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Lactic Acid Bacteria from Tempeh and Their Ability to Acidify Soybeans in Tempeh Fermentation

TATI BARUS[1*], GABRIELA GIOVANIA[2], AND BIBIANA W LAY[2]

[1]*Department of Master of Biotechnology, Faculty of Biotechnology, Universitas Katolik Atma Jaya, Jalan Jenderal Sudirman 51, Jakarta 12930;*
[2]*Department of Biology, Faculty of Biotechnology, Universitas Katolik Atma Jaya, Jalan Raya Cisauk, BSD City, Tangerang, Banten 15345.*

Tempeh is the most famous traditional fermented food in Indonesia. Tempeh fermentation consists of two stages. In the first stage, the acidification of soybeans used bacteria around 24 hours. Lactic acid bacteria are found in tempeh. Therefore, this study is aimed to investigate the diversity of LAB from tempeh based on 16S rRNA gene sequences and to study their function in tempeh fermentation. In this study, twenty-two LAB isolates were obtained from tempeh. The isolates were closely related to *Lactobacillus agilis, Lactobacillus fermentum, Weissella confusa*, and *Lactobacillus delbrueckii*. *L. fermentum* (13 isolates) were the most abundant in tempeh, followed by *L. agilis* (7 isolates). It was found LAB important for the acidification of soybeans which the pH of soybean soaking water decreased from pH 7 to pH 4.4-4.9.

Key words: diversity, LAB, *Lactobacillus*, tempeh, *Weissella*

Tempe merupakan makanan fermentasi dari kedelai asli dari Indonesia. Tahap pertama dalam fermentasi tempe di Indonesia merupakan pengasaman kedelai oleh bakteri yang berlangsung sekitar 24 jam. Salah satu jenis bakteri yang berperan dalam proses pengasaman tersebut adalah bakteri asam laktat (BAL). Oleh karena itu, penelitian ini bertujuan mengkaji keragaman BAL dari tempe berdasarkan sekuens gen 16S rRNA dan mendapatkan potensinya dalam menurunkan pH saat perendaman kedelai pada fermentasi tempe. Dalam penelitian ini, dua puluh dua isolat BAL telah diisolasi dari tempe. Isolat tersebut berkerabat dekat dengan *Lactobacillus fermentum* (13 isolat) paling banyak terdapat pada tempe, disusul *Lactobacillus agilis* (7 isolat), dan yang lainnya *Weissella confusa*, dan *Lactobacillus delbrueckii*. Ditemukan BAL dapat menurunkan pH air rendaman kedelai dari pH 7 menjadi pH 4,4-4,9.

Kata kunci: BAL, keanekaragaman, *Lactobacillus*, tempe, *Weissella*

Tempeh is the most popular fermented soybean product in Indonesia as an important source of protein at low prices for Indonesians. It has been reported that tempeh reduced the adhesion of ETEC to intestinal epithelial cells of pig and human origin (Roubos *et al.* 2009) and showed antibacterial activity against *Bacillus cereus* (Roubos *et al.* 2010). Tempeh can prevent diarrhea (Roubos-van den Hil et al. 2009) and anemia (Astuti 1999). Consuming tempeh is good for the gut microbiota (Stephanie et al. 20170). It has also been reported that tempeh contains vitamin B12 (Keuth and Bisping 1994) and antioxidant compounds (Esaki *et al.* 1996). Anti-nutritional compounds were found to be lower than soybeans (Hong *et al.* 2004). Such beneficial effects of tempeh consumption have been reported and thus it is widely known as part of the vegetarian or vegan diet in many countries, including Japan, the Netherlands, Australia, and the USA (Aderibigbe dan Osegboun 2006).

Tempeh fermentation consists of two stages. The first stage lasts about 24 hours, in which soybean acidification occurs through bacterial activities. Throughout this stage, the pH will decrease from 7 to 4 (Barus *et al.* 2008). This condition is important for the growth of *Rhizopus* spp. in the second stage, which happens ± 48 hours. *Rhizopus* can be obtained from commercial inoculums or traditional inoculums (Barus *et al*. 2019a). One of the most important microorganisms that mediate acidification is the lactic acid bacteria (LAB) group, as they produce lactic acid as their main fermentation product from the culture medium (Konings *et al.* 2000). LAB belongs to a group of Gram-positive bacteria and they are catalase - negative. However only little research on LAB involved in tempeh fermentation has been reported.

