DYNAMICS OF MICROBIAL COMMUNITY DURING TEMPEH FERMENTATION

RAHMADINA RADITA[1], ANTONIUS SUWANTO[1]*, NORIO KUROSAWA[2], ARIS TRI WAHYUDI[1] AND IMAN RUSMANA[1]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia*
[2]*Department of Science and Engineering for Sustainable Innovation, Faculty of Science and Engineering, Soka University, Hachioji, Tokyo 192-8577, Japan*

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ABSTRACT

Tempeh is a sliceable soybean-cake fermented by the bacteria *Rhizopus oligosporus*. Various bacteria are detected during tempeh cultivation, yet limited information is available about their origin or sources. The present study aimed to examine the source/s of bacterial community in tempeh by combining metagenomics analysis and cultivation technique. Samples were obtained from a number of steps in the tempeh production using the double-boiling method for the soybean (WJB tempeh production). All samples were plated on Enterobacteriaceae and Lactic Acid Bacteria media. Total DNA were extracted directly from tempeh for metagenomics analysis, applying the High-Throughput Sequencing (HTS) and cloned 16S rRNA genes. Firmicutes and Proteobacteria were the predominant and second dominant bacteria that existed in the fresh tempeh (FT) obtained by metagenomics analysis. However, cultivation technique showed that Proteobacteria was the predominant phylum, suggesting that most of the Firmicutes were not culturable. FT was dominated by *Lactobacillus* and *Acetobacter*. Both FT and water-soaked tempeh (SW) were dominated by the same species of *Lactobacillus,* i.e., *L. delbreuckii* and *L. mucosae,* indicating that SW was probably the source of bacterial community that was established in the final fermentation product. Predominant bacteria in the starter culture (SC), *Acinetobacter,* was not detected in FT, indicating that the bacteria in SC did not play a significant role in the FT bacterial community development.

Keywords: firmicutes, high-throughput sequencing, Proteobacteria, starter culture

INTRODUCTION

The Indonesian traditional food tempeh is made from soybean fermented by the mold *Rhizopus oligosporus*. However, the microbial composition of tempeh also included bacteria, as the "contaminants" in addition to the mold (Mulyowidarso *et al.* 1990; Barus *et al.* 2008; Efriwati, *et al.* 2013; Nurdini *et al.* 2015). Among the producers in Indonesia, the tempeh production procedures usually differ and depend on the area, experience, and resources. However, the general procedure for tempeh production has few similarities, such as boiling the soybean, dehulling, soaking, mixing with inoculum, and incubation for two days. The producers usually differ in boiling the soybean for the second time after soaking (Barus *et al.* 2008; Efriwati *et al.* 2013; Nurdini *et al.* 2015). Four out of five tempeh producers in Bogor, did not re-boil the soybean after soaking. However, WJB producers applied two-times or double-boiling of soybean (Barus *et al.* 2008). Of all the methods used for tempeh processing, the initial source of the bacteria in the final product fermented has not yet been verified.

Tempeh production in Indonesia is usually done in "open" fermentation using unsterile wooden equipments, where other microbes are probably involved in the production processes. Hence, various bacteria are found in tempeh, such as lactic acid bacteria (LAB) and Enterobacteriaceae (Barus *et al.* 2008; Efriwati *et al.* 2013; Nurdini *et al.* 2015). Lactic acid bacteria

*Corresponding author, e-mail: asuwanto@indo.net.id

are present at the beginning of the fermentation process (water soaking of soybean) until the end of the fermentation process (Efriwati *et al.* 2013). The population of lactic acid bacteria varies according to the stage of fermentation process. Its presence is usually shown by the decrease in pH as a consequence of natural acidification, especially during the soaking processes (Efriwati *et al.* 2013). However, recent studies showed that *laru which was* used as a starter culture in WJB productions, did not contain any Enterobacteriaceae nor lactic acid bacteria (Nurdini *et al.* 2015).

