Bacterial and Fungal Communities in Tempeh as Reveal by Amplified Ribosomal Intergenic Sequence Analysis

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Tempeh is an Indonesian traditional fermented food produced using *Rhizopus* as a starter culture. In practice, however, the starter culture as well as fermentation processes would yield a polymicrobial fermentation, which generated a unique tempeh flavor and texture. This condition makes Indonesian tempeh as one of the most complex fermented food, while at the same time would make it difficult to scale up tempeh production with uniform quality and consistency. The aim of this study was to compare a number of tempeh microbial communities employing Amplified Ribosomal Intergenic Sequence Analysis (ARISA). Fresh tempeh samples were obtained from tempeh producers in Java and Moluccas. 16S rRNA gene libraries and DNA sequencing were employed to analyze further the nature of bacterial diversity in two selected tempeh samples. The results of our study showed that different tempeh producer possessed different Bacterial ARISA (BARISA) or fungi ARISA (FARISA) profiles. However, BARISA profiles were found to be more discriminative than FARISA, and therefore BARISA would be more useful for tempeh genetic fingerprint or barcoding.

Keywords: tempeh, metagenome, ARISA

INTRODUCTION

Tempeh is a traditional fermented food consumed widely in Indonesia which is typically made from soybean with *Rhizopus* as a starter culture. Tempeh is usually produced by household industry with no specific standard process applicable in all areas. It is estimated that there are more than 100.000 tempeh producers spread out all over Indonesia. This is one the primary reasons why there are many variation in tempeh flavor or texture amongst different region or producers (Astuti *et al.* 2000).

Although traditional microbiological studies of tempeh production have been focused mostly on the fungi as starter, the growth of other microbial species such as bacteria and yeast during the process has also been reported (Nout & Kiers 2005; Barus *et al.* 2008). In Indonesia, tempeh is produced by open fermentation which allowed the growth of naturally-occuring bacteria which contributed to variation in sensory quality of tempeh. Starter as primary microbiota, as well as non-starter microorganisms associated with tempeh (secondary tempeh microbiota) could modify the physical and chemical

properties of the fermented food. Knowledge of the structure and dynamics of the whole microbial community would promote better understanding of how characheristics vary with respect to microbial growth and metabolism (Jani & Barbier 2008).

An automated method of ribosomal intergenic spaces analysis (ARISA) was developed for the rapid estimation of microbial diversity and composition in environment. This technique based on PCR amplification of the 16S-23S intergenic spacer region in the rRNA operon with a fluorescence-labeled primer. Because of less selection pressure, the 16S-23S rRNA intergenic spacer sequence seems to be more genetically variable and species specific than that of 16S rRNA and 23S rRNA gene (Fisher & Triplett 1999; Liu *et al.* 2008). This technique could be used for discriminating closely related strains within species and to differentiate microorganism with different genetic community structure in a population system. ARISA, therefore is better applied for fingerprinting microbe communities than T-RFLP. In addition, ARISA also could be used as a molecular fingerprinting for analyzing changes in microbial community structure (Danovaro *et al.* 2006; Ramete 2009).

The aim of the study was to evaluate and compare microbial composition of eight tempeh samples produced in Java and Ambon and find out the specific

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microbe community that could be used as DNA barcode or tempeh fingerprints.

MATERIALS AND METHODS

Sample Treatment. Twenty five gram tempeh was homogenized in 225 ml NaCl 0.9% for 1 min.

The mixture obtained was centrifuged for 1 min at 800 x g and the top solution was moved into new tubes and centrifuged again at 13,000 x g for 5 min. Top solution was discarded and the pellet was washed with TE pH 8.0 prior to DNA extraction.

