International Food Research Journal 25(6): 2313-2320 (December 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Firmicutes is the predominant bacteria in tempeh

[1]Radita, R., [1*]Suwanto, A., [2]Kurosawa, N., [1]Wahyudi, A. T. and [1]Rusmana, I.

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

Article history

Received: 4 August 2017
Received in revised form: 26 February 2018
Accepted: 9 March 2018

Keywords

Tempeh
Metagenome
Firmicutes
Major phylum

Abstract

Tempeh is an Indonesian fermented soybean inoculated with *Rhizopus* spp. A number of culturable bacteria in tempeh had been documented. However, comprehensive study of bacteria population in tempeh has not been reported. This research aimed to analyze species of bacteria in tempeh employing culturable method and metagenomics analysis based on 16S rRNA gene sequences and high-throughput sequencing. Samples were obtained from two tempeh producers in Bogor, Indonesia, i.e. EMP and WJB. Metagenomics analysis indicated that Firmicutes was the predominant phylum in both samples, with Lactobacilalles as the predominant order. The second majority phylum was Proteobacteria. Similarly, the results obtained from culturable technique also showed that Firmicutes was the predominant phylum. One-time boiling of soybean employed for EMP tempeh harbored the highest bacterial diversity than two-times boiling soybean (WJB tempeh). Four orders were the predominant bacteria in EMP tempeh, i.e. Lactobacillales, Clostridiales, Enterobacteriales, and Sphingomonadales, while Lactobacillales and Rhodospirilalles were the only predominant bacteria orders in WJB tempeh.

Introduction

Tempeh is soybean fermented by *Rhizopus* spp. from Indonesia. As a staple food, tempeh contains nutrients advantageous as a daily diet due to its high content of protein, fatty acids, and vitamin B_{12} (Keuth and Bisping, 1993). Utilization of mold in tempeh fermentation changes the indigestible macromolecule in soybean into digestible molecules (Nout and Kiers, 2005). Tempeh also contains variety of vitamins including vitamin B_{12} in higher amount than its raw ingredients. Therefore, this food is very suited for vegetarians who usually deficit of vitamin B12 diet (Watanabe *et al.*, 2013). Moreover, the bacteria present in tempeh could act as immunostimulant which can promote human health (Soka *et al*., 2014).

Basically, tempeh is produced locally through several kinds of processing methods which include cooking the soybean, soaking, dehulling, inoculation of starter culture, and incubation processes. But some tempeh producers employ modified methods which add another boiling step after soaking process (Barus *et al*., 2008; Efriwati *et al.,* 2013). Barus *et al.* (2008) reported that one out of five tempeh producers in Bogor, Indonesia, does the two-times boiling of soybean, such as WJB tempeh producer, while the rest employ one-time boiling soybean, such as EMP tempeh producer. These two types of tempeh has been used as a model for monitoring the microbial community in tempeh fermentation, especially in Indonesia (Barus *et al*., 2008; Efriwati *et al*., 2013; Seumahu *et al.*, 2013; Soka *et al.*, 2014).

Besides *Rhizopus* spp., other microorganisms were also reported present in fresh tempeh, such as lactic acid bacteria (LAB) and Enterobacteriaceae (Moreno *et al*., 2002; Barus *et al*., 2008; Efriwati *et al*., 2013; Nurdini *et al*., 2015). The methods for determining the bacterial communities in tempeh samples so far limited only to culturable 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). Based on culturable methods, the number of bacteria present in tempeh EMP and WJB were different from time to time (Barus *et al*., 2008; Efriwati *et al.*, 2013; Nurdini *et al.*, 2015). Efriwati *et al.* (2013) reported that tempeh made with one-time boiling of soybean, had higher number of lactic acid bacteria than tempeh made with two-times boiling of soybean, but Nurdini *et al*. (2015) reported the opposite. These could explain that tempeh was

*Corresponding author.
Email: *asuwanto@indo.net.id*

a very dynamic fermented food with its bacterial community might change over times. Moreover, these also could be caused by limited amounts of information and unreliable methods of culturable technique in analyzing the microbial community (Hugenholtz *et al*., 1998).

These limitations could be overcome by utilization of metagenomics approach by targeting 16S rRNA gene as a phylogenetic marker for representing the metagenome technique. This technique could be used to examine unculturable bacteria without creating a bias (Kakirde *et al*., 2010) since the data produced using the culturable method is failed to precisely quantify some predominant taxa yet still underestimated biodiversity (Ampe *et al.*, 1999). Moreover, metagenome technique can also provide wealthy information about the microbial community in fermented foods (Kim and Chun, 2005) by sequencing 16S rRNA gene sequences libraries using high-throughput sequencing (HTS). Recently, the development of HTS has led to the large-scale metagenomics sequencing of fermented foods, such as Italian salami (Polka *et al.,* 2015), wine (Bukolich *et al*., 2013), and artisan cheese (Bukolich and Millis, 2013). The aim of the present study was to achieve a fine characterization of bacterial diversity in tempeh. Samples were retrieved from two local producers in Bogor, Indonesia. Culturable technique and cloned 16S rRNA gene were also carried out to compare the result with HTS analysis.