Bacterial community determination in tempeh was only limited to the culture-dependent method (Barus *et al.* 2008; Nurdini *et al.* 2015), T-RFLP (Terminal Restriction Fragment Length Polymorphisms) from culturable bacteria (Efriwati *et al.* 2013), and ARISA (Amplified Ribosomal Intergenic Sequence Analysis) (Seumahu *et al.* 2013). For the starter culture, the method used was only culture-dependent (Nurdini *et al.* 2015). These approaches however, provided only limited information and, therefore, could not reflect the composition of microbial communities. To overcome this limitation, the 16S rRNA gene was used to represent the microbial community in a metagenomics analysis. This study attempted to determine the origin of the bacterial community in tempeh ecosystem by applying the metagenomics approaches derived from cloned 16S rRNAs gene sequences from high-throughput sequencing using the total 16S rRNA gene.

MATERIALS AND METHODS

Sampling and Sample for Analysis

The samples used in this research were obtained from WJB (Warung Jambu) Tempeh Industries (Barus *et al.* 2008), namely raw soybean (RS), fresh water soybean (FW), water-soaked soybean (SW), pre-inoculated dehulled-soybean (BI), commercial *laru* as starter culture (SC), and fresh tempeh (FT). Each sample was taken three times from WJB tempeh production and transported in ice boxes for direct analysis.

Microbiological Analysis

One gram of SC was inoculated into 25 g sterile water-soaked soybean in a 250 mL Erlenmeyer. Incubation was done for 2 days before doing the serial dilution. Twenty-five grams each of RS, BI, SC, FT, or 25 mL of FW and SW, were added into 225 mL 0.85% (w/v) NaCl (Merck, USA) for serial dilution. A 100 µL aliquot of suspension from each dilution were spread on Eosin Methylene Blue Agar (EMB) (Merck, USA) to determine the total count of Enterobacteriaceae and on Man-Rogosa and Sharpe Agar (MRSA) (Merck, USA) with 0.2% of sodium azide (Plengvidhya *et al.* 2007) for enumeration of lactic acid bacteria. Each analysis was done in two replicates and incubated at 30 °C for 3-5 days for mesophilic bacteria? and Enterobacteriaceae; and 5-7 days for lactic acid bacteria.

DNA Extraction for Metagenomics Analysis

Another one gram of SC was inoculated into 25 g sterile soaked soybean in a 250 mL Erlenmeyer and incubated in 30 °C for 48 days to make a sterile tempeh. A hundred gram of sterile tempeh, BI, and FT were homogenized separately in 300 mL of PBS (phosphate buffer saline) for 1 min, following the procedure described in Seumahu *et al.* (2012). Twenty-five grams of RS sample were soaked overnight into 225 mL sterile water in the shaker. A hundred liters of FW and water-soaked RS; and 30 mL of SW were extracted using Power Water Microbial DNA Isolation-Kit (MOBIO, USA) according to the protocol described by the manufacturers. DNA products were initially visualized by electrophoresis using 1% (w/v) agarose gel for further analysis.

PCR Amplification for 16S rRNA Gene

The predominant colonies from each medium and DNA extracted for metagenome analysis were then amplified by using B27F and U1492R (Sakai & Kurosawa 2016). The PCR reaction contained the 25 µL of final solution consisting of 12.5 µL of Emerald Amp MAX PCR Master Mix (TaKaRa, Japan), 1 µL of 10 pmol of each primer, and 1 µL of DNA extract. Samples were amplified in the PCR machine with the following conditions: 94 °C for 3 min; 30 cycles of 94 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 1 min 30 seconds; and continued with 72 °C for 2 min. The PCR products (about 1,500 bp) were purified by

using Illustra GFX PCR DNA and gel band purification kit (GE healthcare, UK). PCR products of predominant colonies were sequenced by custom service provided by Eurofin Genomics (Japan).

Cloned 16S rRNA Gene Analysis

The 16S rRNA gene fragments obtained by PCR were cloned into pT7 Blue T-Vector (Novagen, Germany). The recombinant plasmid were transformed into *Escherichia coli* DH5α (TaKaRa, Japan) plated onto Luria-Bertani (LB) plates including 100 μg/mL ampicillin (Wako) and 40 μg/mL X-gal (TaKaRa, Japan). The inserted 16S rRNA gene in the plasmid was amplified by PCR using 1 μL of the culture as template with the primers T7P-F and T7U-R with the following conditions; 94 °C for 3 min; 35 cycles 94 °C for 30 seconds, 51 °C for 30 seconds, 72 °C for 2 min; followed by 72 °C for 5 min (Nishiyama *et al.* 2013; Sakai & Kurosawa 2016).