DNA Extraction. Microbial DNA was extracted from tempeh employing PowerFood Microbial DNA

Table 1. Different tempeh processing profiles in eight tempeh producers in Ambon, Bogor, Malang, and Sidoarjo

Sample*	Treatment before processing	Cooking	Fermentation Treatment	Fermentation time	Water resources	Starter	Soybean type
WJB	cooked	twice	Once	1 night	PAM	Raprima	Jempol
EMP	cooked	once	Once	2 days	Sumur	Raprima	Jempol, Gunung
WHR	soaking in hot water for 1 night	once	Once	3 days	PAM	Raprima	Jempol
HTN	soaking in hot water for 1 hour	once	Once	2-3 days	PAM	Raprima	Jempol
MLGS	cooked but not boiled	twice	Once	2 days	Sumur	Daun Waru	Jempol, GCU
MLGA	cooking until ½ cooked	twice	Once	2 days	Sumur	Daun Waru	Jempol
SDJD	cooking for 1 night	twice	Twice	2 x 1 days	Sumur	Jago & Raprima	Jempol, Bola
SDJK	soaking for 8 hour	once	Twice	2 x 1 days	Sumur	Jago	Jempol, Bola

*EMP, WJM = tempeh from Bogor; WHR, HTN = tempeh from Ambon; MLGA, MLGS = tempeh from Malang; and SDJD, ADJK = tempeh from Sidoarjo.
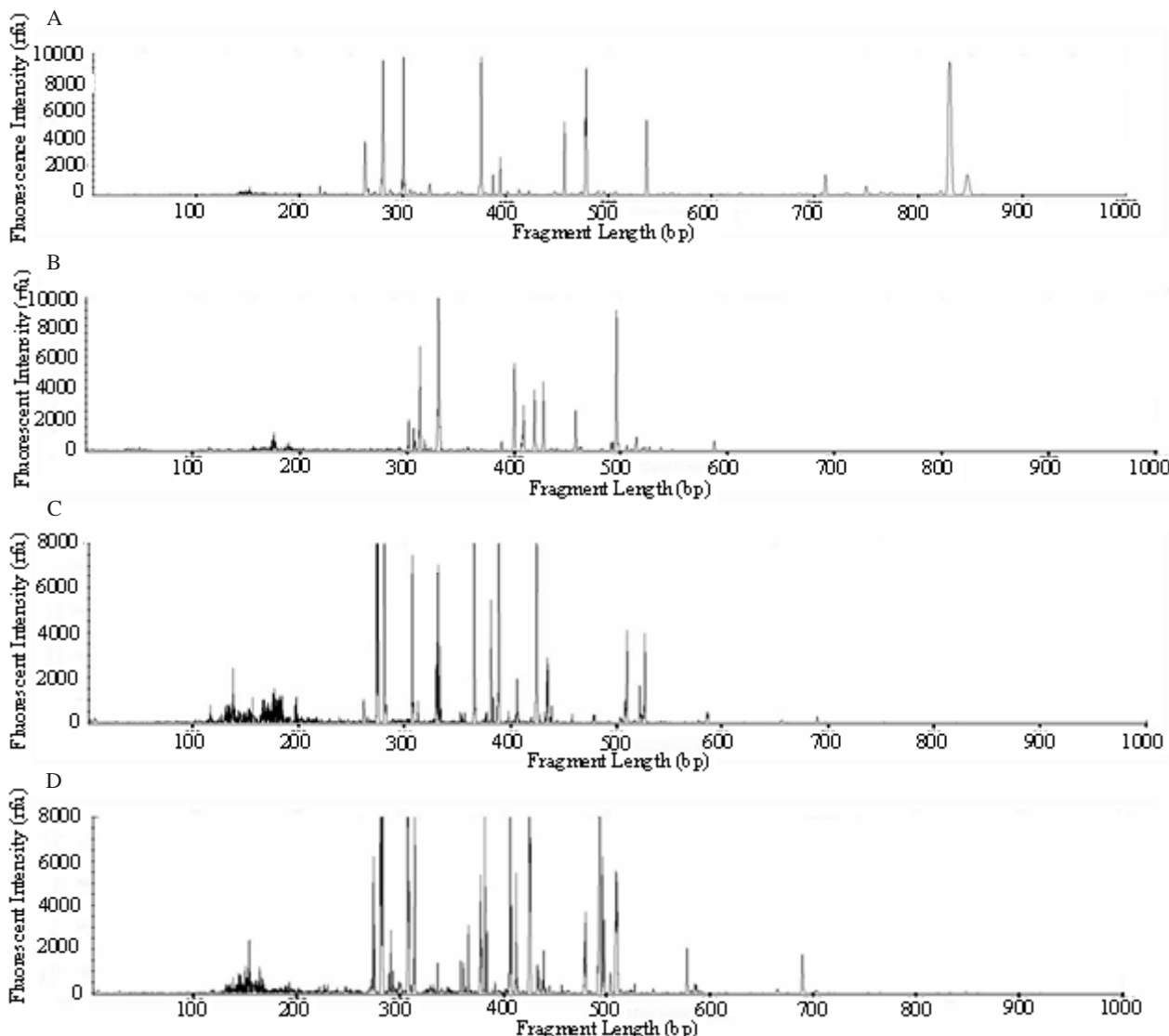