Materials and Methods

Enumeration and microbiological analysis

Samples for this study were fresh tempeh obtained from two tempeh industries, designated each as EMP and WJB tempeh (Barus *et al*., 2008; Efriwati *et al*., 2013; Soka *et al.*, 2014). Sampling were taken three times from different tempeh production and transported in ice box for direct processing.

25 gram of each samples were homogenized in 225 mL of NaCl 0.85%. A 100 µL of aliquot was plated into modified MRSA (Man Rogosa-Sharpe Agar) (Merck, US) with 1% of calcium carbonate supplementation (Efriwati *et al*., 2013) for enumerating lactic acid bacteria, EMB (Eosin Methylene Blue) (Merck, USA) for enumerating Enterobacteriaceae, and PCA (Plate Count Agar) (Merck, USA) for enumerating mesophilic aerobic bacteria. Enumeration was done in replicate and the plates were incubated at 26-30^{0}C for two days for all media. The total numbers of colonies on plates were expressed as number of cfu per gram sample. Representative colonies from each medium and sample were further analyzed for identification based on 16S rRNA gene sequence.

Identification based on 16S rRNA gene.

Representative colonies from each medium were identified through their 16S rRNA gene. A 1.5 mL of aliquot bacterial suspension which had been grown for one night in their respective medium was centrifuged for 3 mins at 10 000 x *g*. Pellet formed then extracted according to the procedure of Presto Mini gDNA Bacterial Kit (Geneaid, Taiwan). The PCR reaction contained 25 µL of final solution consisting of: 12.5 µL of EmeraldAmp MAX PCR Master Mix (TaKaRa, Japan); 1 µL of 10 ng.µL^{-1} of each universal primer: B27F and U1492R; and 1 µL of DNA extraction. Samples were amplified in PCR machine (Applied biosystem 2720 Thermal cycler) with the following condition: 94°C for 3 mins; 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 mins 40 seconds; and continued with 72°C for 2 mins. Sequencing was carried out by Eurofin Genomics (Japan). Sequences from representative colonies were submitted for BLASTN database searching for 16S rRNA gene to identify individual sequence (BLAST, *http://www.ncbi.nlm.nih.gov/BLAST/*).

DNA extraction for metagenomics analysis

A hundred gram of tempeh from each tempeh samples were homogenized separately in 300 mL PBS (phosphate buffer saline) for 1 mins. The mixture later blended using blender (Maspion, Indonesia) for 5 mins. Then, centrifugation was done for 1 mins at 1,000 rpm and the supernatant were moved into new tubes and centrifuge again at 10,000rpm for 3 mins. Supernatant were discarded and the pellet were washed with TE (pH 8.0) prior to DNA extraction. Microbial DNA was extracted by using PowerFood Microbial DNA Isolation kit (MOBIO, USA) (Seumahu *et al*., 2012) according to the protocol described by the manufacturer. The DNA products were visualized by electrophoresis on 1% (w/v) agarose gel before being used for next step of analysis.

Cloned 16S rRNA gene

DNA extractions from previous section were amplified employing universal primer B27F and U1492R (Lane, 1991). PCR products (approximately 1,500bp) were purified by using Illustra GFX PCR DNA and gel band purification kit (GE healthcare, UK). The 16S rRNA gene fragments were cloned into pT7 Blue T-Vector (Novagen, Germany). Recombinant plasmid were transformed into

Escherichia coli DH5α (TaKaRa, Japan) plated onto Luria-Bertani (LB) plates supplemented with 100 µg. mL^{-1} ampicillin (Wako, Japan) and 40 µg.mL^{-1} X-gal (TaKaRa, Japan). The inserted 16S rRNA gene in the plasmid was amplified by PCR using 1 µL culture as template which mixed along with T7P-F and T7U-R primers. PCR products were sequenced employing a custom service provided by Eurofin Genomics (Japan) (Nishiyama *et al.*, 2013; Sakai and Kurosawa, 2016).

