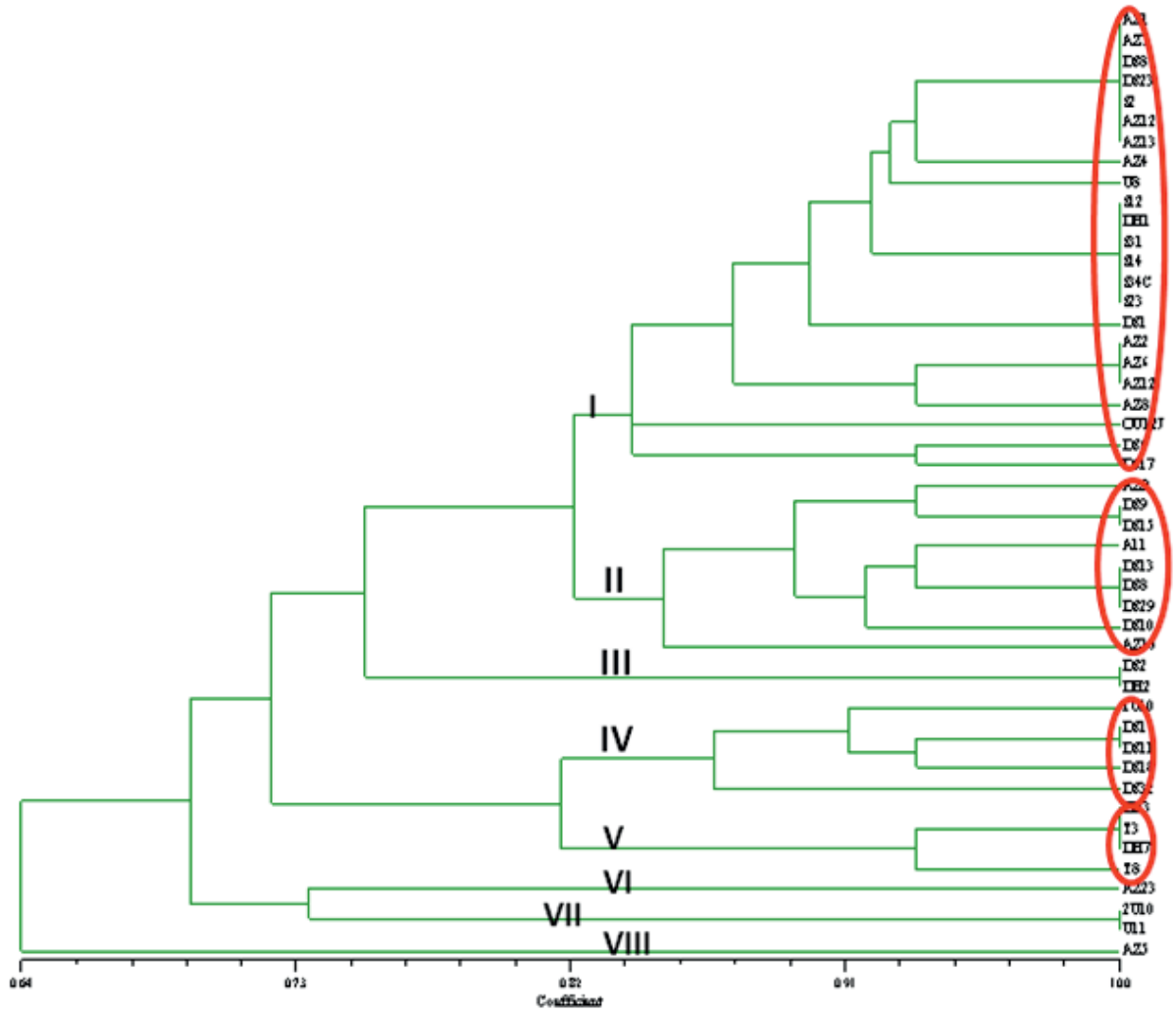

Fig 3 Dendrogram generated after cluster analysis of the digitized RAPD Lb2 primer of the LAB isolated from Indonesia traditional fermented foods. Majority of isolates classified into four major groups (GI, GII, GIV and GV). GI contained 10 isolates from Tempoyak and GII contained 7 isolates from dadih.