Identification of 16S rRNA Gene Clones

All the cloned 16S rRNA gene and predominant colonies were checked for chimeric sequences using the GENETYX ver. 11.0.1 software homology search program (Japan). Sequences with similarity of 98% and above were grouped together into the same phylotype (Watanabe *et al.* 2008). Sequences were submitted for BLASTN database, searching for 16S rRNA gene for identifying analysis (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/).

High-Throughput Sequencing Analysis

For the amplification of the V4 domain in bacteria 16S rRNA, the primers 515f and 806r were modified to contain the MiSeq Illumina adapter region. PCR reaction using TaKaRa ExTaq HS (TaKaRa, Japan) with PCR condition consisting of an initial 94 °C for 2 min followed by 20 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 30 sec, and final extension of 72 °C for 5 min. The PCR product was purified with AMPure XP beads (Belkman Counter, Japan) before applying the tailed-PCR using 15-index2 and 17-index1 primers. The PCR reaction consisted of an initial 94 °C for 2 min, followed by 8 cycle of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec, and final extension of 72 °C for 5 min. Sequencing of the PCR products was done using the custom service provided by the Bioengineering Lab (Japan) and Fastmac (Japan).

Data Analysis

Raw fastq files were demultiplexed, quality filtered, and analyzed with QIIME 1.6.0. The bacterial 16S rRNA gene sequences clustered using the QIIME subsampled reference OTU-picking pipeline with UCLUST-reference against the Greengenes 16S rRNA database, were clustered at 97% pairwise identity (Bokulich & Millis 2013). Shannon-Wiener index (H'), Simpson index (D), and the Equitability (E) were calculated to describe community diversity, similarity between species, and relative importance of each operational taxonomic unit (OTU) within the entire assemblages (Pangastuti *et al.* 2010).

RESULTS AND DISCUSSION

Culturable Enterobacteriaceae and Lactic Acid Bacteria

Proteobacteria, represented by Enterobacteriaceae, were predominant in the FT and SC samples (Fig. 1). Enterobacteriaceae presented in the raw material RS was 3.6×10^5 CFU/g, and decreased to 2.4×10^2 CFU/g in BI after being boiled twice, then reached its maximum value in FT with 2.9×10^7 CFU/g. *Klebsiella pneumoniae* were the predominant Proteobacteria in FT samples, while previous study reported *Pseudomonas putida* and *Acinetobacter* sp. as the predominant bacteria in the WJB tempeh (Barus *et al.* 2008). On the other hand, lactic acid bacteria reached its maximum value in SW, 10^7 CFU/g, where the natural acidification occurred with the presence of lactic acid bacteria (Efriwati *et al.* 2013) and in addition of 1.4×10^2 CFU/g of Enterobacteriaceae. Previous study reported similar number of lactic acid bacteria detected in water-soaked soybean at 6.0×10^5 CFU/g and fresh tempeh from WJB producer at 4.3×10^5 CFU/g (Barus *et al.* 2008). Whereas in this current study, lactic acid bacteria were 7.7×10^4 CFU/g in the FT sample predominantly the *Lactobacillus lactis, L. fermentum,* and *Weissella confusa.*

