Metagenome analysis of tempeh production: Where did the bacterial community in tempeh come from?

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ABSTRACT

Aims: Tempeh is a soy-based traditional food fermented by *Rhizopus oligosporus.* Although this mold is the main microorganism responsible for tempeh fermentation, various unknown bacteria presence in tempeh could enhance tempeh's nutritional value. This study is aimed to examine the identity of bacteria in tempeh bacterial community by combining metagenomics analysis and culturable technique.
Methodology and results: Samples were obtained from a tempeh producer which consists of raw soybeans, fresh water used to soak the beans, soaking water after the beans were soaked for 18 h, dehulled-soybean before inoculation, starter culture, and fresh tempeh. All samples were plated onto Enterobacteriaceae and Lactic Acid Bacteria agar media, and the total DNA was extracted for metagenomics analysis based on 16S rRNA gene cloning and High-Throughput Sequencing (HTS). Metagenomic analysis indicated that Firmicutes and Proteobacteria were the predominant and sub-dominant bacteria, respectively, while the culturable technique showed Proteobacteria were the predominant bacteria. Firmicutes species detected in tempeh were similar to the ones in the soaking water, which were populated by *Lactobacillus.* However, another predominant bacteria from tempeh, *Enterococcus,* was similar to minor population of *Enterococcus* detected in dehulled-soybean before inoculation. Based on the cloned 16S rRNA genes, we observed *L. agilis*, *L. fermentum*, and *E. cecorum* as the predominant bacteria in tempeh. The starter culture, which was dominated by *Clostridium,* did not alter bacterial community in tempeh, since its proportion was only 2.7% in tempeh clean reads.
Conclusion, significance and impact of study: The dominant bacteria in tempeh was *Lactobacillus* from Firmicutes. The bacterial community in tempeh was not affected by the starter culture used, but mainly because of the soybean soaking process.

Keywords: tempeh, starter culture, bacterial community, high-throughput sequencing

INTRODUCTION

Tempeh is an emblematic traditional Indonesian food that has been consumed worldwide. In Indonesia, tempeh is routinely consumed by lower-income people due to its economic value. Approximately 27 different types of tempeh are made with various legumes, including soybean, velvet bean, winged bean, mung bean, fava bean, grains and coconut pressed cake (Shurtleff and Aoyagi, 1979), yet soybean-based tempeh is the most popular type of tempeh among Indonesians. Tempeh is also popular among vegans due to its beneficial properties, such as high content of vitamin B_{12}, bioavailable and bioactive aglycones, calcium, and lower phytic acid level than its raw ingredient. Those traits are mostly generated by various microbial activities in tempeh (Murakami *et al.*, 1984; Sutardi and Buckle 1985; Keuth

and Bisping, 1993; Tsangalis *et al.*, 2002; Haron *et al.*, 2010; Haron *et al.*, 2011). Furthermore, tempeh also contains a high amount of lactic acid bacterium, which can increase its nutritional value (Soka *et al.*, 2014).

Tempeh fermentation is comprised of two stages, including lactic acid fermentation during the preparatory soaking of soybeans by naturally-occuring microbiota and a fungal solid state fermentation stage by *Rhizopus oligosporus* (Nout and Kiers, 2005; Barus *et al.*, 2008; Seumahu *et al.*, 2012; Efriwati *et al.*, 2013). *Rhizopus oligosporus* was inoculated onto cooked dehulled-soybeans. Aside from the presence of mold, lactic acid bacteria and Enterobacteriaceae were reported as a colony forming unit (CFU) (Nurdini *et al.*, 2015). However, information about its role in the bacterial communities' formation in tempeh is still limited. During the soaking process, in which acidification occurred, the bacterial

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population was dominated by 10^6-10^9 CFU/g of lactic acid bacteria (Moreno *et al.*, 2002; Efriwati *et al.*, 2013). This process was preferable, since it can increase the shelf life of tempeh and its safety by reducing the number of pathogenic bacteria in the fermentation product (Nout and Rombouts, 1990; Mulyowidarso *et al.*, 1990; Nurdini *et al.*, 2015). Tempeh fermentation occurs in an uncontrolled environment, which applied to its raw material (soybean, water), starter culture (*laru*) and environmental conditions (temperature, humidity). Those uncontrolled conditions could affect the microbial community in tempeh, which could consequently influence the quality of tempeh (Barus *et al.*, 2008).