The bekasam isolates S12, S14 and S34, which were clustered together in group VI using rep-PCR analysis with primer (GTG)5, all clustered together with the *L. plantarum* type strain using rep-PCR fingerprinting, indicating that on the basis of these genotypic typing methods the strains can be characterised as *L. plantarum*. The Dadih isolates (DH1, DH2, and DS11*)* clustered together with *Lactobacillus fermentum* subgroup IA strain. The Tempoyak isolate (U8, U11, AZ2 and AZ4) clustered together with *Lactobacillus fermentum* type strain. The rep-PCR indicated there were isolates clustered together into subgroup IIA strain. The isolate U10, AZ6, AZ8, AZ9, AZ10, AZ11, AZ23 and AZ23 clustered together with *Lactobacillus plantarum* type strain. The rep-PCR indicated there were isolates clustered together into subgroup IIB strain (Fig 2).

Identification of the Lactic Acid Bacteria. The partial 16S rRNA gene sequences (1490 bp) of all the strains were determined. Then, the sequences were compared with related bacteria in GenBank and sequence similarities were determined using the BLAST program.The result confirmed that 12 isolates belong to 2 genera (*Lactobacillus* and *Pediococcus*), 2 species groups, and 4 species: *L. plantarum* group, *L. fermentum, Pediococcus pentosaceus, Pediococcus acidilactici* (Table 2). Isolates S12, S14, S31 and S34 isolated from bekasam fermented meat showed similarity to *L. plantarum.* They grouped on the phylogenetic tree together with the corresponding type strain.