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

High-throughput sequencing (HTS) analysis

HTS conducted employing MiSeq Illumina. For amplification of the V4 domain of bacterial 16S rRNA gene sequence, modified F515 and R806 primers were used to contain Illumina adapter region. PCR reactions were conducted using TaKaRa ExTaq HS (TaKaRa, Japan) at initial 94°C for 30 sec followed by 20 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 5 mins. PCR products were purified through AMPure XP beads (Belkman Counter, Japan) and subsequently used as templates for tailed-PCR with step as follows: 94°C for 2 mins, followed by 8 cycle of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 5 mins. Sequencing of the PCR products were done using a custom service provided by Fastmac (Japan).

Data analysis

Raw fastq files were demultiplexed, quality filtered, and analyzed with QIIME 1.6.0. Bacterial 16S rRNA gene sequences were clustered using QIIME subsampled reference OTU-picking pipeline with UCLUST-reference against the Greengenes 16S rRNA gene sequences database, clustered at 97% pairwise identity. From this alignment, chimeric sequences were identified and removed with QIIME. Sequences failing alignment or identified as chimera were removed prior to downstream analysis (Bokulich and Millis, 2013). Shannon-Wiener index (H'), Simpson index (D), and the Equitability (E) were calculated to describe the diversity of community, similarity between species, and relative importance of each OTU within the entire assemblages. H' was calculated as follows: $H' = -\Sigma (pi) (\ln pi)$ where pi is the relative abundance of fragment i. D was measured based on equation: $D = \Sigma n_i(n_i-1)/N(N-1)$, where n_i was the relative abundance of fragment i and N was the total sequence from all OTU. Meanwhile E was calculated by the following equation: $E = H'/Hmax$ where $Hmax = \ln S$ (Pangastuti *et al*., 2010).

Results and Discussion

Basic analysis of high-throughput sequencing

A total of 808,348 sequencing reads were generated from two DNA pools derived from EMP and WJB tempeh. A number of 681,888 sequencing reads were yielded after quality screened of 340 bp length on average. With an average number of sequence reads, tempeh had the highest bacterial abundance than any other fermented foods, such as salami (Polka *et al*., 2015), surimi (Zhao *et al*., 2015), cassava beer (Colehour *et al.*, 2014), and sourdough (Minervini *et al.*, 2015). The clean high-quality reads were the sequences screened through quality issues (length, homopolymers, ambiguous bases) and chimera (<16.5%). Another 1% was discarded because of miss amplified taxa (chloroplast, mitochondria, and unassigned sequences). Diversity indices as described by the number of OTU, Shannon-Wienner index, Simpson index, and equitability index were listed in Table 1. As shown by Shannon-Wiener and Simpson indices, the bacterial diversity in EMP tempeh was higher than in WJB tempeh samples. Moreover, a number of each OTU in EMP tempeh was also distributed more equally as the indices of equitability index higher than WJB tempeh. This result suggested that EMP tempeh had more diverse bacteria on its community level than WJB tempeh.

Diversity of bacterial community structure in tempeh samples

16S rRNA gene sequencing reads were classified into different taxonomies. The taxon abundance of EMP tempeh and WJB tempeh were generated into phylum, class, order, family, and genera levels by employing HTS, while its species dominance was acquired by cloned 16S rRNA gene. The phyla of bacterial community existed in both samples was composed of two predominant phyla, Firmicutes and Proteobacteria, as shown in Figure 1. Other phyla acquired were Actinobacteria and Bacteroidetes which grouped into "Others", as its abundance in total reads were less than 1%.

At the order level, a total of 21 orders were found in both samples, EMP and WJB tempeh with each sample consist of 19 orders, while other three orders were different to each other. Having higher diversity,

Table 1. Statistical analysis of the bacterial 16S rRNA gene sequencing data sets of the tempeh samples

Samples	Total Reads	High-Quality Reads	Number of OTUs	Shannon-Wiener Index (H')	Simpson Index (D)	Equitability (E)
EMP tempeh	425,570	361,492	1,003	2.11	0.76	0.31
WJB tempeh	382,778	319,293	539	1.65	0.75	0.26

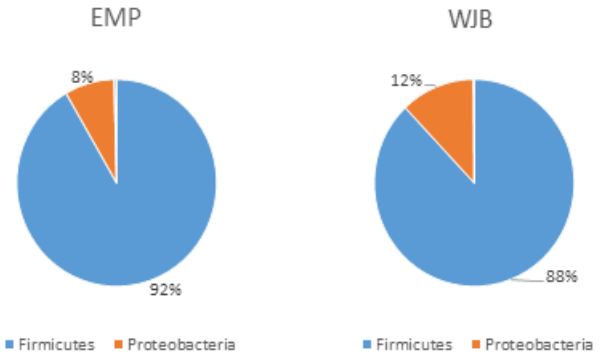

Figure 1. Bacterial phyla in tempeh as revealed by total 16S rRNA gene sequence analysis. Phyla that were acquired in less than 1% in samples were gropued as "Others".