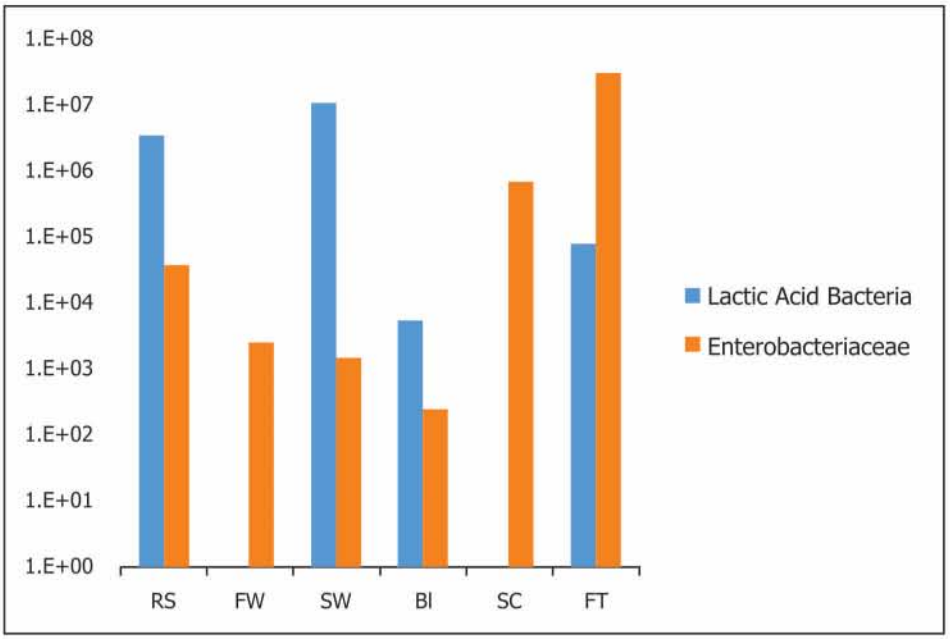

Figure 1 Number of total bacteria populations based on culture-dependent technique from WJB production
Notes: RS = raw soybean; FW = fresh water soybean; SW = water-soaked soybean; BI = soybean before inoculation; SC = starter culture; FT = fresh tempeh.

High-Throughput Sequencing Data Processing

Sequencing of the amplicon pool resulted in 971,785 paired-ends assembled reads after the assembly and sample index-based demultiplexing, which were reduced to 804,136 cleaned reads after being sorted for quality issues (homopolimers, length), chimera sequence, and miss amplified taxa (chloroplast, mitochondria, archaea, or unassigned kingdom) with average length of 340 bp. All of these sequences were used for taxonomy-based analysis, which consisted of kingdom, phylum, class, ordo, family, and genus level. Indices regarding diversity parameters were measured with the number of OTUs (Operational Taxonomic Unit) showing generally high values, from a minimum of 303 in RS to a maximum of 2,705 in FW, thus indicating the richness of the samples (Table 1). Shannon-Wiener Index, Simpson Index, and Equitability also showed similar patterns among the samples. FW and enriched SC had the highest values among the indices, indicating that the number of individual species in each OTU was even. However, the BI samples had the lowest Simpson Index for the predominant bacteria obtained in greater number than other species.

Table 1 Number of sequences, observed diversity richness (OTUs), and diversity estimates of bacteria

Samples	Total Reads	Clean Reads	Total OTUs	Shannon-Wiener Index (H')	Simpson Index (D)	Equitability (E)
RS	53,609	47,161	303	1.401	0.435	0.245
FW	64,614	55,576	2,705	3.218	0.803	0.407
SW	57,563	51,709	528	0.640	0.231	0.102
BI	70,830	51,879	803	0.807	0.066	0.120
SC	342,391	278,518	1,095	2.818	0.893	0.403
FT	383,778	319,293	539	1.649	0.754	0.262

Notes: RS = raw soybean; FW = fresh water soybean; SW = water-soaked soybean; BI = pre-inoculated soybean; SC = starter culture ; FT: fresh tempeh.

Bacterial Communities Structure from WJB Production

Taxa present in 1% or more in at least one sample are identified and listed, while rarer taxa were grouped under the "other" label (Fig. 2). HTS data analysis showed that WJB tempeh was produced by only four phyla, including Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes. The latter mentioned only showed in the FW sample, with its participants in other samples less than 1%. Firmicutes and Proteobacteria were the predominant and second dominant bacteria that existed in the WJB tempeh. In the raw material used for tempeh production, Firmicutes was dominant in RS (93%) while Proteobacteria was dominant in FW (73%) and SC (96%) with proportion from each clean reads. Firmicutes were also the predominant bacteria in FT (88%) and SW (96%), while the dehulled-soaked and pre-inoculated soybean, BI, was dominated by Proteobacteria (98%). Firmicutes population in FT presumably originated from RS and/or SW.