Currently, various molecular biology approaches are available and applied to study bacterial diversity. Among these molecular methods, the 16S rRNA gene sequences have been used extensively to study the

*Corresponding author: Phone: +62-21-5703306; Fax: +62-21-5719060 ; Email: tati.barus@atmajaya.ac.id

diversity of bacteria in the environment (Pisa et al. 2011; Koike et al. 2003; Barus et al. 2013; Barus et al. 2017). In this study, we explored the diversity of LAB from tempeh based on the 16S rRNA gene sequences and then used for the analysis of the role of LAB in determining the quality of tempeh.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria. The sample of tempeh was collected from the local market in Jakarta, Lembang, and Tangerang, Indonesia. Aseptically, 5 g of each sample was added into 45 mL of sterile 0.85% (w/v) NaCl (Oxoid) solution and mixed thoroughly. Then, it was made a dilution series of 10-1 to 10-7. Furthermore, dilutions in the series 10-5-10-7 were taken as 1 mL in each and directly poured in duplicate on the de Man Rogosa Sharpe (MRS) agar (Oxoid Ltd, Basingstoke, UK) with 1% calcium carbonate (CaCO3) (w/v) and were incubated at 37°C for 48 h (Assohoun-Djeni et al. 2016; Sirichokchatchawan et al. 2018) under aerobic conditions. Each olony with a halo zone was isolated and observed in terms of characters: non-motile, endospores negative, catalase negative, and Gram-positive bacteria. Furthermore, fresh overnight cultures of each isolate were grown in Luria-Bertani (LB) broth and stored in the freezer at −80 °C in 20% glycerol for further tests.

Genomic Extraction. LAB isolates were grown for 18 hours at 30^0C on Luria-Bertani broth. Cells were harvested by centrifugation at 12,000 × g for 4 min. The cell pellet was resuspended in 1 mL of 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/ v) SDS, followed by genomic extraction using the Genomic DNA Purification Kit (Fermentas®, Lithuania) based on the manufacturer's protocol. Genomic extracts were stored in TE 1X buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) at -20°C.

Amplification of 16S rDNA. For molecular identification of LAB isolates based on the 16S rDNA gene, we amplification of partial 16S rRNA gene was conducted via the GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) using the universal primer pair 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-CAG GCC TAA CAC ATG CAA GTC-3′) (Marchesi *et al.* 1998). The PCR reaction mixture (25 µL) was prepared as follows: 12.5 µL GoTaq Green (Promega, Madison, USA), 9.5 µL Nuclease Free Water (Promega, Madison, USA), 1 µL of 10 pmol of each primer, and 1 µL DNA extraction (±100 ng). PCR conditions were as follows: initial denaturation at 95^0C for 5 minutes; 30 cycles consisting of denaturation at 95^0C for 1 minute, annealing at 57^0C for 1 minute, extension at 72^0C for 1 minute; and post extension at 72^0C for 10 minutes. PCR products were visualized on 1% (w/v) agarose gel (Promega, Madison, USA) and then stained with ethidium bromide (Sigma Aldrich, USA).

Sequence Analysis. PCR products of all LAB isolates were then partially sequenced using the 63F and 1387R primer at Macrogen Inc., South Korea. DNA sequences were matched to GenBank through base using Nucleotide Basic Local Alignment Search Tool (BLASTN) at the National Biotechnology Information Center (NCBI) at www.ncbi.nlm.nih.gov to the database of 16S ribosomal RNA sequences (Bacteria and Archaea). Phylogenetic tree analysis using Molecular Evolutionary Genetics Analysis X (MEGA version X).

Determined pH of Soybeans Soaking Water. The soybean soaking was done using a method described by Barus *et al.* (2019b) with modification. Modification in terms of replacing chemical acidification using acetic acid with acidification using the species of lactic acid bacteria found in this study. Each of the soybeans (80 grams of wet weight) soaked in 240 ml of sterile water then inoculated with 1.8 mL suspension of each LAB (10^7cells ml^-) (Keuth and Bisping 1994).