Figure 1. BARISA profile from four tempeh A. EMP (Bogor), B. WJB (Bogor), C. MLGA (Malang), and D. MLGS (Malang).

Isolation kit-MOBIO (PFMDIK) according to the protocol described previously (Seumahu *et al.* 2012). The quality of DNA was analyzed using electrophoresis on 1% (w/v) agarose gel in TAE buffer and stained with ethidium bromide.

Automated Ribosomal Intergenic Spacer Analysis (ARISA). Bacterial and fungal DNA from the DNA extract was amplified using primers that amplify the intergenic spacer region (ITSF and ITSReub for bacteria and 2234C and 3126T for fungi (Ranjard *et al.* 2001; Cardinale *et al.* 2004). The 5' end of the ITSReub and 2234C were labeled with phosphoramidite dye HEX (6-carboxy-1,4-dichloro-2,4,5,7-tetra-chlorofluorescein).

PCR mixture contains GoTaq Green mastermix (Promega) and primers for BARISA or FARISA. PCR condition consisted of an initial denaturation step (94 °C, 3 min) followed by 30 cycles of 94 °C for 45 sec, 56.8 °C for Bacterial ARISA (BARISA) or 60.7 °C for Fungi ARISA (FARISA) for 1 min and 72 °C for 2 min, followed by a terminal extention step at 72 °C for 7 min. ARISA sequencing service was conducted in PT Wilmar Benih Indonesia, Cikarang as described by Cardinale *et al.* 2004. Peak size (bp), height and area were estimated by comparison with the internal size standard LIZ 1200 fragment. All measurement were done in triplicate. Tempeh was purchased from producer in Ambon, Bogor, Malang and Sidoarjo. Daily samplings were done for three days to validate reproducibility of microbial communities analysis. The ARISA profile were same for three days. We chose one profile as a representative data in this paper for each producer. Each peak was referred as operational taxonomic unit (OTU) and this was not referred to single species. Similarities between eight communities were analyzed by manually calculating Sorensen's coefficients. Cluster analysis was conducted with the MEGA5.1 program using the Sorensen's coefficient of similarity and clustering analysis was conducted via the unweighted-pair-group mean-average method.
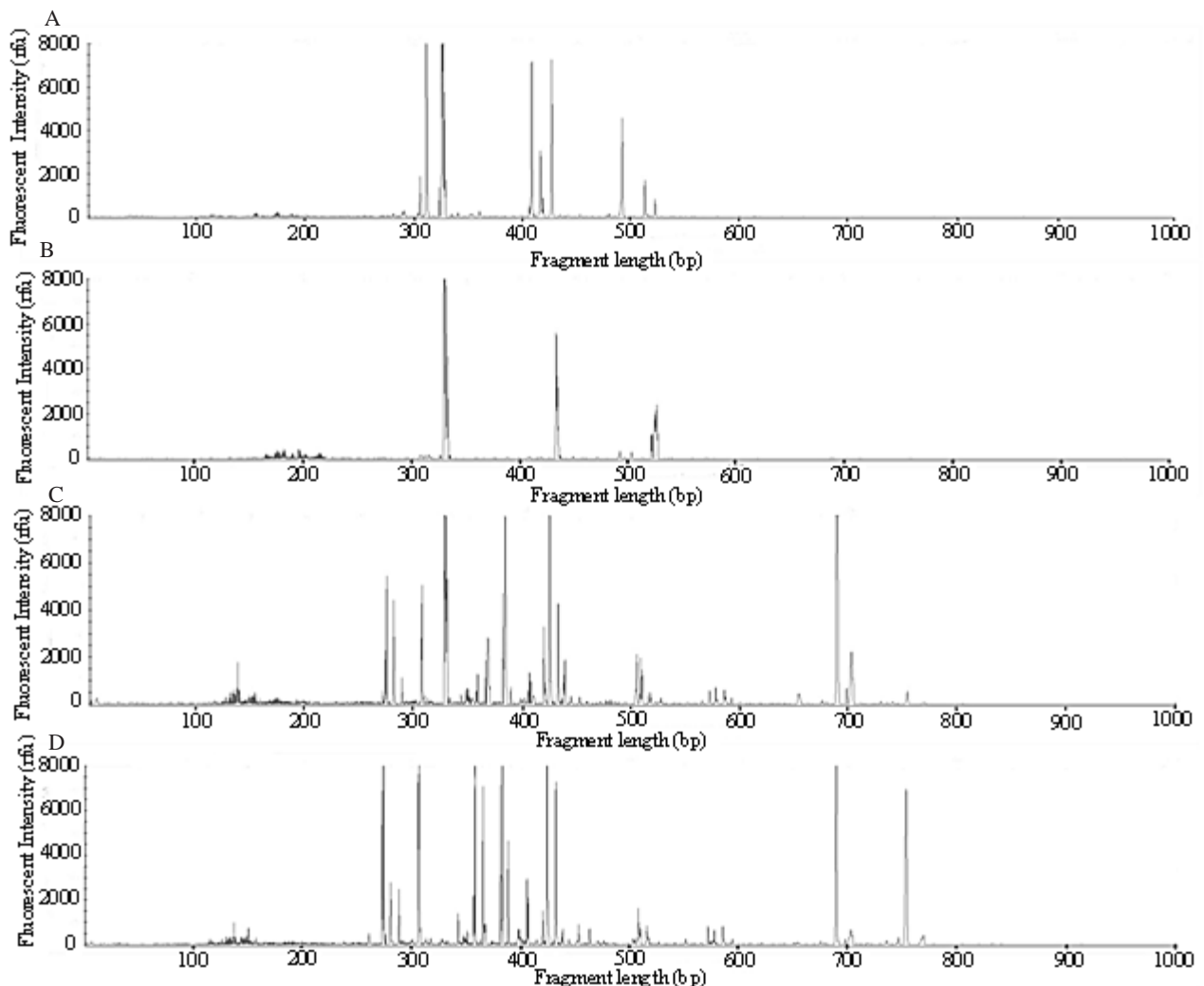