Lactobacillales was the predominant ordo in all samples populated by Firmicutes, namely; RS, SW, and FT, while predominant orders representing Proteobacteria varied in their number in other samples (Figs. 3 & 4). With 57% abundance, Burkholderiales was predominant in FW, but these microbes vanished once the soybeans were soaked and was not detected in the next step. However, the double boiling before the inoculation of the soaked soybean, resulted in the decrease of its species richness and decline of its number (Efriwati *et al.* 2013). Rhodospirilales vastly increased as the predominant Ordo detected in the BI sample with 97% abundance, while Lactobacillales significantly diminished from the soaking process. However, Rhodospirilales abundance was decreased in FT until 11% and the bacterial predominance shifted to Lactobacilalles (88%). Unlike the result shown by culturable technique, the sole Ordo signifying Proteobacteria in SW was Rhodospirilales (1.6%), while Enterobacteriaceae occurrence itself was less than 1%. Other predominant Proteobacteria orders that occurred were Enterobacteriales (34%) and Pseudomonadales (36%) in the SC samples. As expected, neither of these orders occurred more than 1% in the final fermentation product, FT, due to its small amount of administration for tempeh fermentation. Enterobacteriales, which harbored *K. pneumoniae* as vitamin B_{12} producer in tempeh (Ashok *et al.* 2012), was detected in FT with 0.05% from the entire clean reads. With 340 bp average length sequence, it was not adequate enough to identify Enterobacteriales until genera level, unlike the other orders (Humblot & Guyot 2009; Roh *et al.* 2010). Thus, information about the presence of *K. pneumoniea* in FT was only achieved by culture technique.

Figure 2 Phyla of bacteria present in the WJB sample analyzed with total 16S rRNA sequence
Notes: Phyla acquired in samples less than 1% was put under "Others" label.
RS = raw soybean; FW = fresh water; SW = water-soaked soybean; BI = pre-inoculated soybean; SC = starter culture; FT = fresh tempeh.

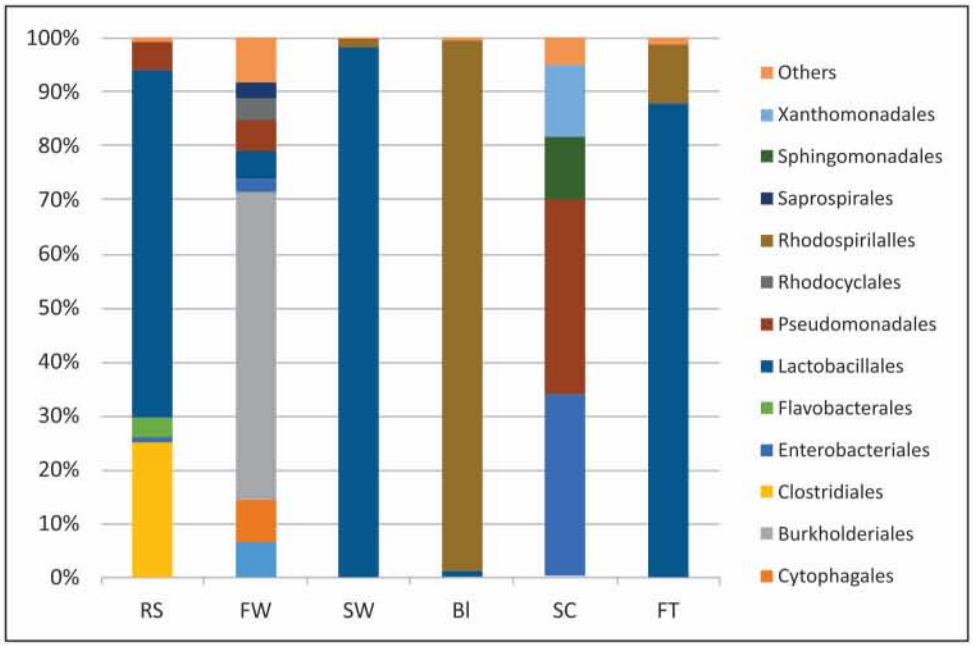

Figure 3 Major Orders detected from WJB samples applied with total gene 16S rRNA sequences analysis
Notes: Major Orders that occurred in less than 1% of samples were put under "Others" label.
RS = raw soybean; FW = fresh water; SW = soaked water soybean; BI = pre-inoculated soybean; SC = starter culture; FT = fresh tempeh.

