Dynamics of Microbial Community during Tempeh Fermentation

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DYNAMICS OF MICROBIAL COMMUNITY DURING TEMPEH FERMENTATION AS REVEALED BY HIGH-THROUGHPUT SEQUENCING

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ABSTRACT

Tempeh is sliceable soybean-cake fermented by Rhizopus oligosporus. Various bacteria were detected in tempeh employing cultivation technique with limited information 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 tempeh production employing two-times boiling of soybean (WJB tempeh production). All samples were plated on Enterobacteriaceae and Lactic Acid Bacteria medium. Total DNA were extracted directly from tempeh for metagenomics analysis, employing High-Throughput Sequencing (HTS) and cloned 16S rRNA genes. Firmicutes and Proteobacteria were the predominant and second dominant bacteria existed in fresh tempeh (FT) obtained by metagenomics analysis. In contrast, cultivation technique showed that Proteobacteria was the predominant phylum, suggested that most of the Firmicutes were not culturable. FT was dominated by Lactobacillus and Acetobacter. Both FT and soaking water (SW) were dominated by same species of Lactobacillus, i.e. L. delbreuckii and L. mucosae, indicated that SW was probably the source of bacterial community established in the final product of fermentation. Predominant bacteria in starter culture (SC), Acinetobacter, was not detected in FT, indicating that bacteria in SC might not play significant role in bacterial community development in FT.

Keywords: Firmicutes, high-throughput sequencing, Proteobacteria, starter culture, tempeh.

INTRODUCTION

Tempeh is traditional food from Indonesia which made from soybean fermented by mold Rhizopus oligosporus. However, the microbial composition in tempeh also includes bacteria, as the “contaminants” in addition to mold (Mulyowidarso et al. 1990; Barus et al. 2008; Efriwati, et al. 2013; Nurdini et al. 2015). The procedure of tempeh production among the producers in Indonesia are usually different, depends on the area, experience, and resources. But generally, the procedure for tempeh production has a few similarities, such as boiling the soybean, dehulling, soaking, mixing with inoculum, and incubation for two days. The differences among producers usually 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, not boiling the soybean for the second time after soaking it, whether the rest, such as WJB production, employs two-times boiling of soybean (Barus et al.
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Despite the method used for tempeh processing, the initial source of bacteria in final product of fermentation has not yet been verified.

Tempeh production in Indonesia usually done in “open” fermentation using unsterile wooden equipments, where other microbes could enter into production processes. Therefore, various bacteria in different number 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 present in the beginning of fermentation process (soaking water of soybean) until the last process of fermentation (Efriwati et al. 2013). The population of lactic acid bacteria was dynamic according to the stage of fermentation process. Its presence is usually shown by decrease in pH as a consequences of natural acidification, especially during soaking processes (Efriwati et al. 2013). However, recent studies showed that laru used as starter culture in WJB productions, did not contain any Enterobacteriaceae nor lactic acid bacteria (Nurdini et al. 2015).

Methods used for determining bacterial community in tempeh so far limited only to 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). But, these approaches were only producing limited amounts of information and, therefore, cannot reflect the composition of microbial communities. To overcome this limitation, 16S rRNA gene could be used to represent microbial community in a metagenomics analysis. In this study, we attempted to determine the origin of the bacterial community in tempeh ecosystem employing metagenomics approaches, derived from cloned 16S rRNAs gene sequences from high-throughput sequencing employing total 16S rRNA gene.

MATERIALS AND METHODS

Sampling and Sample for Analysis

Samples conducted for this research obtained from WJB (Warung Jambu) tempeh industries (Barus et al. 2008), such as raw soybean (RS), fresh water (FW), soybean soaking water (SW), dehulled-soybean before inoculation (BI), commercial laru as starter culture (SC), and fresh tempeh (FT). Each sample was taken three times from WJB tempeh production and transporting in ice box for direct analysis.

Microbiological Analysis

One gram of SC was inoculated into 25 g sterile soaked-soybean in a 250 mL Erlenmeyer. Incubation for 2 days were done before doing the serial dilution. Twenty-five gram 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 replicate and incubated at 30°C for 3-5 days for mesophilic and Enterobacteriaceae; and 5-7 days for lactic acid bacteria.

**DNA Extraction for Metagenomics Analysis**

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 procedure described in previous study (Seumahu et al. 2012). 25 g RS sample were soaked into 225 mL sterile water for overnight in the shaker. A hundred liter of FW and soaked water of RS; and 30 mL of SW were extracted using PowerWater Microbial DNA Isolation-Kit (MOBIO, USA) according to the protocol described by the manufacturers. DNA products were visualized by electrophoresis using 1% (w/v) agarose gel before using for next step of analysis.