RESULTS

After the purification process, as much as twenty-two LAB isolates with form halo zone have been successfully isolated from tempeh samples in the MRS agar medium supplemented with 1% CaCO3 incubated at 37^0C (Table 1). The genome of 22 LAB isolates and amplification of 16S rDNA have been successfully obtained from the isolates. The amplicon of 16S rDNA sequences using primer pair 63F and 1387R yielded a single DNA band (±1,300 bp) for each isolate (Figure 1 as representative).

BLASTN results of the partial sequence of 16S rRNA genes (approximately 950 nucleotides) showed among our isolates with various LAB (Table 1). Seven isolates similar to *Lactobacillus agilis* 96%-99% (TBTE1-TBTE4, TBTE11-TBTE12, TBTE18). Thirteen isolates similar to *Lactobacillus fermentum* 97% -100% (TBTE5, TBTE7-BTE10, TBTE13-TBTE17, TBTE19-TBTE21). Two isolates similar to *Weissella confusa* 96% (TBTE6), and *Lactobacillus delbrueckii* 98% (TBTE22).

Filogram genetic diversity among LAB isolated

from tempeh based on the 16S rDNA sequence has been successfully constructed (Figure 2). The LAB isolates were divided into four major clusters. The first and second clusters were related to *W. confusa* and *L. delbrueckii*, respectively. The third cluster was formed with *L. fermentum*, while the fourth cluster was related to *L. agilis* (Figure 2).

DISCUSSION

The growth temperature of LAB in the spontaneous fermentation was reported 35-37^{0}C (Manini *et al.* 2016). All LAB isolates in this study could grow to a temperature of 37°C. The appearance of halo zones was due to the dissolution of $CaCO_3$ in the MRS agar

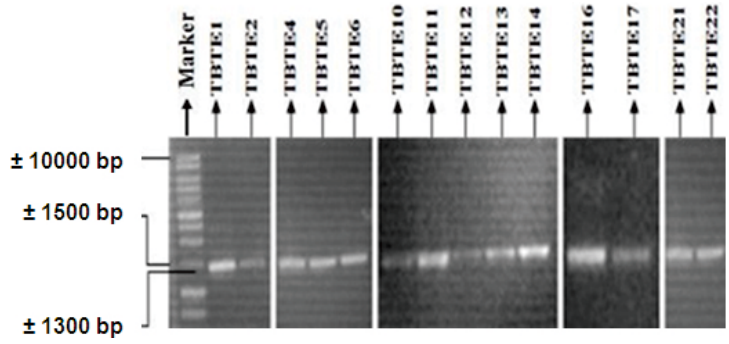

Fig 1 PCR amplification sequences of 16S rDNA sequences of LAB isolates from tempeh using primer 63F and 1387. Marker: 1 kb DNA lambda ladder.