Twelve major genera from predominant Orders were detected from the WJB samples (Fig. 4). Major Lactobacillales genera attained from RS was different from SW's and FT's predominant genera. *Clostridium* (43% abundance) and *Lactococcus* (28% abundance) dominated the RS sample. Yet, these bacteria were not detected in the next step. Both SW (97%) and FT (92%) were populated by solely *Lactobacillus*. Being the major bacteria in both samples, *Lactobacillus* abundance was actually increased in FT, after vanishing in BI until 272 sequences of BI total reads (0.5%). These indicated that some *Lactobacillus* were still alive during the second-boiling of soybean, thus they dominated once more in the final fermentation product. The second boiling of the soybean did not only shift the bacterial community presence in BI, but also slightly decreased its total number (Efriwati *et al.* 2013), especially *Lactobacillus* genera. Apparently, the tempeh fermentation environment was very suited for *Lactobacillus* growth, since it remarkably increased during the two days fermentation, 800 times higher than in its original number in BI.

Changes in bacterial community through the fermentation process was expected, since bacterial communities within ecosystem respond to the environmental conditions. The shifting of bacteria community also happened to food-based fermented product, such as *kimchi* (Park *et al.* 2012) and *dongchimi* (Jeong *et al.* 2013), and now in the soybean soaking process in tempeh production. Abundance of some environmental bacteria which might have contributed to the ecology of the initial fermentation has shifted to lactic acid bacteria as affected by the accompanying pH decreases and temperature increase (Yan *et al.* 2013). Moreover, lactic acid bacteria could produce antimicrobial compound aside from the organic acid that could eliminate pathogen and spoilage bacteria (Trias *et al.* 2010).

Predominant ordo in BI sample was solely *Acetobacter* from Rhodospirilalles with 99% relative abundance. These bacteria already existed in the SW processing with 1.5% abundance. Its existence was also reported when using the cultivation technique in water-soaked soybean in different tempeh production (Barus *et al.* 2008). *Acetobacter* was known as acetic acid producer from alcohol substrate in the presence of excess oxygen (Purwoko 2009). These bacteria also existed in the pre-inoculated dehulled-soybean without the second boiling of soybean, even though its relative abundance and number were lower, yet the species richness was higher than BI from WJB production (data not shown). This is probably because the BI from