**PCR Amplification for 16S rRNA Gene**

Predominant colonies from each medium in preceding section and DNA extraction for metagenome analysis were amplified by using B27F and U1492R (Sakai & Kurosawa 2016). 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 pmol of each primer, and 1 µL of DNA extraction. Samples were amplified in PCR machine with the condition were as follows: 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 product (about 1,500 bp) were purified by using Illustra GFX PCR DNA and gel band purification kit (GE healthcare, UK). PCR product 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⁻¹ ampicillin (Wako) and 40 µg.mL⁻¹ 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 reaction as followed: 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
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129 min (Nishiyama et al. 2013; Sakai & Kurosawa 2016).

130

Identification of 16S rRNA Gene Clones

131

All the cloned 16S rRNA gene and predominant colonies were checked for chimeric sequences
132 by using GENETYX ver. 11.0.1 software homology search program (Japan). Sequences with
133 similarity of 98% and above group together into the same phylotype (Watanabe et al. 2008).
134 Sequences were submitted for BLASTN database, searching for 16S rRNA gene for identifying

136

High-Throughput Sequencing Analysis

137

For amplification of the V4 domain in bacterial 16S rRNA, we used primers 515f and 806r
138 modified to contain MiSeq Illumina adapter region. PCR reaction using TaKaRa ExTaq HS (TaKaRa,
139 Japan) with PCR condition consisted of an initial 94°C for 2 min followed by 20 cycles of 94°C for
140 30 sec, 50°C for 30 sec, 72°C for 30 sec, and final extension of 72°C for 5 min. PCR product was
141 purified with AMPure XP beads (Belkman Counter, Japan) before employed the tailed-PCR using
142 15-index2 and 17-index1 primers. PCR reaction consisted of an initial 94°C for 2 min, followed by 8
143 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.
144 Sequencing of the PCR products was done using custom service provided by Bioengineering Lab
145 (Japan) and Fastmac (Japan).

146

Data Analysis

147

Raw fastq files were demultiplexed, quality filtered, and analyzed with QIIME 1.6.0. Bacterial
148 16S rRNA gene sequences were clustered with the QIIME subsampled reference OTU-picking
149 pipeline with UCLUST-reference against the Greengenes 16S rRNA database, clustered at 97%
150 pairwise identity (Bokulich & Millis 2013). Shannon-Wiener index (H’), Simpson index (D), and the
151 Equitability (E) were calculated to describe the diversity of community, similarity between species,
152 and relative importance of each OTU within the entire assemblages (Pangastuti et al. 2010).

153

RESULTS AND DISCUSSION

154 Culturable Enterobacteriaceae and Lactic Acid Bacteria.

155 Microbial counts of Enterobacteriaceae and lactic acid bacteria from WJB tempeh production
156 are reported in Figure 1. Proteobacteria represent by Enterobacteriaceae were predominant in FT and
157 SC. Even so, Enterobacteriaceae presented since raw material RS, 3.6 x 10^5 CFU.g^-1 and decreased
158 in BI after being boiled for two-times until 2.4 x 10^2 CFU.g^-1, then reached its maximum value in FT
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with $2.9 \times 10^7$ CFU.g$^{-1}$. *Klebsiella pneumoniae* were the predominant Proteobacteria in FT samples, while previous study reported *Pseudomonas putida* and *Acinetobacter* sp. as predominant bacteria from tempeh WJB (Barus et al. 2008). On the other hand, lactic acid bacteria were reached its maximum value in SW, $10^7$ CFU.g$^{-1}$, where the natural acidification occurred with the presence of lactic acid bacteria (Efriwati et al. 2013) and in addition of $1.4 \times 10^2$ CFU.g$^{-1}$ of Enterobacteriaceae. Previous study reported similar number of lactic acid bacteria were detected in soaking water of soybean and fresh tempeh from WJB producer, $6.0 \times 10^5$ CFU.g$^{-1}$ and $4.3 \times 10^5$ CFU.g$^{-1}$, respectively (Barus et al. 2008). Whereas from our study, lactic acid bacteria were $7.7 \times 10^4$ CFU.g$^{-1}$ in FT sample. *Lactobacillus lactis*, *L. fermentum*, and *Weissella confusa* were mainly predominant lactic acid bacteria in FT sample.