Asides from the general methods, some tempeh producers employ modified methods and starter cultures which are unique from producer to another (Barus *et al.*, 2008; Efriwati *et al.*, 2013). Barus *et al.* (2008) reported that four out of five tempeh producers in Bogor, Indonesia, employ one-time boiling of soybean, such as EMP tempeh producer. Another considerable interest in tempeh EMP is the adulteration of commercial starter culture with cassava solid waste (*onggok*), namely *laru onggok,* in 1:10 ratio. Microbiological studies employing culturable approaches to study microbial community in tempeh EMP have been reported before (Barus *et al.*, 2008; Efriwati *et al.*, 2013; Nurdini *et al.*, 2015), however, the results of these studies were still preliminary, partial and can sometimes be contradictory. Therefore, bacterial communities and dynamics in tempeh were not clearly understood. Recently, the retrieval and sequence analysis of 16S rRNA gene has become a standard approach for the investigation of microbial diversity in food ecosystems in addition to conventional culturable approaches (Hugenholtz *et al.*, 1998). Culture-independent technique could be used to examine unculturable bacteria without creating a bias, since the data produced using the culturable technique may underestimate microbial diversity (Ampe *et al.*, 1999; Kakirde *et al.*, 2010). In the present paper, we applied high-throughput sequencing (HTS), which has been used for metagenomics studies in other fermented foods, including Italian salami (Polka *et al.*, 2015), wine (Bokulich *et al.*, 2013), and artisan cheese (Bokulich and Mills, 2013). In addition, HTS was used to determine the succession of bacterial community during the fermentation process.

MATERIALS AND METHODS

Sampling and sample for analysis

Samples used for this research were obtained from the EMP tempeh producer in Bogor, Indonesia (Barus *et al.*, 2008; Efriwati *et al.*, 2013). Sample were taken from three different steps in tempeh production, which include raw soybeans (SS), fresh water used to soak the bean (FW), soaking water after the beans were soaked for 18 h (SW), soybean before inoculated (BI), starter culture (SC), and fresh tempeh (FT).

Total bacterial count analysis

One gram of SC was inoculated into 25 g sterile soaked-soybean in a 250 mL Erlenmeyer flask. Incubation for 2 days was done at room temperature (27-29 °C) before doing the serial dilution. Approximately, 25 g of each SS, BI, SC, FT, or 25 mL of FW and SW was added into 225 mL 0.85% (w/v) NaCl (Merck, USA). The serial dilution was made for each sample. From each dilution, a 100 µL aliquot of suspension was 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 the plates were incubated at 30 °C for 3-5 days to allow Enterobacteriaceae growth or 5-7 days to allow lactic acid bacteria growth.

DNA extraction for metagenomics analysis

One gram of SC was inoculated into 25 g sterile soaked-soybean in a 250 mL Erlenmeyer flask and incubated in 30 °C for 48 h to make a sterile tempeh. A hundred grams 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 a previous study (Seumahu *et al.*, 2012). Approximately, 25 g of SS samples were soaked into 225 mL sterile water for 24 h in the shaker. A 1000 mL of soaking water of SS, FW, and 300 mL of SW, were then 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 being used for the next step of analysis.

PCR amplification for 16S rRNA gene

Dominant colonies formed in EMB and MRSA agar were collected for further identification. Each DNA extraction from dominant colonies and metagenomics analysis were amplified by PCR using universal primers: B27F and U1492R (Lane, 1991). 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. Amplification by PCR comprised 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min 30 sec; and continued with 72 °C for 2 min. The PCR products were visualized on 1% agarose gel (w/v). Sequencing was carried out by Eurofin Genomics (Japan).