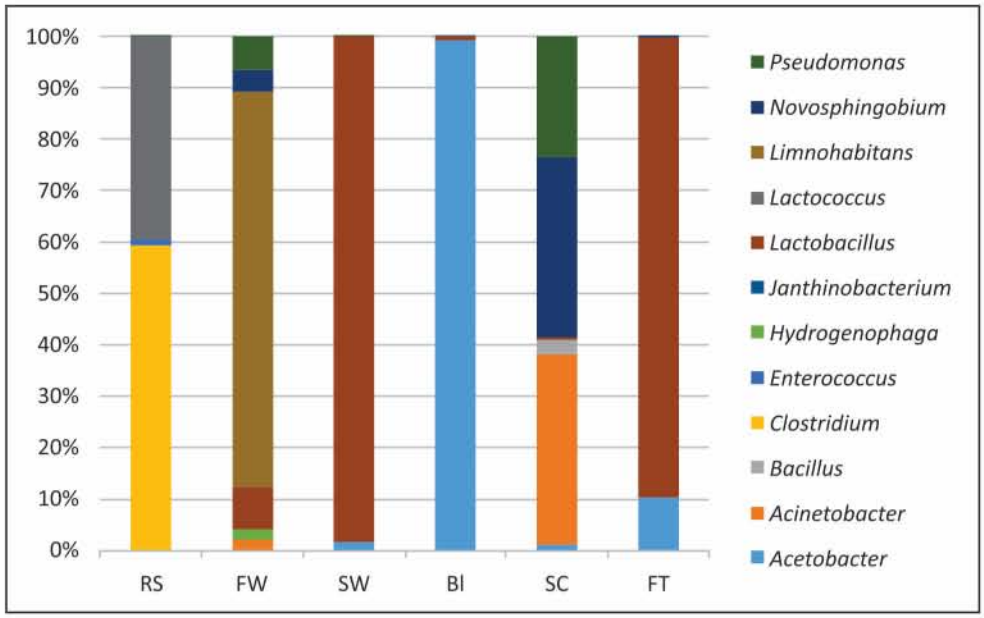

Figure 4 Predominant genera attained from WJB samples, employing total 16S rRNA gene analysis
Notes: Major genera that occurred less than 1% in all samples were put under "Others".
RS = raw soybean; FW = fresh water; SW = water-soaked soybean; BI: soybean before inoculation; SC = starter culture; FT = fresh tempeh.

WJB production was not washed with water after the second boiling process. Moreover, the second boiling process used the soaking water, not the fresh water. Thus, excessive ethanol from heterofermentative lactic acid fermentation in the soaking process was used as the substrate for *Acetobacter* growth (Purwoko 2009). The bacteria itself presumably originated from the environment where the tempeh production occurred. However, these bacteria later were reduced to 65% of its original number in FT sample, indicating that tempeh fermentation condition was not suited for *Acetobacter* growth, thus consequently shifting to *Lactobacillus*. In another food fermentation process, *Acetobacter* was responsible for the fermentation of vinegar (Wu *et al.* 2010), *nata* (Jagannath *et al.* 2010), and chocolate (Papalexandratou *et al.* 2011), with different *Acetobacter* species.

Pseudomanadales was the predominant bacteria in the SC (35%), while Enterobacteriales was the second predominant bacteria in SC with its sole family, Enterobacteriaceae (34%). Due to its inaccurate identification data, Enterobacteriaceae cannot be identified further until genera level, thus the predominant genera that existed in SC remained unknown. Pseudomonadales was represented by *Acinetobacter* (37%) and *Pseudomonas* (23%) as the predominant genera in SC used by WJB. Only 1 g of SC was added to 250 g of BI, thus the abundance of predominant bacteria in SC were detected in FT as less than 1%. SC contained *Lactobacillus* with 0.4% abundance. However, the involvement of *Lactobacillus* from SC to establish *Lactobacillus* as predominant bacteria in FT was ignored, since its abundance in SC was less than 1%. Bacterial community in the fermented product was not affected by bacterial community in the starter culture (Park *et al.* 2012; Polka *et al.* 2015). Most probably many of bacteria contained in starter culture did not play an ecological role in tempeh fermentation. Some identified genera, such as *Acinetobacter* was identified as contaminant in rye and wheat sourdough (Ercolini *et al.* 2013); *Pseudomonas* was commonly found in fermented shrimp (Park *et al.* 2011), while *Novosphingobium* was frequent in Chinese traditional sourdough (Zhang & He 2013).

Cloned 16S rRNA Gene Analysis

For better assessment of the bacterial species involved in the bacterial community in tempeh products, cloned 16S rRNA genes were used from the same pool of DNA as previously analyzed. A total of 126 clones was obtained

from six samples and divided into two major phyla, Firmicutes and Proteobacteria (Table 2). The predominant genera obtained through this method coincided with previous result (Fig. 2), yet contradicted with the results of the cultivation technique (Fig. 1). Both SW and FT were dominated by the same species *Lactobacillus, L. delbrueckii* and *L. mucosae*. The similar sequences probably indicated that predominant bacteria in FT probably originated from SW processes. Moreover, *Lactobacillus, Enterococcus* and *Pediococcus* genera were also present in the WJB tempeh analyzed using T-RFLP (Terminal Restriction Fragment Length Polymorphisms). But their position in the tempeh dominant bacteria were not reported (Nurdini 2015). The possibility of drawbacks using PCR-based community analysis technique, such as biases from DNA extraction and amplification; and the presence of amplified dead cells (Yan *et al.* 2013; Jeong *et al.* 2014) were eliminated during the HTS analysis. As previously mentioned, the abundance of *Lactobacillus* genera in SW decreased after the second boiling of soybean, but it vastly increased again after 48 hours' incubation process in the tempeh fermentation. Thus, *Lactobacillus* sequences amplified by both methods were not a bias and not dead cells.