![Figure 1](image_url) Number of total bacteria population based on culture-dependent technique from WJB production. RS: raw soybean, FW: fresh water, SW: soaked water 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 assembly and sample index-based demultiplexing, which were reduced to 804,136 cleaned reads after sorted for quality issues (homopolimers, length), chimera sequence, and miss amplified taxa (chloroplast, mitochondria, archaea, or unassigned kingdom) with average length was 340 bp. All of these sequences were used for taxonomy-based analysis, which consists of kingdom, phylum, class, ordo, family, and genus level. Measurement of indices regarding diversity parameters is shown in Table 1, with the number of OTUs (Operational Taxonomic Unit). The latter showed 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. Shannon-wiener index, Simpson index, and Equitability are reported as well, and showed
similar pattern among samples. FW and enriched SC were covered the highest value among those indices, indicating that the number of individual species in each OTU was in even number. On the other hand, BI sample covered the lowest value for Simpson index, as the predominant bacteria obtained in greater number than other species.

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


Bacterial Communities Structure from WJB production

The abundances of HTS-derived sequences assigned to defined bacterial phyla in each sample are reported in Figure 2 as relative percentage. Data refer to taxa participating for the 1% or more in at least one sample, while rarer taxa were grouped under the “other” label. HTS data analysis showed that WJB tempeh production only occupied by four phyla, including Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes. The latter mentioned only showed in FW sample, with its participants in other samples were less than 1%. Firmicutes and Proteobacteria were predominant and second dominant bacteria existed in WJB production. In raw material used for tempeh production, Firmicutes was dominant in RS (93%) while the other material, FW and SC were dominated by Proteobacteria with 73% and 96% proportion from each clean reads, respectively. Firmicutes also predominant bacteria in FT (88%) and SW (96%), while sample dehulled-soaked-soybean ready for inoculation, BI, was dominated by Proteobacteria (98%). With these result, Firmicutes populated in FT was presumably originated from RS and/or SW.
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Figure 2 Phyla of bacteria present in sample taken from WJB production analyzed with total 16S rRNA sequence. Phyla acquired in samples less than 1% was put into Others label. RS: raw soybean, FW: fresh water, SW: soaked water soybean, BI: soybean before inoculation, SC: starter culture, FT: fresh tempeh.

The predominant ordo and genera involved in WJB tempeh production were shown in Figure 3 and Figure 4, respectively. Lactobacilalles was the predominant ordo in all samples populated by Firmicutes, RS, SW, and FT, while predominant orders represent Proteobacteria were varied in the rest of sample. With 57% abundance, Burkholderiales was predominant in FW, but its presence was vanished once in soaking the soybean process and not detected anymore in the next step. Afterward, soaked soybean was boiled for the second times before ready for inoculation. Two-times boiling of soybean was indeed decreasing the species richness from tempeh in addition to its degrading number (Efriwati et al. 2013). Rhodospirilales was vastly increased as the predominant Ordo detected in BI sample with 97% abundance, while Lactobacillales from the soaking process was significantly diminished. However, Rhodospirilales abundance was decreased in FT until 11% and its bacterial predominance was shifted into Lactobacilalles (88%). Unlike the result shown by culturable technique, the sole Ordo signifies Proteobacteria in SW was Rhodospirilales (1.6%), while Enterobacteriaceae occurrence itself was less than 1%. Another predominant Proteobacteria orders was Enterobacteriales and Pseudomonadales in SC samples with 34% and 36% occurrence, respectively. As we expected, neither of this orders occurrence were more than 1% in final product of fermentation, FT, due to its small amount of administration for tempeh fermentation. Enterobacteriales, which harbored *K. pneumoniae* as vitamin B₁₂ 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. pneumoniae* in FT was only gained by culturable technique.
Figure 3 Major Orders attained from WJB production, employing total gene 16S rRNA sequences analysis. Major Orders occurrence less than 1% in all samples was put into Others label. RS: raw soybean, FW: fresh water, SW: soaked water soybean, BI: soybean before inoculation, SC: starter culture, FT: fresh tempeh.

Twelve major genera from predominant Orders were acquired from WJB production (Figure 4). Major Lactobacillales genera attained from RS was different with SW and FT predominant genera. *Clostridium* and *Lactococcus* were dominated RS sample with 43% and 28% abundance, respectively. 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 vanished in BI until 272 sequences of BI total reads (0.5%). These indicated that some *Lactobacillus* still alive during the second-boiling of soybean, thus dominated once more in final product of fermentation. Second-times boiling of soybean was not only shifted the bacterial community presence in BI, but also slightly decreasing its total number (Efriwati *et al.* 2013), especially *Lactobacillus* genera. The environment of tempeh fermentation apparently was very suited for *Lactobacillus* growth, since it was vastly increasing through two days fermentation, 800 times higher than in its original number in BI.