EMP tempeh had four orders which its abundance was more than 1% in total reads. Those four orders consisted of Lactobacillales and Clostridiales from Firmicutes phylum; and Enterobacterales and Sphingomonadales from Proteobacterium phylum with each proportion from clean reads was 87.8%, 2.9%, 3.9%, and 2.7%, respectively. With 84.8% and 11% proportion, classified Lactobacilalles and Rhodospirilalles (Proteobacteria phylum) as the dominated orders existed in WJB tempeh (data not shown). Even though both samples was dominated by Lactobacilalles, its abundance in total sequence reads was different. In the EMP tempeh, Lactobacilalles was 320,121 sequence reads, while in WJB tempeh was 280,854 sequence reads. Lactobacilalles or lactic acid bacteria (LAB) was classified as Generally Recognized as Safe (GRAS) bacteria with numerous functional ingredients, such as probiotic, anti-microbial agents, a source of vitamin, enzyme, low-sweetener, etc. (Florou-Paneri *et al.,* 2013). Thus, sample with more sequence reads of Lactobacilalles was considered to have more benefit for consumer health.

In order to understand better, predominant family and genera under Firmicutes phylum which more than 1% abundance were shown in Figure 2. With the total of 64 families from both samples, WJB tempeh apparently had more families than EMP tempeh, with total 34 families. Unlike EMP tempeh which had two predominant families with nearly even abundance proportion, i.e. Lactobacillaceae (42.8%) and Enterococcaceae (47.8%), WJB tempeh only had Lactobacillaceae (88%) as predominant family. Other families from Proteobacteria phylum were Enterobacteriaceae and Sphingomonadaceae from EMP tempeh, and Rhodospirillaceae from WJB tempeh.

Almost all of the genera acquired in WJB tempeh also existed in EMP tempeh, except for *Staphylococcus*. With an uneven proportion of bacteria, WJB tempeh only had one major genera as the most abundant bacteria, i.e. *Lactobacillus* with its occurrence in Lactobacilalles reads was 99.3%. The rest of orders which less than 1% percentage were divided evenly to *Weissella, Lactococcus, Paenibacillus, Streptococcus, Bacillus, Clostridium, Staphylococcus, Anoxybacillus,* and *Enterococcus.* Beside *Lactobacillus*, EMP tempeh samples also had *Enterococcus* as the predominant genera, with nearly similar proportion, i.e. 42.8% and 47.8%, respectively. With less than 10% abundance, similar genera found in WJB tempeh were also acquired from EMP tempeh, except for *Staphylococcus. Leuconostoc, Cohnella, Brevibacillus,* and *Pediococcus* were additional genera which also acquired from EMP tempeh samples.

Different bacterial community compositions acquired between tempeh samples were possible. Even though tempeh was primarily fermented by mold, microbial composition in tempeh also includes other bacteria as the "contaminants" (Barus *et al.*, 2008; Efriwati *et al*., 2013). This might be due to the fact that tempeh fermentation occurs mainly as open fermentation, where other microbes could enter into the production process. Furthermore, the involvement of second-times boiling soybean could reduce the bacterial abundances and composition, as in WJB tempeh producers (Figure 1). Therefore, different producers could yield different composition of microbial communities. Other than procedure used, environment condition where the fermentation occurred, (temperature and RH), the raw material used, and water source (Kim *et al*., 2012; Jung *et al.*, 2013), have high possibility to alter the bacterial composition in tempeh.

Predominant species analysis

More than 80% of total clean reads yielded by HTS did not identify the species of sequence reads. This might be due to the inaccuracy of sequencing

Table 2. Species identification based on 16S rRNA derived from EMP and WJB

Tempeh Samples	Number of Total Clones	Species	Similarity (%)	Accession Number
EMP	28	*Enteroccocus cecorum*	99	AB932534
	4	*Lactobacillus agilis*	99	M58803
	1	*Lactobacillus fermentum*	99	AP008937
WJB	13	*Lactobacillus fermentum*	98	JN188384
	9	*Lactobacillus mucosae*	99	JQ805645
	1	*Acetobacter indonesiensis*	97	KJ469771
	2	*Lactobacillus agilis*	96	LC065041
	8	*Lactobacillus delbrueckii*	99	FJ915697

approach as only 340 bp of 16S rRNA gene was amplified (Humblot and Guyot, 2009). However, 95% of total clean reads were sufficient to identify until genera level, while 5% of the rest were identified up to family level (data not shown). This result suggested that another method than HTS, should be applied to cover the absence of species identity. Therefore, cloned 16S rRNA gene was used to obtain species identity since it can detect the predominant bacteria in one sample, culturable or unculturable species (Park *et al*., 2010).