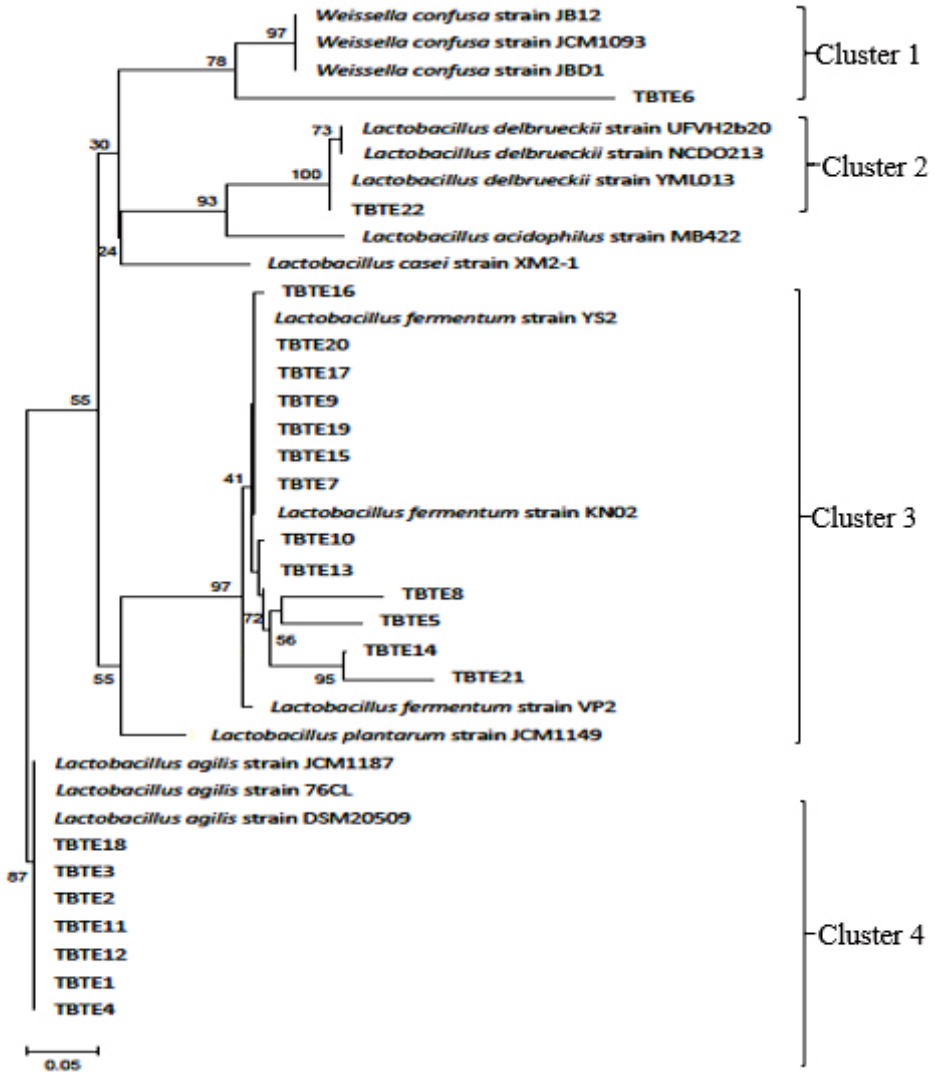

Fig 2 Filogram genetic diversity among LAB isolated from tempeh based on 16S rDNA..

Table 1 Identity of LAB isolates from tempeh based on 16S rDNA sequences

	Isolate code	Organism	Accession number	Identity (%)
Jakarta	TBTE1	*Lactobacillus agilis* strain C1-3-2	KP979478.1	98
Jakarta	TBTE2	*L. agilis* strain AAC59	KY810569.1	99
Jakarta	TBTE3	Source	AB911458.1	97
Tangerang	TBTE4	*L. agilis* strain M17	KC561119.1	98
Jakarta	TBTE11	*L. agilis* strain DSPV009P	KU295178.1	99
Jakarta	TBTE12	*L. agilis* strain TB-A07	AB425914.1	96
Jakarta	TBTE18	*L. agilis* strain DSPV005C	GQ231439.1	98
Jakarta	TBTE5	*Lactobacillusfermentum* strain EIPW-5A	KF932274.1	97
Lembang	TBTE7	*L. fermentum* strain SM44	KJ690759.1	98
Jakarta	TBTE8	*L. fermentum* strain LAB-21-ITTG	KY574532.1	98
Jakarta	TBTE9	*L. fermentum* strain IMAU60221	FJ915666.1	98
Jakarta	TBTE10	*L. fermentum* strain 10	MF066936.1	99
Jakarta	TBTE13	*L. fermentum* strain NL31	KJ958422.1	99
Jakarta	TBTE14	*L. fermentum* strain Chchm7.1an (JQ	JQ446535.1	97
Jakarta	TBTE15	*L. fermentum* strain 6704	KX218444.1	100
Jakarta	TBTE16	*L. fermentum* strain KLAB15	KM485578.1	95
Jakarta	TBTE17	*L. fermentum* strain KF5	KT159934.1	99
Jakarta	TBTE19	*L. fermentum* strain KLDS 1.06	EU419592.1	98
Jakarta	TBTE20	*L. fermentum* strain BCS27	EU547298.1	98
Jakarta	TBTE21	*L. fermentum* strain SABA5	KX599357.1	97
Lembang	TBTE6	*Weissella confusa* strain SL3	KU060304.1	96
Jakarta	TBTE22	*Lactobacillusdelbrueckii* strain SMN1-6	KY007528.1	98

medium by acid production (Pisol *et al.* 2015). All isolates were negative endospores, non-motile, negative catalase, and Gram-positive bacteria. The character of all isolates in this study is in line with the character of lactic acid bacteria (LAB) were reported by Nurhikmayani *et al.* (2019) and Pisol *et al.* (2015).