Changing of bacterial community through fermentation process was not surprising, since bacterial communities within ecosystem corresponded to its environmental conditions. The shifted of bacteria community also happened to food-based fermentation product, such as *kimchi* (Park *et al.* 2012) and *dongchimi* (Jeong *et al.* 2013), and the soybean soaking process in tempeh production. Yan *et al.* (2013) reported that some environmental bacteria which might be contributed to the ecology of fermentation at the start, was shifting to lactic acid bacteria accompanying with pH decrease and
temperature increase. Moreover, lactic acid bacteria can produce antimicrobial compound aside from organic acid that can eliminate pathogen and spoilage bacteria existence (Trias et al. 2010).

Predominant ordo in BI was solely *Acetobacter* from Rhodospirilalles with 99% of relative abundance. These bacteria already existed in SW process with 1.5% abundance. By cultivation technique, Barus et al. (2008) also reported its existence in soybean soaking water in different tempeh production. *Acetobacter* was known as acetic acid producer from alcohol substrate in the presence of excess oxygen (Purwoko 2009). These bacteria also existed in dehulled-soybean ready for inoculation without second times 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 might be because BI from WJB production was not washed with water once it finished the second boiling process. Moreover, second-times boiling process was used the soaking water, not fresh water. Thus, excessive ethanol from heterofermentative lactic acid fermentation in the soaking process was used as substrate for *Acetobacter* growth (Purwoko 2009). The bacteria itself presumably originated from the environment where the tempeh production was 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 shifted sequentially to *Lactobacillus*. In another food fermentation, *Acetobacter* was responsible for fermentation of vinegar (Wu et al. 2010), *nata* (Jagannath et al. 2010), and chocolate (Papalexandratou et al. 2011), with different *Acetobacter* species.

Figure 4 Predominant genera attained from WJB production, employing total 16S rRNA gene analysis. Major genera occurrence less than 1% in all samples was put into Others label. RS: raw soybean, FW: fresh water, SW: soaked water soybean, BI: soybean before inoculation, SC: starter culture, FT: fresh tempeh.
Pseudomonadales was predominant bacteria in SC (35%), while Enterobacteriales was second predominant bacteria in SC with its solely family, Enterobacteriacea (34%). Due to its inaccuracy identification, Enterobacteriacea cannot be identified further until genera level, thus the predominant genera existed in SC was remain unknown. Pseudomonadales was represented by Acinetobacter (37%) and Pseudomonas (23%) as the predominant genera in SC used for WJB tempeh fermentation. The administration of SC was 1 gram to 250 grams of BI, thus the abundance of predominant bacteria in SC were detected in FT less than 1%. SC also contained Lactobacillus with 0.4% abundance. However, the involvement of Lactobacillus from SC to establish Lactobacillus as predominant bacteria in FT could be ignored, since its abundance in SC was less than 1%. Bacterial community in product of fermentation was not affected by bacterial community in starter culture (Park et al. 2012; Polka et al. 2015). Most probably many of bacteria contained in starter culture were 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 is commonly found in fermented shrimp (Park et al. 2011), while Novosphingobium is frequently found in Chinese traditional sourdough (Zhang & He 2013).

Cloned 16S rRNA Gene Analysis

For better assessment of bacterial species involved in bacterial community in tempeh production, cloned 16S rRNA genes were employed from the same pool of DNA as previous analysis. A total of 126 clones was obtained from six samples and divided into two majored phyla, Firmicutes and Proteobacteria (Table 2). Predominant genera obtained with this method coincided with previous result (Figure 2), yet contradicted with cultivation technique result (Figure 1). Both SW and FT were dominated by the same species of Lactobacillus, L. delbrueckii and L. mucosae. The similar sequences might be indicated that predominant bacteria in FT were originated from SW processes. Besides Lactobacillus, Enterococcus and Pediococcus genera were also reported present in WJB tempeh acquired by T-RFLP (Terminal Restriction Fragment Length Polymorphisms) analysis. But its position in tempeh dominant bacteria was 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 said, the abundance of Lactobacillus genera in SW was indeed decreased after second-boiling of soybean, but it vastly increased again through 48 hours’ incubation process in tempeh fermentation. Thus, Lactobacillus sequences amplified by both methods were not a bias and not dead cells.
The variations in bacterial community structures that occur on tempeh production, including the ordering of abundant and rare phylotypes, have implications for understanding how bacterial community composition in tempeh production are affected by processes involved. According to the previous study, tempeh produced by WJB was preferable and had less bitter taste than tempeh produced by other producers in Bogor, due to its lower bacterial number (Barus et al. 2008). However, the lower bacterial number also correspond to lower lactic acid bacteria which could act as immunostimulant (Soka et al. 2014), lower Enterobacteriales which represent K. pnemoniae as vitamin B₁₂ producer (Ashok et al. 2012), and no Bifidobacteriales sequences obtained as isoflavone aglycone producer (Raimondi et al. 2009). Therefore, two-times boiling of soybean was good to lessen the bitterness in tempeh flavor, yet it also decreasing the nutritive value that could be obtained from tempeh consumption. Information about bacterial community composition is poor for food ecosystem, even though the diversity and composition of microbial communities in food are thought to have a direct influence on the gut microbial consortia in humans (Hehemann et al. 2010; Park et al. 2012).