As shown in Table 2, predominant species in 33 clones obtained from EMP tempeh samples were coincided with HTS result, *Enterococcus cecorum* and *Lactobacillus agilis.* As in HTS, cloned 16S rRNA gene result did not yield similar proportion for both species, but rather 85% to 15% for *E. cecorum* and *L. agilis,* respectively. The dissimilar result could occur between metagenome approaches since there was strictly correlated with ratio of 16S rRNA gene used for clone analysis to the total DNA extracted and the random colony picked during the transformation step (Lee *et al.*, 2000). 16S rRNA gene clones 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). *Lactobacillus fermentum* was predominant species of Lactobacilalles found in WJB tempeh samples, together with *Acetobacter indonesiensis* from Rhodospirilalles.

E. cecorum, L. agilis, and *L. fermentum* were the member of lactic acid bacteria that producing lactic acid as the major metabolic end-product of carbohydrate fermentation. The presence of lactic acid could extend the shelf-life of food, improve its organoleptic properties, produce bacteriocins, and inhibit other pathogenic bacteria multiplication (Pesavento *et al.*, 2014). These bacteria obtained

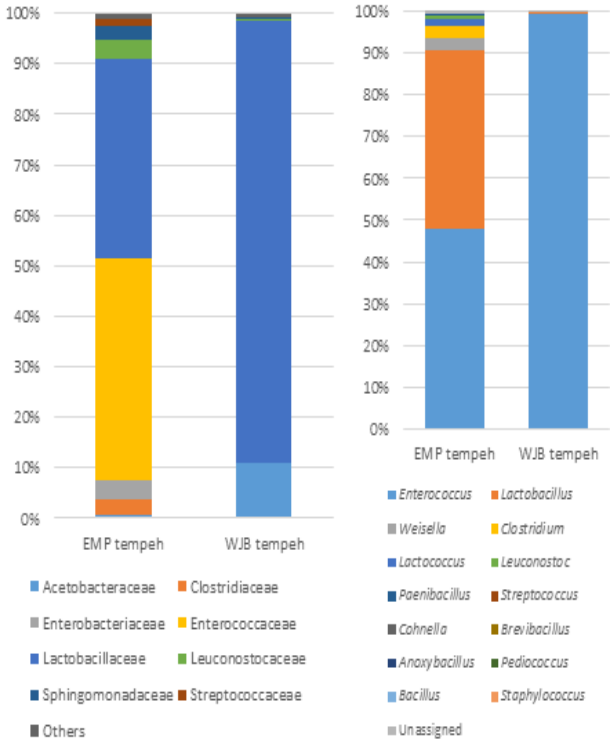

Figure 2. Predominant families of total bacteria (left) and genera of Firmicutes phylum (right) obtained from EMP tempeh and WJB tempeh samples based on total 16S rRNA gene sequence analysis. Major families that were detected in less than 1% in both samples were grouped as "Others". Operational Taxonomic Unit (OTU) with no assigned genera were grouped as "Unassigned".

from cloned 16S rRNA gene were only a small part of bacterial community in tempeh compared with preceding result. Many minor bacteria population had been missed. Therefore, 16S rRNA gene cloned result generated might not describe the entire composition of microbial communities. In this case, HTS analysis was preferable to examine the microbial communities of tempeh sample, since it can be used to detect minor population (Kiyohara *et al.*, 2012).

Culturable bacteria

Each phylum was represented by certain kind of bacteria. Microaerophilic lactic acid bacteria (LAB) in Man-Rogosa Sharpe Agar (MRSA) medium was used to represent Firmicutes phylum, while Enterobacteriaceae in Eosin Methylene Blue Agar (EMB) medium was used to represent Proteobacteria phylum. Similar to metagenome results (Figure 2), culturable bacteria enumeration in both tempeh samples showed that Firmicutes was the predominant phylum (Figure 3). Separate study also reported that predominant colonies present in tempeh was Firmicutes (Nurdini *et al.*, 2015). These results suggested that culturable technique could be used to predict microbial community in tempeh, even though

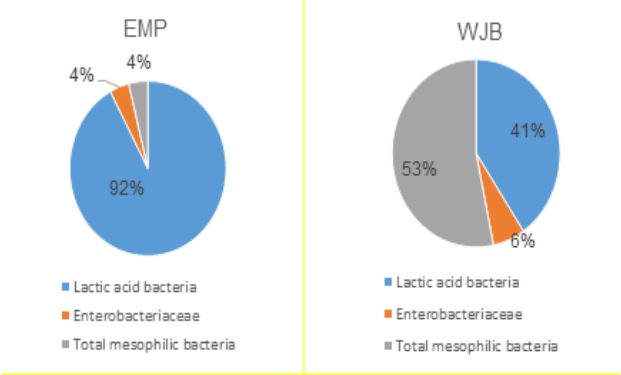

Figure 3. Bacterial communities in tempeh based on culturable technique

its relative abundance can not be compare with metagenome result.