Cloned 16S rRNA gene analysis

Cloning of 16S rRNA gene was conducted according to Sakai and Kurosawa (2016). Briefly, the 16S rRNA gene fragments for metagenomic analysis obtained by PCR were cloned into pT7 Blue T-Vector (Novagen, Germany). The recombinant plasmid was transformed into

Escherichia coli DH5α plated onto Luria-Bertani (LB) plates including 100 µg/mL ampicillin (Wako, Japan) 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 a template with the primers T7P-F and T7U-R. PCR reactions were as followed: 94 °C for 3 min; 35 cycles 94 °C for 30 sec, 51 °C for 30 sec, 72 °C for 2 min; followed by 72 °C for 5 min. 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 using GENETYX ver. 11.0.1 software homology search program (Japan). Sequences with similarity of 98% and above were grouped together as one phylotype (Watanabe *et al.*, 2008). Sequence of representative clones were submitted to BLASTN database (http://www.ncbi.nlm.nih.gov/BLAST/) and EzTaxon server 2.1 (http://eztaxon-e.ezbiocloud.net) (Kim *et al.*, 2012).

High-throughput sequencing analysis

For amplification of the V4 domain in bacterial 16S rRNA, we used primers: 515F and 806R modified to contain MiSeq Illumina adapter region. PCR reaction were conducted using TaKaRa ExTaq HS (TaKaRa, Japan) 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 a final extension of 72 °C for 5 min. The PCR products were purified with AMPure XP beads (Belkman Counter, Japan) and further used as templates for tailed-PCR using primers: 15-index2 and 17-index1. PCR reactions in this step were as follows: 94 °C for 2 min, followed by 8 cycles 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 custom service provided by Bioengineering Lab (Japan) and Fastmac (Japan).

Data analysis

Raw fastq files were de-multiplexed, quality filtered, and analyzed with QIIME 1.6.0. Bacterial 16S rRNA gene sequence were clustered with the QIIME subsampled reference OTU-picking pipeline with UCLUST-reference against the Greengenes 16S rRNA gene 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 Mills, 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: $H' = -\Sigma (p_i)(\ln p_i)$ where p_i is the relative abundance of fragment i. D was measured based on equation: $D = \Sigma\ n_i(n_i\text{-}1)/N(N\text{-}1)$, 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: $E = H'/H_{max}$ where $H_{max} = \ln S$ (Pangastuti *et al.*, 2010).

RESULTS AND DISCUSSION

High-throughput analysis

A total of 1,099,495 total reads were obtained from six samples taken from EMP producer. 13.9% of the reads discarded based on several factors: quality (length, homopolymers and ambiguous base), miss-amplified taxa (chloroplast, mitochondria and unassigned kingdom) and chimera formation. As a result, 946,094 clean reads of 340 bp length on average were retained and analyzed. Each sample harbored different total reads (Table 1). Enriched SC and FT samples covered the highest total reads among them. The statistical bacterial diversities for each sample were calculated, and diversity parameters were presented in Table 1. Shannon-Wiener Index shows the species diversity, while Simpson Index gives the probability that any two individuals are different species. Based on these indices, the bacterial community was less diverse in SW and SC, indicating that less species of bacteria were present. Equitability indices also showed that SW and SC had a lower value than other samples. This indicates that the quantity distribution of each species was not equal, and only one or two taxa qualified as the dominant species.

Table 1: Number of bacterial 16S rRNA gene sequences, operational taxonomic unit (OTUs); and diversity estimation.

Samples	Total Reads	Clean Reads	Total OTUs	Shannon-Wiener Index (H')	Simpson Index (D)	Equitability Index (E)
SS	67,371	58,939	640	2.146	0.841	0.332
FW	67,599	58,436	1,650	2.717	0.823	0.367
SW	60,534	54,370	413	0.445	0.145	0.074
BI	54,492	50,873	723	2.309	0.819	0.351
SC	423,929	361,984	1,137	1.097	0.390	0.156
FT	425,570	361,492	1,002	2.108	0.763	0.305

Note: SS, soybeans; FW, fresh water used to soak the bean; SW, soaking water after the beans were soaked for 18 h; BI, soybeans before inoculated; SC, starter culture; FT, fresh tempeh.