Table 2 Cloned bacterial 16S rRNA sequences during tempeh production.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Species</th>
<th>Similarity (%)</th>
<th>Phylum</th>
<th>No. Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS</td>
<td><em>Clostridium beijerinckii</em></td>
<td>99</td>
<td>Firmicutes</td>
<td>KJ957170</td>
</tr>
<tr>
<td></td>
<td><em>Lactococcus taiwanensis</em></td>
<td>96</td>
<td>Firmicutes</td>
<td>NR_114327</td>
</tr>
<tr>
<td>FW</td>
<td>Acidovorax sp.</td>
<td>98</td>
<td>Proteobacteria</td>
<td>JX177703</td>
</tr>
<tr>
<td></td>
<td>Curvibacter sp.</td>
<td>99</td>
<td>Proteobacteria</td>
<td>JN679217</td>
</tr>
<tr>
<td>SW</td>
<td><em>Lactobacillus delbrueckii</em></td>
<td>98</td>
<td>Firmicutes</td>
<td>FJ915697</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus mucosae</em></td>
<td>99</td>
<td>Firmicutes</td>
<td>JQ805650</td>
</tr>
<tr>
<td>BI</td>
<td><em>Acetobacter indonesiensis</em></td>
<td>99</td>
<td>Proteobacteria</td>
<td>AJ419841</td>
</tr>
<tr>
<td></td>
<td><em>Acetobacter aceti</em></td>
<td>99</td>
<td>Proteobacteria</td>
<td>KR261398</td>
</tr>
<tr>
<td>SC</td>
<td><em>Acinetobacter ursingii</em></td>
<td>99</td>
<td>Proteobacteria</td>
<td>LC014147</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>100</td>
<td>Proteobacteria</td>
<td>KP866890</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus fermentum</em></td>
<td>98</td>
<td>Firmicutes</td>
<td>JN188384</td>
</tr>
<tr>
<td>FT</td>
<td><em>Lactobacillus delbrueckii</em></td>
<td>98</td>
<td>Firmicutes</td>
<td>FJ915697</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus mucosae</em></td>
<td>99</td>
<td>Firmicutes</td>
<td>JQ805650</td>
</tr>
</tbody>
</table>


The dissimilar result happened between cultivation technique and metagenomics analysis in this study. Most of the predominant bacteria existed in tempeh production was not detected by using...
cultivation technique, especially lactic acid bacteria. This indicated that tempeh ecosystem dominated by bacterial communities which cannot be cultured by traditional methods, thus bias toward the predominant bacterial community in tempeh could be occurred. Consequently, the result obtained by cultivation technique cannot be used to reflect the real microbial communities in tempeh (Aslam et al. 2010). But, using metagenomics technique without combining with cultivation technique was also not preferable, since metagenomics employing 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 microbial community in food ecosystem, even for minor population of bacteria. Metagenomics analysis of bacterial community in tempeh improves the understanding of bacterial ecology in tempeh limited in the classical knowledge of the tempeh ecosystem.

CONCLUSION

Firmicutes and Proteobacteria were the predominant and second dominant bacteria existed in WJB fresh tempeh as demonstrated by metagenomics analysis. However, cultivation technique showed the opposite significantly, which might underestimated Firmicutes population. Two-times boiling of soybean employed in WJB production resulted in the low number of bacteria existed in fresh tempeh (FT) which was dominated by Lactobacillus. Both FT and soybean soaking water (SW) harbored Lactobacillus delbreuckii and L. mucosae, indicated that SW probably was the main source of bacterial community established in fresh tempeh. Bacteria in starter culture (SC) might not play essential role in bacterial community development in FT.

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