Similar to previous study, *Klebsiella pneumoniae* (Barus *et al.,* 2008) and *K. variicola* were the most abundant bacteria among any other Enterobacteriaceae in EMP tempeh. Its existence could increase nutrient value in tempeh, since it can produce vitamin B_{12} (Keuth and Bisping, 1993). However, two-times boiling soybean used for tempeh production in WJB, was not affecting the number of its mesophilic bacteria, 2.5 x 108 CFU.g^{-1} (Figure 3), which was higher than one-time boiling soybean, EMP tempeh (2.5 x 10^6 CFU.g^{-1}). Predominant mesophilic bacteria found in WJB tempeh samples was *Bacillus pumilus* and *Bacillus safensis*. Other than *Bacillus* genera, Barus *et al*. (2008) also found *Pseudomonas putida* and *Acinetobacter* sp. as the predominant species in tempeh WJB. While in tempeh EMP, predominant species reported were *Acetobacter indonesiensis, Bacillus subtilis,* and *Flavobacterium* sp. Mesophilic LAB species predominant in both samples were in line with the result from cloned 16S rRNA gene analysis. The species of *Lactobacillus* obtained by MRSA medium in EMP tempeh was *L. fermentum,* while in WJB tempeh combined together with *Lactococcus lactis*. Other than *Lactobacillus* genera, *Weissella confusa* and *Staphylococcus warneri* were also found in those samples. *Lactobacillus casei, Streptococcus faecium* and *Enterococcus faecium* in tempeh were also reported previously (Mulyowidarso *et al.*, 1990; Moreno *et al.,* 2002).

There were several differences between EMP and WJB tempeh production, including the number of boiling the soybean (one-time or two-times), the order of soaking and dehulling the soybean, and starter culture used. Despite those differences, this study focused on the influence of the number of boiling. Based on previous study, tempeh made by two-times boiling soybean had lower number of bacteria, approximately 10^5 – 10^6 CFU.g^{-1} (Barus *et al*., 2008; Efriwati *et al.,* 2013), which is similar to our finding. Despite its limitation in precise identification to species level, HTS analysis proved to be a useful tool for monitoring population dynamics of a complex bacterial community. Since it is a current technology used for detecting microbial community, its limitation eventually could surpass for better analysis.

Conclusion

Bacterial diversity in EMP and WJB tempeh, were analyzed by combined metagenome employing HTS and cloned 16S rRNA gene, as well as employing culturable technique. The result shown by metagenome analysis were in line with culturable technique, suggested that culturable technique could be use to predict microbial community in tempeh. HTS and culturable technique yielded Firmicutes followed by Proteobacteria were the predominant phyla in both tempeh samples. HTS result showed that tempeh produced by two-times boiling of soybean showed less bacterial diversity than the one-time boiling of soybean.

Acknowledgement

This work was supported by Program Magister Doktor Sarjana Unggul (PMDSU) bagi Dosen Perguruan Tinggi, tahun anggaran 2014 and 2015 and Peningkatan Kualitas Publikasi Internasional (PKPI) tahun 2015. We also acknowledged Laboratory of Extremophilles, Department of Science and Engineering for Sustainable Innovation, Faculty of Science and Engineering, Soka University in Japan, for providing laboratory facilities.

References

Ampe, F., Omar, N. B. and Guyot, J. P. 1999. Culture-independent quantification of physiologically active microbial group in Mexican pozol, a lactic acid fermented dough, using rRNA-targeted oligonucleotide probes. Journal of Applied Microbiology 87: 131-140.

Aslam, Z., Yasir, M., Khaliq, A., Matsui, K. and Chung, Y. R. 2010. Mini review: Too much bacteria still unculturable. Biosciences 1: 59-60.

Barus, T., Suwanto, A., Wahyudi, A. T. and Wijaya, H. 2008. Role of bacteria in tempeh bitter taste formation: Microbiological and molecular biological analysis based on 16S rRNA gene. Microbiology Indonesia 2:17-21.

Bokulich, N. A., Joseph, M. L., Allen, G., Benson, A. K. and Mills, D. A. 2013. High-throughput sequencing reveals significant bacterial diversity of botrytized wine. PLOS one 7(5): e36357.