Bacterial communities structure from EMP tempeh producer

Bacterial community composition of tempeh production might be contributed by many factors, including raw material, water source, environmental conditions, and traditional tools of manufacture (Jung *et al.*, 2013). In this study, we examined bacterial communities in raw material used for tempeh production, including soybean and starter culture, and also the water source. Taxonomical assignment for all sequences derived in this study was carried out in QIIME, which is based on alignments with the GreenGenes database. Obtained results consisted of kingdom, phylum, class, order, family and genus level. Additional information about species level was done by employing the 16S rRNA gene cloning as discussed in the subsequent section. The predominant phyla of SS, SW, SC, and FT were composed of Firmicutes with different proportions (Figure 1). Other samples, FW and BI were populated by Proteobacteria with 88% and 69% abundance, respectively. With Firmicutes proportion nearly 100%, indicating that SW and SC only had one predominant phylum, unlike SS or FT that also consisted of 27% and 8% of Proteobacteria abundance, respectively. To our knowledge, tempeh is the only mold-fermented food product which bears Firmicutes as its predominant bacteria (Tamang *et al.*, 2016). Another sample, FW, used for soaking and washing dehulled-soybean, and BI, dehulled-soybean ready for inoculation, was populated by Proteobacteria, with Firmicutes only as a sub-dominant phylum. Rarer phyla acquired from FT were Actinobacteria and Bacteroidetes, which was further categorized as "Others", as its abundance in total reads were less than 1%.
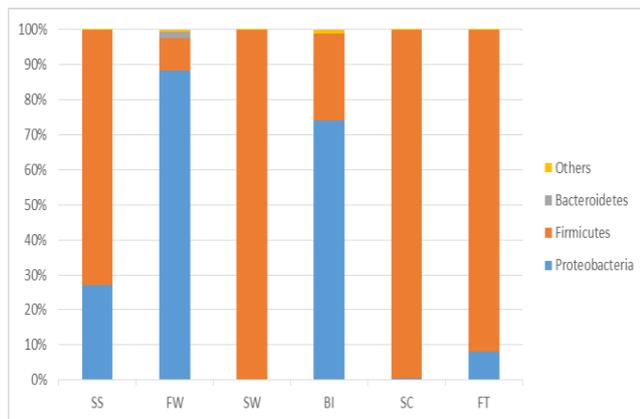
The most abundant (> 1% in at least one sample) bacterial order found throughout tempeh production is shown in Figure 2, while the major genus composition is shown in Figure 3. At the order level, a total of 202 order were classified and 14, 106, 22, 23, 19 and 18 were detected from tempeh production such as SS, FW, SW, BI, SC, and FT, respectively. Lactobacillales, which belongs to the Firmicutes, was the predominant order in three samples, SS, SW, and FT, with each sample proportion being 60%, 100%, and 88%, respectively. The dominant orders from Proteobacteria found in FT were Sphingomonadales and Enterobacteriales (Figure 2). Relative abundance of each order in FT were 2.8% and 3.9% respectively. But in term of their relative abundance in Proteobacteria, each order were 37% and 52.5%, respectively. Sphingomonadales contained one dominant genera, i.e. *Novosphingobium* (Figure 3). However, Enterobacteriales, in which *Klebsiella pneumoniae* belongs to (Keuth and Bisping, 1993), can be differentiated only up to the family level, which is Enterobacteriaceae. The low discriminatory power might be due to the short length of sequence (340 bp) associated with the HTS technique employed in this study (Humblot and Guyot, 2009). Bifidobacteriales was also detected in BI and FT, although its abundance in both samples was less than 1%. Members of the Bifidobacteriales are known as probiotic bacteria which can transform biologically inactive isoflavone found in soybean into bioavailable and bioactive aglycones (Kailasapathy and Chin, 2000; Tsangalis *et al.*, 2002; Atkinson *et al.*, 2005).
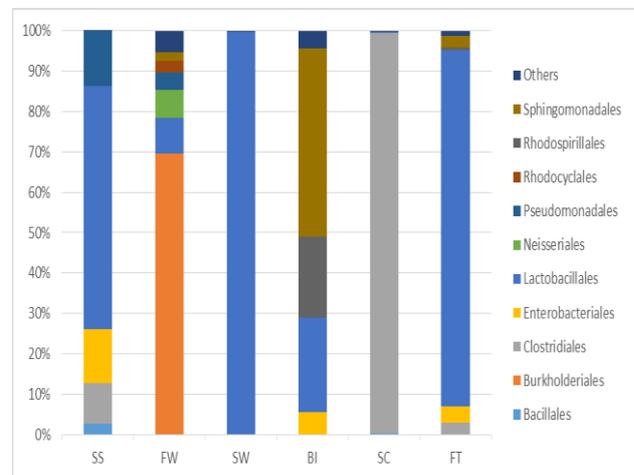