Figure 2. BARISA profile from four tempeh A. HTN (Ambon), B. WHR (Ambon), C. SDJD (Sidoarjo), and D. SDJK (Sidoarjo).
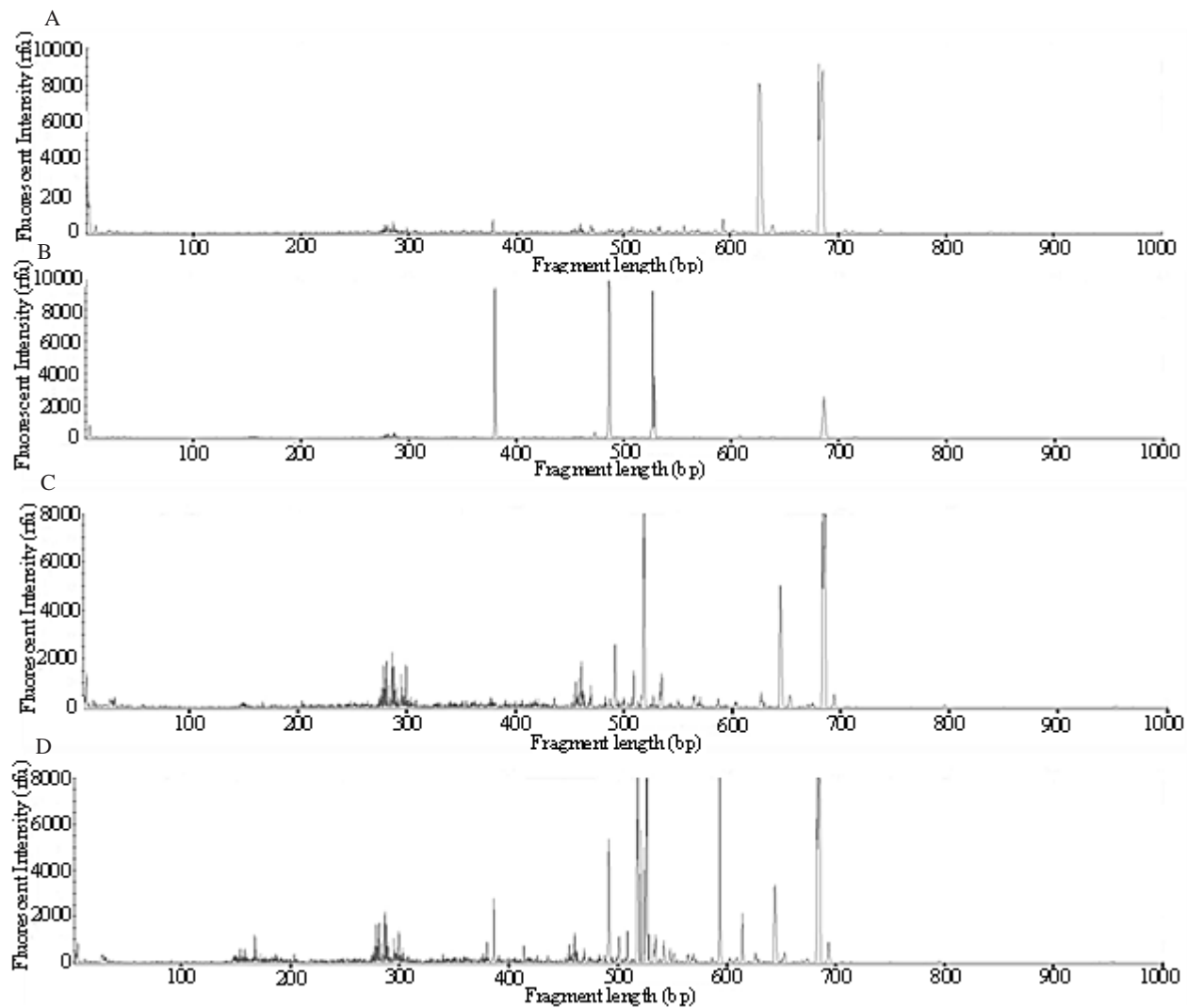
Figure 3. FARISA profile from four tempeh A. EMP (Bogor), B. WJB (Bogor), C. MLGA (Malang), and D. MLGS (Malang).