Bokulich, N. A. and Mills, D. A. 2013. Facility-specific

"house" microbiome drives microbial landscape of artisan cheesemaking plants. Applied Environmental Microbiology 79(17): 5214-5223.

Colehour, A. M., Meadow, J. F., Liebert, M. A., Cepon-Robins, T. J., Gildner, T. E., Urlacher, S. S., Bohannan, B. J. M., Snodgrass, J. J. and Sugiyama, L. S. 2014. Local domestication of lactic acid bacteria via cassava beer fermentation. PeerJ 2: e479.

Efriwati, Suwanto, A., Rahayu, G. and Nuraida, L. 2013. Population dynamics of yeasts and lactic acid bacteria (LAB) during tempeh production. HAYATI Journal of Biosciences 20(2): 57-64.

Florou-Paneri, P., Christaki, E. and Bonos, E. 2013. Lactic acid bacteria as source of functional ingredients. INTECH Open Access Publisher.

Hugenholtz, P., Goebbel, B. M. and Pace, N. R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology 180: 4765-4774.

Humblot, C. and Guyot, J. P. 2009. Pyrosequencing of tagged 16S rRNA gene amplicons for rapid deciphering of the microbiomes of fermented foods such as pearl millet slurries. Applied Environmental Microbiology 75(13): 4354–4361.

Jeong, D. W., Kim, H. R., Jung, G., Han, S., Kim, C. T. and Lee, J. H. 2014. Bacterial community migration in the ripening of Doenjang, a traditional Korean fermented soybean food. Journal of Microbiology and Biotechnology 24(5): 648-660.

Jung, J. Y., Lee, S. H., Kim, M. J., Park, M. S., Bae, J. W., Hahn, Y., Madsen, E. L. and Jeon, C. O. 2011. Metagenomics analysis of kimchi, a traditional Korean fermented food. Applied Environmental Microbiology 77: 2264-2274.

Kakirde, K. S., Parsley, L. C. and Liles, M. R. 2010. Size does matter: Application-driven approaches for soil metagenomics. Soil Biology and Biochemistry 21: 1-13.

Keuth, S. and Bisping, B. 1993. Formation of vitamins by pure culture of tempeh molds and bacteria during the tempeh solid substrate fermentation. Journal of Applied Bacteriology 75:427-434.

Kim, M. and Chun, J. 2005. Microbial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16s rRNA gene analysis. International Journal of Food Microbiology 103: 91-96.

Kim, B., Seo, W. T., Kim, M. G., Yun, H. D. and Cho, K. M. 2012. Metagenomics lactic acid bacterial diversity during Mulkimchi fermentation based on 16S rRNA sequence. Journal of the Korean Society for Applied Biological Chemistry 55: 787-792.

Kiyohara, M., Koyanagi, T., Matsui, H., Yamamoto, K., Take, H., Katsuyama, Y., Tsuji, A., Miyamae, H., Kondo, T., Nakamura, S., Katayama, T. and Kumagai, H. 2012. Changes in microbiota population during fermentation of Narezushi as revealed by pyrosequencing analysis. Biosciences, Biotechnology, and Biochemistry 76(1): 48-52.

Lane, D. J. 1991. 16S/23S rRNA sequencing. Nucleic Acid Technic in Bacterial Systemics 125-175.

Lee, H. J., Park, S. Y. and Kim, J. 2000. Multiple PCR-based detection and identification of Leuconostoc species. FEMS Microbiology Letter 193: 243-247.

Leite, A. M. O., Mayo, B., Rachid, C. T. C. C., Peixoto, R. S., Silva, J. T., Paschoalin, V. M. F. and Delgado, S. 2012. Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. Food Microbiology 31(2): 215-221.

Lui, Y., Liu, C., Zheng, X., Yu, J., Gao, Q., Hou, Y. and Huang, X. 2008. PCR detection of *Klebsiella pneumoniae* in infant formula based on 16S-23S internal transcribed spacer. International Journal of Food Microbiology 125: 230-235.

Masoud, W., Takamiya, M., Vogensen, F. K., Lillevang, S., Al-Soud, W. A., Sorensen, S. J. and Jakobsen, M. 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturing gradient gel electrophoresis and pyrosequencing. International Dairy Journal 21(3): 142-148.

Minervini, F., Lattanzi, A., Angelis, M. D., Celano, G. and Gobbeti, M. 2015. House microbiotas as sources of lactic acid bacteria and yeasts in traditional Italian sourdough. Food Microbiology 52: 66-76.