Figure 1: Bacterial phyla in samples collected from EMP tempeh producer. Relative abundance of each phylum was defined as the percentage of the same phylum to the corresponding total 16S rRNA gene sequences for each sample. Phyla that were acquired at less than 1% in samples were grouped as "Others". SS: soybeans, FW: fresh water used to soak the bean, SW: soaking water after the beans were soaked for 18 h, BI: soybeans before inoculated, SC: starter culture, FT: fresh tempeh.

Figure 2: Major bacterial orders attained from EMP tempeh producer. Relative abundance of each order was defined as the percentage of the same order to the corresponding total 16S rRNA gene sequences for each sample. Order that were detected at less than 1% in all samples were grouped as "Others". SS: soybeans, FW: fresh water used to soak the bean, SW: soaking water after the beans were soaked for 18 h, BI: soybeans before inoculated, SC: starter culture, FT: fresh tempeh.

A majority of the genera identified in the six samples were mostly composed of species from the Lactobacilalles genera, i.e. *Enterococcus, Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Weissella*. *Enterococcus* and *Lactobacillus* were the vast majority of FT sample, with 46.3% and 41.5% abundance, respectively, while the other genera proportion was less than 3%, including *Novosphingobium* as the next sub-dominant genus in FT. *Lactobacillus* was also the major genus in SW with 98% abundance, confirming its role as an organic acid producer in the spontaneous fermentation that occurred during the soybean soaking process (Barus *et al.*, 2008; Efriwati *et al.*, 2013). In the following step, BI, the *Lactobacillus* population was decreased by 3.5% before they re-increased post *Rhizopus oligosporus* fermentation. This reduction might be caused by dehulled-soaked soybean and washing process afterwards, which eliminated most of the *Lactobacillus* population and shifted the bacterial community into the environmental bacteria, *Novosphingobium* and *Acetobacter* (Figure 3). Therefore, *Lactobacillus* which existed in FT might survive through dehulling and washing processes, since its number was increased in FT.
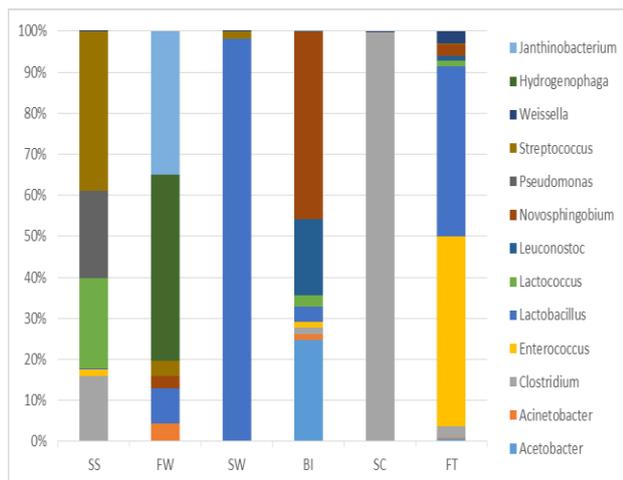

Figure 3: Predominant bacterial genera attained from EMP tempeh producer. Relative abundance of each genus was defined as the percentage of the same genus to the corresponding total 16S rRNA gene sequences for each sample. SS: soybeans, FW: fresh water used to soak the bean, SW: soaking water after the beans were soaked for 18 h, BI: soybeans before inoculated, SC: starter culture, FT: fresh tempeh.