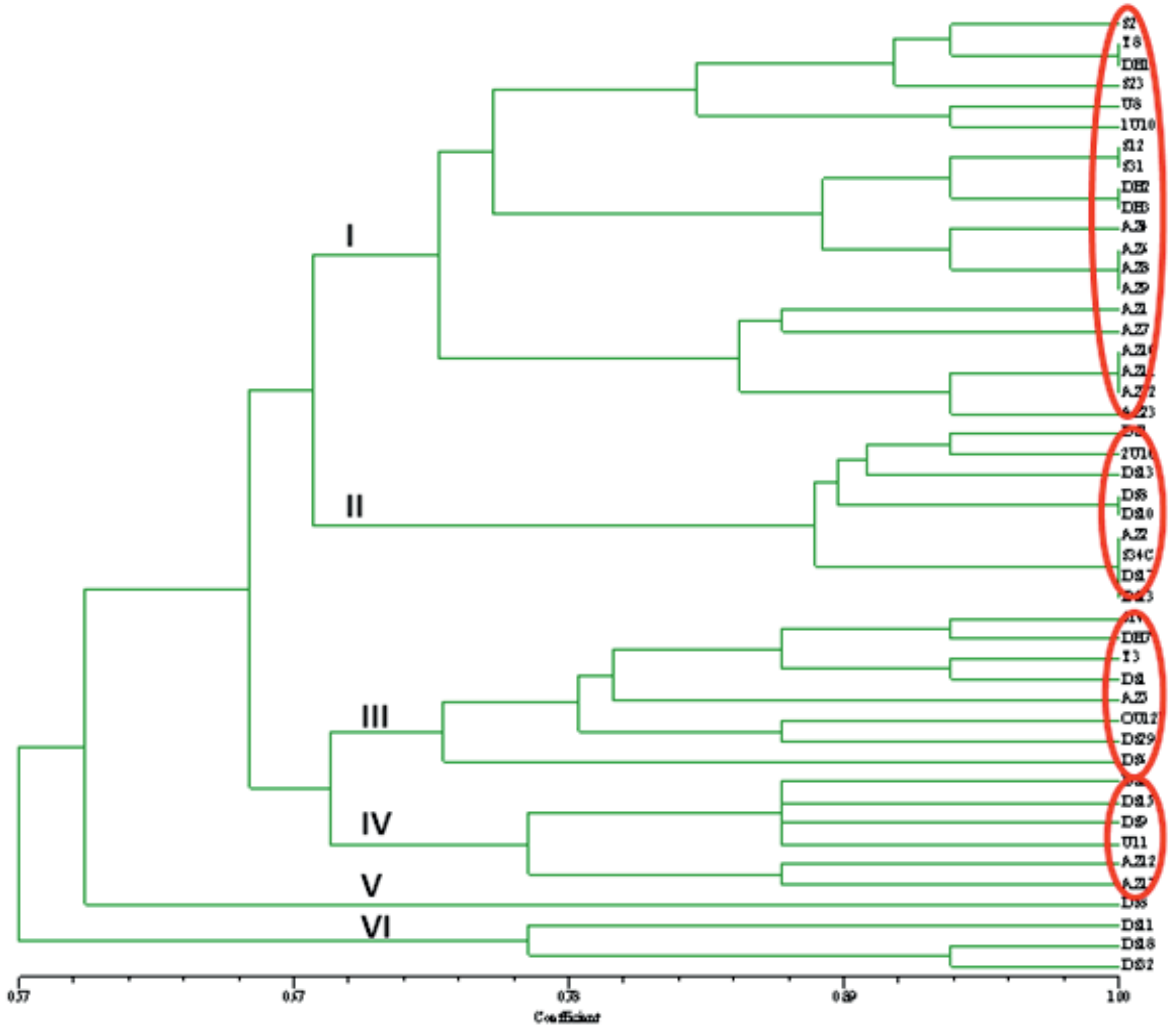

Fig 4 Dendrogram generated after cluster analysis of the digitized RAPD M13 primer of the LAB isolated from Indonesia traditional fermented foods. Majority of isolates classified into four major groups (GI, GII, GIII and GIV). GI contained 12 isolates from Tempoyak and GII contained 6 isolates from dadih.

The bekasam strains clustered with the *Lactobacillus plantarum* type strain. The almost complete 16S rRNA gene sequence of some of these strains (S12, S14 and S34) was determined and showed high homology (98-99%) to that of the *L. plantarum*. The complete 16S rRNA sequence of DH1 strain was high homology (98%) to *L. fermentum*. The Tempoyak isolate 16S rRNA gene sequencing showed that one of these strains (U11) could also be identified as *L.plantarum* (99 % similarity in 16S rRNA gene sequence). The almost complete 16S rRNA gene sequence of strain U10 showed high homology (99%) with *Lactobacillus plantarum* type strain.

Phylogenetic Relationships. FASTA analysis of the 16S rRNA gene sequence of strain S34 (a continuous stretch of 1561 bp) revealed that *Lactobacillus plantarum* were the closest relatives (with 99% sequence similarity). The phylogenetic tree of the genus Lactobacillus consisted of two separate clades (Fig 6). The clade containing strain S12, T8, S34, DH7, S23, DS13, DH1, U11, T3 and S14 also included *Lactobacillus plantarum* WCSF1 and *Lactobacillus plantarum* subsp *plantarum* STIII, *Lactobacillus fermentum* MTCC 8711 and *Lactobacillus fermentum* BCS36, *Pediococcus pentosaceus* KT3CE27, *Pediococcus acidilactici* UL5. The second clade comprised isolate U10 and S31.