Knowledge on the variations in the bacterial community structures that occur during tempeh production, including the order of abundant and rare phylotypes, are important in understanding how bacterial community composition in tempeh production are affected by each step in the processes involved. In another study, the tempeh produced by WJB was preferable and had less bitter taste than the tempeh produced by other producers in Bogor, was probably due to its lower bacterial number (Barus *et al.* 2008). However, the lower bacterial number also corresponds to lower lactic acid bacteria which is an imuno-stimulant (Soka *et al.* 2014), lower Enterobacteriales which represent *K. pnemoniae* as vitamin B_{12} producer (Ashok *et al.* 2012), and no Bifidobacterales which is an isoflavone aglycone producer (Raimondi *et al.* 2009). Consequently, the double boiling of soybean was good to lessen the bitterness in tempeh, yet it also decreased the nutritive value that could be obtained from tempeh. Limited information is available about bacterial community composition in food production, even though the diversity and composition of microbial communities in food are thought to have a direct influence on the human guts' microbial consortia (Hehemann *et al.* 2010; Park *et al.* 2012).

The dissimilar result occurred between cultivation techniques and metagenomics analysis in this study. Most of the predominant bacteria that existed in tempeh products were not detected by the cultivation technique, particularly the lactic acid bacteria. This indicated that tempeh ecosystem dominated by bacterial communities could not be cultured by traditional methods, thus a bias occurred in favor of the predominant bacterial community found in tempeh. Consequently, the results obtained by cultivation technique could not reflect the real microbial communities in tempeh (Aslam *et al.* 2010). Using metagenomics technique without the cultivation technique was neither preferable. Metagenomics employing

Table 2 Cloned bacterial 16S rRNA sequences during tempeh production

Sample	Species	Similarity (%)	Phylum	No. Accsession
RS	*Clostridium beijerinckii*	99	Firmicutes	KJ957170
	Lactococus taiwanensis	96	Firmicutes	NR_114327
FW	*Acidovorax* sp.	98	Proteobacteria	JX177703
	Curvibacter sp.	99	Proteobacteria	JN679217
SW	*Lactobacillus delbrueckii*	98	Firmicutes	FJ915697
	Lactobacillus mucosae	99	Firmicutes	JQ805650
BI	*Acetobacter indonesiensis*	99	Proteobacteria	AJ419841
	Acetobacter aceti	99	Proteobacteria	KR261398
SC	*Acinetobacter ursingii*	99	Proteobacteria	LC014147
	Pseudomonas aeruginosa	100	Proteobacteria	KP866890
FT	*Lactobacillus fermentum*	98	Firmicutes	JN188384
	Lactobacillus delbrueckii	98	Firmicutes	FJ915697
	Lactobacillus mucosae	99	Firmicutes	JQ805650

Notes: RS = raw soybean; FW = fresh water; SW = water-soaked soybean; BI = pre-inoculated soybean; SC = starter culture; FT = fresh tempeh.

PCR-based community analysis might be selective for some population over others, due to the selective extraction of nucleic acids, selective amplification of 16S rRNA gene, and the presence of dead cells (Yan *et al.* 2013; Jeong *et al.* 2014). However, up to date, metagenomics analysis employing HTS was the most extensive sequencing tool to study the microbial community in food production processes, even for minor population of bacteria. Metagenomics analysis of bacterial community in tempeh improves the understanding of bacterial ecology in tempeh which was limited in the classical knowledge of the tempeh ecosystem.

CONCLUSION

Firmicutes and Proteobacteria were the predominant and second dominant bacteria that existed in the WJB fresh tempeh as indicated by the metagenomics analysis. However, the cultivation technique showing the significantly opposite results, might have under-estimated the Firmicutes population. Double boiling of soybean applied in the WJB products resulted in the low number of bacteria existing in the fresh tempeh (FT) which was dominated by *Lactobacillus*. Both FT and water-soaked soybean (SW) harbored *Lactobacillus delbreuckii* and *L. mucosae*, indicating that SW probably was the main source of the bacterial community established the in fresh tempeh. Apparently, the bacteria in the starter culture (SC) did not play an essential role in the bacterial community development in FT.

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