Moreno, M. F., Leisner, J. J., Tee, L. K., Ley, C., Radu, S., Rusul, G., Vancanneyt, M. and De Vuyst, L. 2002. Microbial analysis of Malaysian tempeh and characterization of two bacteriocins produced by isolates of Enterococcus faecium. Journal of Applied Microbiology 92: 789-805.

Mulyowidarso, R. K., Fleet, G. H. and Buckle, K. A. 1990. Association of bacteria with the fungal fermentation of soybean tempe. Journal of Applied Microbiology 68: 43-47.

Nishiyama, M., Yamamoto, S. and Kurosawa, N. 2013. Microbial community analysis of a coastal hot spring in Kagoshima, Japan, using molecular- and culture-based approaches. Journal of Microbiology 51(4): 413-422.

Nout, M. J. R. and Kiers, J. I. 2005. Tempe fermentation, innovation, and functionality: update into the third millennium. Applied Environmental Microbiology 98:789-805.

Nurdini, A. L., Nuraida, L., Suwanto, A. and Suliantari. 2015. Microbial growth dynamics during tempe fermentation in two different home industries. International Food Research Journal 22(4): 1668-1674.

Park, J. M., Shin, J. H., Lee, D. W., Song, J. C., Suh, H. J., Chang, U. J. and Kim, J. M. 2010. Identification of lactic acid bacteria in kimchi according to initial and over-ripened fermentation using PCR and 16S rRNA gene sequence analysis. Food Science and Biotechnology 19(2): 541-546.

Pangastuti, A., Suwanto, A., Lestari, Y. and Suhartono, M. T. 2010. Bacterial communities associated with white shrimp *(Litopenaeus vannamei)* larvae at early developmental stages. Biodiversitas 11(2): 65-68.

Pesavento, G., Calonico, C., Ducci, B., Magnanini, A. and Lo Nostro, A. 2014. Prevalence and antibiotic

resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. Food Microbiology 41: 1–7.

Plengvidhya, V., Breidt, F. Jr., Zhongjing, L. U. and Fleming, H. P. 2007. DNA fingerprinting of lactic acid bacteria in Sauerkraut fermentations. Applied Environmental Microbiology 73: 7697-7702.

Polka, J., Rebecchi, A., Pisacane, V. and Morelli, L. 2015. Bacterial diversity in typical salami at different ripening stage as revealed by high-throughput sequencing of 16S rRNA amplicons. Food Microbiology 46: 342-356.

Sakai, H. D. and Kurosawa, N. 2016. Exploration and isolation of novel thermophiles in frozen enrichment cultured derived from a terrestrial acidic hot spring. Extremophilles 20 (2): 207-214.

Sakamoto, N., Tanaka, S., Sonomoto, K. and Nakayama, J. 2011. 16S rRNA pyrosequencing-based investigation of the bacterial community in nukadoko, a pickling bed of fermented rice bran. International Journal of Food Microbiology 144(3): 352-359.

Seumahu, C. A., Suwanto, A., Rusmana, I. and Solihin, D. D. 2012. Comparison of DNA extraction methods for microbial community analysis in Indonesian tempeh employing amplified ribosomal intergenic spacer analysis. HAYATI Journal of Biosciences 19(2): 93-98.

Seumahu, C. A., Suwanto, A., Rusmana, I. and Solihin, D. D. 2013. Bacterial and fungal communities in tempeh as reveals by amplified ribosomal intergenic sequence analysis. HAYATI Journal of Biosciences 20(2): 65-71.

Soka, S., Suwanto, A., Sajuthi, D. and Rusmana, I. 2014. Impact of tempeh supplementation on gut microbiota composition in Sprague-Dawley rats. Research Journal of Microbiology 9(4): 189-198.

Watanabe, F., Yabuta, Y., Tanioka, Y. and Bito, T. 2013. Biologically active vitamin B12 compounds in food for preventing deficiency among vegetarian and elderly subjects. Journal of Agriculture and Food Chemistry 232(10): 1266-1274.

Watanabe, K., Nagao, N., Toda, T. and Kurosawa, N. 2008. Changes in bacterial communities accompanied by aggregation in a fed-batch composting reactor. Current Microbiology 56: 458-467.

Yan, Y., Wolkers-Rooijackers, J., Nout, M. J. R. and Han, B. 2013. Microbial diversity and dynamics of microbial communities during back-slop soaking of soybeans as determined by PCR-DGGE and molecular cloning. World Journal of Microbiology and Biotechnology 29(10): 1969-1974.

Zhao, D., Lu, F., Qiu, M., Ding, Y. and Zhou, X. 2016. Dynamics and diversity of microbial community succession of surimi during fermentation with high-throughput sequencing. Journal of Food Safety: 308-316.