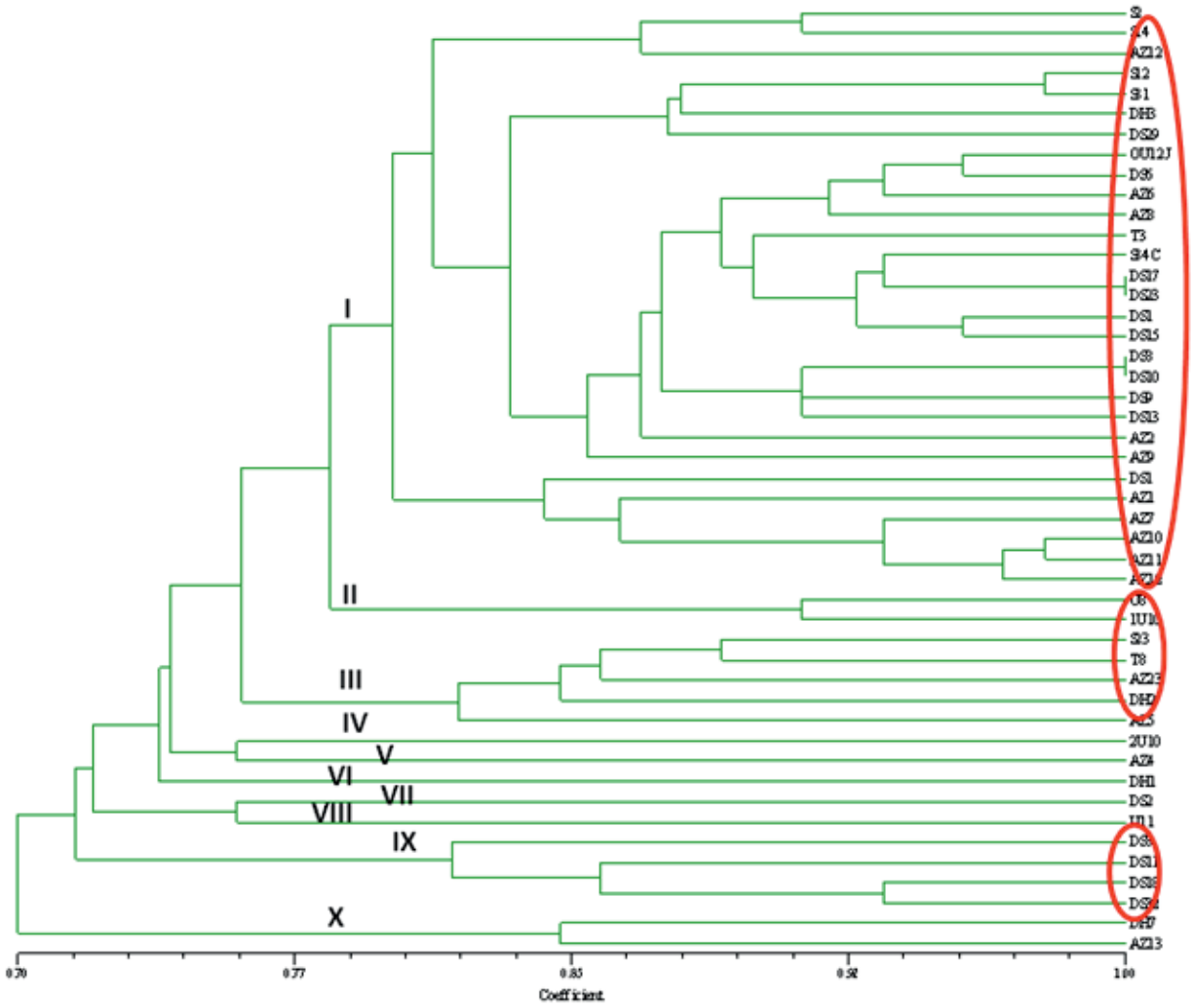

Fig 5 Dendrogram generated after cluster analysis of the digitized RAPD A, B, C primer of the LAB isolated from Indonesia traditional fermented foods. Majority of isolates classified into three major groups (GI, GIII, and GIX). GI contained 10 isolates from Tempoyak.

Table 1 Screening of LAB Isolated from Indonesian fermented foods

Fermented foods	Sources	Location	Total LAB isolated	Code of isolate
Dadih	Fermented buffalo milk	Padang, West Sumatera	4	DH
		Solok, West Sumatera	16	DS
Tempoyak	Durian meat	Palembang, South Sumatera	4	U
		Musi Banyuasin, South Sumatera	14	AZ
Bekasam	Fermented meat	Way Kanan, Lampung, Sumatera	6	S
Tape ketan	Glutinous rice	Kuningan, West Java	2	T
Total			120	

Table 2 The lactic acid bacteria from Indonesian tradisional fermented foods

No	Isolate	Spesies	Identity	Accession Number
1	S12	*Lactobacillus plantarum*	99%	JN560843.1
2	S14	*Lactobacillus plantarum*	97%	ACGZ01000098.1
3	S23	*Pediococcus acidilactici*	100%	FJ844982.1
4	S31	*Lactobacillus plantarum*	98 %	ACGZ01000098.1
5	S34	*Lactobacillus plantarum*	99%	AL935263.2
6	T3	*Lactobacillus plantarum*	99%	AL935263.2
7	T8	*Lactobacillus plantarum*	100%	AL935263.2
8	DH1	*Lactobacillus fermentum*	98%	GU213430.1
9	U10	*Lactobacillus plantarum*	99%	AL935263.2
10	U11	*Lactobacillus fermentum*	99%	FJ462686.1
11	DS13	*Pediococcus pentosaceus*	100%	AB481102.1
12	DH7	*Pediococcus acidilactici*	97%	EF059987.1