The amplicon of 16S rDNA sequences using primer pair 63F and 1387R yielded a single DNA band (±1,300 bp) for each isolate (Figure 1 as representative). The size of this amplicon is in line with Wahyudi *et al.* (2011) and Barus *et al.* (2017). BLASTN results of the partial sequence of 16S rRNA genes (approximately 950 nucleotides) showed among our isolates with various LAB (Table 1). Schlaberg *et al.* (2012) explained that the percentage of similarity of isolates was categorized in the taxonomic level of the same species, genus, family if the similarity values were ≥99%, 97% to <99%, 95% to <97% respectively.

The 16S rDNA sequence variations can show evolution among bacterial species because each *W. confuse*, *L. delbrueckii*, *L. fermentum* and *L. agilis* occupy in different clusters. The 16S rRNA gene sequence is currently the most used in assessing bacterial diversity in a particular habitat because the 16S rRNA gene is distributed in all bacteria. (Větrovský *et al.* 2013). The 16S rDNA sequences also have variable and conserved regions, and this region reflects the phylogenetic relationship between species. However, variations in the 16S rDNA sequence are not enough to compare bacteria to strain levels. This can be seen in Figure 2 that each cluster is occupied by bacteria with different strains. Three strains of *W. confusa* (Cluster 1), three strains of *L. delbrueckii* (Cluster 2), two strains of *L. fermentum* (Cluster 3), and three strains of *L. agilis* are exactly occupied the same cluster. The results of this study are in line with those previously reported (Clarridge *et al.* 2004; Sapalina *et al.* 2020).

LAB plays an important role in many fermented foods in the world. BAL in fermented foods is important for health (Caggianiello *et al.* 2016; Choi *et al.* 2015; Adeniyi *et al.* 2015; Nuraida 2015). LAB has been reported to play a role in determining the quality of fermented food (Han *et al.* 2016; O'sullivan *et al.* 2002; Settanni *et al.* 2010) and is important as a probiotic for health (Angmo *et al.* 2016; Mokoena *et al.* 2016; Argyri *et al.* 2016). Huang *et al.* (2018) reported that tempeh

contains important LAB to prevent diabetes mellitus.

Each of 80 g wet weight of soybeans soaked in 240 mL of sterile water then inoculated with 1.8 mL suspension of each isolate LAB (10^7 cell mL^{-1}). All the isolates were added for acidification in soaking soybeans. It was found that the acidity of soybean soaking water decreased from pH 7 to pH 4.4 - 4.9. In this condition, tempeh will be successfully produced in good quality because this acidic condition is suitable for the growth of *Rhizopus*. Barus *et al.* (2008) reported that pH 7 of soybean soaking water will drop to around pH 4 after 24 hours. This acidic condition is needed for the germination of *Rhizopus* as the main microbe of tempeh. Medwid *et al.* (1984) have reported that *Rhizopus olygosporus* germinates well under acidic conditions with pH 4-5.

In tempeh fermentation, acidic conditions can be made by adding organic acids such as lactic acid (Huang *et al.* 2018) or acetic acid (Kartawiria *et al.* 2018). However, tempeh fermentation in Indonesia does not use organic acids but acidic conditions occur naturally by various types of bacteria. This can cause the quality of tempeh to be inconsistent and sometime it can cause a bitter taste of tempe (Barus *et al.* 2008). Therefore, to get a consistent quality of tempeh, it is necessary to add a good selection of microbes to obtain good tempeh quality. Therefore, LAB can add to create acidifications in tempeh fermentation. Abundant bacteria have been reported in tempeh (Barus *et al.* 2008; Barus *et al.* 2017; Barus *et al.* 2013; Ayu *et al.* 2014; Efriwati *et al.* 2013; Nurdini *et al.* 2015). Therefore, research on the role of bacteria in tempeh still needs to be continued.

CONFLICT OF INTEREST

The author honestly declare no conflict of interest. All experiments in this study comply with the applicable laws of the country in which they are conducted.

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