Other than *Lactobacillus*, *Enterococcus* was the dominant bacterial group detected in FT. Unlike *Lactobacillus*, *Enterococcus* was not detected in SW. It was detected in BI samples with 1.3% proportion before becoming the predominant bacteria in FT. *Enterococcus* availability in tempeh sample had been reported in the previous study, but its position as the dominant bacteria was not described (Moreno *et al.*, 2002; Mo *et al.*, 2013).

Since *Enterococcus* was detected in BI and not in SW, a possible source for this bacterial group was from the process in between, dehulling and washing with FW. This bacterium may only grow in condition suitable for tempeh fermentation, but remained dormant in the water. However, BI is critical for the establishment of *Enterococcus* as a predominant member of the bacterial population during tempeh fermentation. Some *Enterococcus* species can produce bacteriocin, which might extend tempeh shelf life (Moreno *et al.*, 2002; Giraffa, 2014). A previous study reported that *Enterococcus* together with lactic acid bacteria may play major roles in protein degradation, flavor formation, and acid production in the ripening process of *meju*, a fermented soybean paste from Korea (Jeong *et al.*, 2014). Therefore, this bacteria might also contribute to the bitter taste formation in tempeh made by EMP producer, in addition to *Bacillus* (Barus *et al.*, 2008).

The dominant genera in FW sample were *Hydrogenophaga* and *Janthinobacterium,* members of Burkholderiales*,* with 45.2% and 35% abundance, respectively. Though dominant, their presence disappeared in the subsequent step of tempeh production, i.e. SW, BI, and FT. FW was used to soak the soybean overnight and to wash dehulled soybeans, prior to inoculation with SC. The loss of Burkholderiales and several other orders (i.e. Neisseriales, Pseudomonadales, and Rhodocyclales) from FW in SW was possibly due to acidification by lactic acid fermentation during the soybean soaking process. Lactic acid fermentation produces organic acid and antimicrobial compounds that may inhibit the growth of pathogen and spoilage bacteria, which plays a role in ensuring the safety of fermentation products (Mulyowidarso *et al.*, 1990). Tempeh fermentation that is done without the soaking process often fails due to this reason. Two other orders, Sphingomonadales and Lactobacillales, were also found in FW, with 8.9% and 2.3% abundance, respectively.

Unlike Lactobacillales as the dominant order in SW, Sphingomonadales was found in a BI sample with *Novosphingobium* (46.6%) and *Acetobacter* (24.7%) as its dominant genera. But, their respective dominance was decreased by 58% and 27.5% after two days incubation in FT. *Novosphingobium* are often associated with the biodegradation of aromatic compounds, which frequently are isolated from aquatic environments (Vaz-Moreira *et al.,* 2011). Therefore, *Novosphingobium* in BI was presumably coming from FW used for washing the soybean. On the other hand, *Acetobacter* presumably originated from the environment where the tempeh production was occurring. In another food fermentation study, *Acetobacter* was indigenous acetic acid bacteria which responsible for the fermentation of vinegar (Wu *et al.*, 2010).

Not only in FT and SW, Lactobacillales was also present in SS. However, its predominant genera was different than in FT and SW. Dominant genera obtained from SS were *Streptococcus* and *Lactococcus,* with 39% and 22% abundance, followed by *Pseudomonas* and *Clostridium,* with 21% and 16% abundance, respectively.

Throughout the boiling and soaking process, the population of *Pseudomonas* altogether with the *Streptococcus* and *Lactococcus* decreased in FT sample, even though their 16S rRNA gene sequences can still be retrieved from FT in lower numbers (< 1.5%). On the other hand, *Clostridium* was the only genus in SS and its population was increased in FT, despite the relative abundance was showed otherwise (Figure 3).