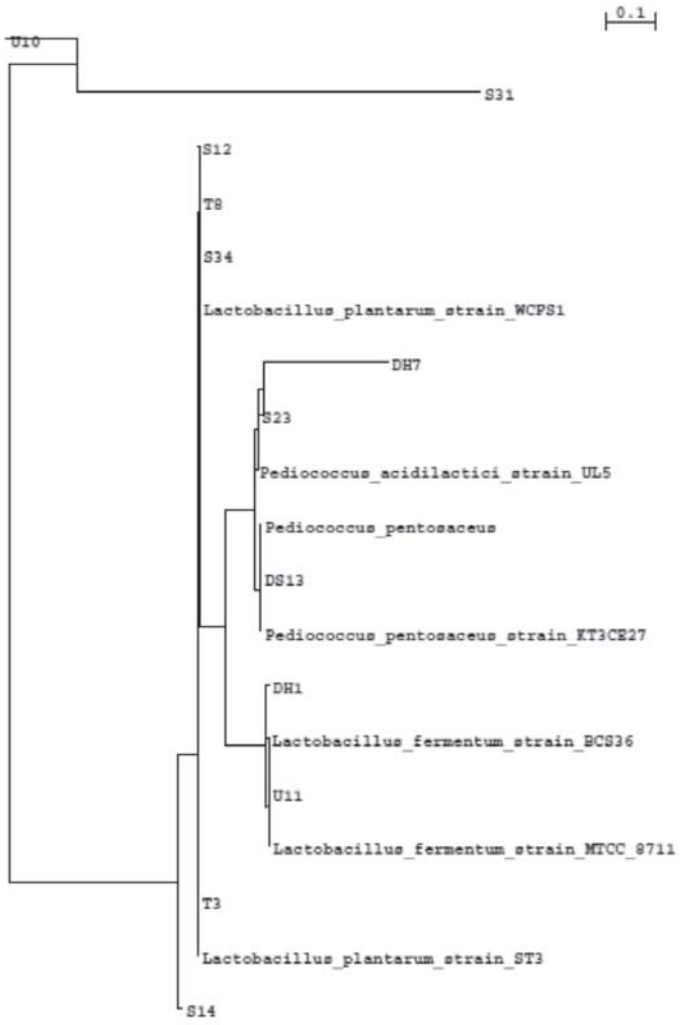

Fig 6 Phylogenetic tree based on 16S rRNA sequence analysis, showing the phylogenetic placement of strains isolated Indonesian fermented food. The tree was constructed by the neighbor-joining method.

DISCUSSION

Lactic acid bacteria biodiversity was evaluated from Indonesian fermented foods such as dadih (buffalo fermented milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous rice). Forty-six of LAB were isolated from Indonesian fermented foods and were phylogenetically characterized based on their diversity. The greatest majority of these active colonies was originated from dadih (43.48%), tempoyak (39.13%), bekasam (13.04%), and tape (4.35). Predominant strains was well characterised based on a genotypic methods such as RAPD and rep-PCR as well as 16S rRNA gene sequencing of representative strains. Identification using 16S rRNA showed that the majority of strains were *Lactobacillus plantarum, Lactobacillus fermentum,* and *Pediococcus pentosaceus* strains.

A study conducted by Leisner *et al.* (2001), a total of 38 strains of LAB were selected for comparison by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of their whole cell protein patterns. These strains were also examined for their carbohydrate fermentation patterns by use of API 50 CH. Isolates belonging to the *Lactobacillus plantarum* group were shown to be the predominant members of the LAB flora. In addition, isolates belonging to the *Lactobacillus brevis* group, *Leuconostoc mesenteroides*, *Lactobacillus mali*, *Lactobacillus fermentum,* and an unidentified *Lactobacillus* sp. were also observed.

LAB isolated from tempoyak was made in Indonesia and Malaysia. It was expected that strains of LAB and other microorganisms varied depending on the place where the product was prepared. The identified two isolates from tempoyak that showedsimilarity to *Lactobacillus plantarum for U10 and* similarity to *Lactobacillus fermentum, L. plantarum, L. brevis, L. mali, L. fermentum for U11* were also found in tempoyak from Malaysia (Issa 2000; Leisner *et al.* 2001), while Wirawati (2002) and Ekowati (1998) had isolated *L. plantarum, L. casei, L. corynebacterium* and *L. fermentum, L. casei*, respectively, from tempoyak in Indonesia. Leisner *et al.* (2002) reported the new species of *Lactobacillus, L. durianis sp*., isolated from Malaysian tempoyak. Other LAB presented in tempoyak from Malaysia was *Leuconostoc mesenteroides* (Leisner *et al.* 2001)

The identified three isolates from dadih originated from West Sumatera showed that *DH1 had similarity to L. fermentum, DH7 had similarity to Pediococcus acidilactici and DS13 had similarity to Pediococcus pentosaceus.* Surono and Nurani (2001) reported *Lactobacillus sp*, *Lactococcus* sp., and *Leuconostoc* sp. were dominant in dadih from Bukit Tinggi and Padang Panjang, West Sumatera. *Leuconostoc paramesenteroides* was the dominant strain of lactic acid bacteria encountered in dadih originated from Bukit Tinggi (Hosono *et al*. 1989). Among tten lactic strains from Bukit Tinggi-originated dadih previously identified by the API 50CH system (Api Products, Bio Merieux, Marcy l'Etoile, France), (Torshizi *et al*. 2008), 5 strains were re-identified by PCR as *Lactobacillus plantarum* strains IS-10506 and IS-20506; *Enterococcus faecium* strains IS-27526, IS-23427, and IS-16183 showed potential probiotic properties with good survival rate at low pH value and in the presence of lysozyme, and short lag time in the presence of 0.3% oxgal (Surono 2003).

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