The starter culture used by EMP tempeh producer is *laru onggok* (SC). It is made from cassava solid waste (*onggok*) which inoculated by commercial starter culture (Raprima brand). Its bacterial community was greatly influenced by *onggok,* since Raprima starter culture itself was not dominated by *Clostridium* (data not shown). Clostridiales was the dominant order found in SC (99.2%). *Clostridium* was the major genus existing in SC, yet its abundance was decreased to 96.9% in FT, suggesting that the bacteria in SC did not survive post inoculation to BI due to the aerobic fermentation processes. Solely, lactic acid bacteria, mostly those belonging to the *Lactobacillus* genus, were detected in *laru onggok* by traditional culturable technique (Nurdini *et al.*, 2015).

Cloned 16S rRNA gene analysis

To obtain a complete identification of species involved in tempeh production, cloned 16S rRNA gene analysis was conducted on all samples. A total of 135 clones were obtained from the EMP tempeh producer. Similar to HTS results, SS, SW, SC and FT harbored Firmicutes as the dominant bacteria, while FW and BI were populated by Proteobacteria (Table 2). As observed in previous section, the dominant genera detected in SS, SW, SC and FT were different, even though we observed a similar phylum in those samples, Firmicutes. *Weissella cibaria* and *Streptococcus lutetiensis* were the dominant bacteria in SS, while the same species were not detected in both SW and FT. The dominant bacteria detected in SW, *Lactobacillus agilis* and *L. fermentum,* also existed in FT samples. This indicates that *Lactobacillus* in tempeh were probably originating from the soaking process. Meanwhile, bacteria detected in SC was *Clostridium beijerinckii*. This bacterium is rarely found in the starter culture where its fermented product's bacterial community is lactic acid bacteria (Leroy and De Vuyst, 2004). Usually, *Clostridium* is found in fermented fish products, such as *ngari* (Devi *et al.*, 2015) and *balacan* (Salampessy *et al.*, 2010).

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

Sample	Species	Percentage of similarity (%)	Phylum	Accession number
SS	*Weissella cibaria*	100	Firmicutes	KC110687
	Streptococcus lutetiensis	100	Firmicutes	AF429763
	Clostridium diolis	100	Firmicutes	AJ458418
	Lactococcus lactis	99	Firmicutes	AB100804
FW	Uncultured *Tepidimonas* sp.	97	Proteobacteria	HF912322
	Duganella sp.	99	Proteobacteria	AM389091
	Lactobacillus delbrueckii	100	Firmicutes	AB680073
SW	*Lactobacillus agilis*	99	Firmicutes	AB911458
	Lactobacillus fermentum	99	Firmicutes	KF149390
SC	*Clostridium beijerinckii*	99	Firmicutes	LC071789
	Weissella confusa	100	Firmicutes	KT260733
	Sphingomonas sp.	100	Proteobacteria	AB6967751
BI	*Acetobacter cibinongensis*	99	Proteobacteria	AB9064051
	Leuconostoc pseudomesenteroides	99	Firmicutes	LT2235931
	Lactococcus hircilactis	99	Firmicutes	NR136465
	Enterococcus cecorum	99	Firmicutes	AB932534
FT	*Lactobacillus agilis*	99	Firmicutes	M58803
	Lactobacillus fermentum	99	Firmicutes	AP008937

Note: SS, soybeans; FW, fresh water used to soak the bean; SW, soaking water after the beans were soaked for 18 h; BI, soybeans before inoculated; SC, starter culture; FT, fresh tempeh.

Culturable Enterobacteriaceae and lactic acid bacteria

The numbers of culturable Enterobacteriaceae and lactic acid bacteria colonies on the plate during tempeh processing were varied from one stage to another (Figure 4). Inconsistent to the HTS results, the results of culturable technique demonstrated that FT was dominated by Enterobacteriaceae (Proteobacteria).

During tempeh fermentation, lactic acid bacteria had been always present in higher numbers than Enterobacteriaceae, but not in the resulting FT. Lactic acid bacteria were already detected and higher than Enterobacteriaceae as early as in the raw material used, i.e. SS, and they could be identified in almost every step during tempeh production. However, its number was considerably decreased in FT (3.3×10^6 CFU/g to 6.4×10^4 CFU/g) compared to Enterobacteriaceae. SC also

harbored lactic acid bacteria (4.0×10^4 CFU/g), and thus may contribute to the total amount of this bacterial group in FT. The dominant culturable lactic acid bacterium in FT was *L. fermentum,* which is similar to the results obtained from cloned 16S rRNA gene analysis. The abundance of lactic acid bacteria in Indonesian soybean was higher than the previous report that detected lactic acid bacteria in Malaysian soybean (less than 10^2 CFU/g) (Moreno *et al.*, 2002). The starter culture used in EMP tempeh production was *laru onggok.* Previous study reported that *laru onggok* contained 3.9 log CFU/g of lactic acid bacteria, while *onggok* itself contained 2.2 log CFU/g (Nurdini *et al.*, 2015). With the same method, we reported that the predominant lactic acid bacterium detected in the starter culture was *Weissella confusa.* However, our HTS analysis showed that *Weissella* was the sub-dominant bacteria (1.5%), after *Clostridium* (Figure 3). Thus, culturable technique could generate bias if we did not combine with metagenome analysis, since *Clostridium,* as the dominant bacteria in SC, would not grow well under semi-anaerobic condition use in this study.

Figure 4: Abundance of total culturable bacterial population in various stages of tempeh fermentation. SS, soybeans; FW, fresh water used to soak the bean; SW, soaking water after the beans were soaked for 18 h; BI, soybeans before inoculated; SC, starter culture; FT, fresh tempeh.

FT contained culturable Proteobacteria with Firmicutes as the sub-dominant bacterial group, while HTS analysis showed the opposite. This contrasting results indicated that the culturable technique was biased in the observation of bacterial community in tempeh. Thus, the result obtained by culturable technique did not reflect the real microbial communities and thus it is not preferable to be used to study the composition of microbial community (Aslam *et al.*, 2010). By culturable technique, previous study also reported that the the dominant bacteria in EMP tempeh belong to the Proteobacteria group, i.e. *Klebsiella pneumoniae* and *Acetobacter indonesiensis*; and the Bacteroidetes group, i.e. *Flavobacterium* sp. (Barus *et al.*, 2008). The culturable technique may be better for investigating the viable cells among the microbial communities in food fermentation. In addition, it also could be used to detect any desirable bacteria such as *K. pneumoniae* that cannot be observed by metagenomics analysis. However,

this method is not suitable for studying the composition of microbial community as a whole.

As opposed to what was reported based on HTS analysis, the maximum number of culturable Enterobacteriaceae found in the final product of fermentation was 2.54×10^7 CFU/g, higher than culturable lactic acid bacterium, thus Enterobacteriaceae were the predominant bacteria in FT. Similar with the previous finding, dominant Enterobacteriaceae detected in FT was *K. pneumoniae* (Barus *et al.*, 2008) and *K. variicola.* Those bacteria were failed to be detected by HTS analysis. It might be due to the short length of 16S rRNA gene sequence generated.

Previous study reported that *laru onggok* contained 1.6 log CFU/g Enterobacteriaceae (Nurdini *et al.*, 2015), though our culturable technique and HTS analysis failed to detect any Enterobacteriaceae in SC. Such discrepancies may arise of the difference in environmental conditions in which the fermentation and starter culture production occurred. Tempeh starter culture is fermented in the open air, where other microbes could easily be exposed to the processes. Therefore, tempeh starter culture produced from different batches may yield different composition of microbial communities.

Random analysis of 16S rRNA library might be too tedious for the examination of the entire composition of microbial communities, but it may still be useful to detect some predominant bacteria in tempeh. Therefore, HTS was preferable to study bacterial communities in tempeh samples, since it could detect minor populations (Kiyohara *et al.*, 2012) and thus could reveal deeper information on tempeh bacterial diversity.

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