RESULTS

Tempeh Production and its Implication on the Microbial Diversity. The data in Table 1 showed that tempeh was prepared employing different procedures from one producer to the others. Soaking processes were done after boiling the soybean, or direct soaking in hot water. Fermentation processes were also different between tempeh producers. One of the tempeh producers in Sidoarjo (SDJD) used combined starter, while producers from Malang used fungal spore obtained from Waru leafs (*Hibiscus tiliaceus*). Variation in tempeh making could result in the naturally-occurring microbial community (Figure 1-4). The Shannon-Wiener index (H') and Simpson Index (D) were calculated to showed the diversity of peaks (OTU) for barcode system. The Shannon-Wiener index (H) were almost same for bacterial than fungi community but Simpson Index (D) was low for bacteria than fungi community in tempeh (Table 2).

Clustering Analysis. Clustering of 'BARISA type' (Figure 5) and 'FARISA type' (Figure 6) from the tempeh BARISA and FARISA fingerprinting were constructed to show the difference between bacterial and fungal communities. Our analysis showed that Bacterial community in eight tempeh were significantly different compared to fungi community according to Sorensen's similarity coefficient. Sorensen's similarity coefficient was chosen because microbial community in tempeh could be more diverse than the sample taken. Tempeh from the same area were grouped into distinct clusters except for tempeh from Sidoarjo.

DISCUSSION

Fermentation process that allow naturally occurring microbe makes it difficult to produce food with uniform quality (Jung *et al.* 2012). Different producers with different procedure (soaking, dehulling and cooking) could yield different composition of microbial communities in tempeh Unique microbial
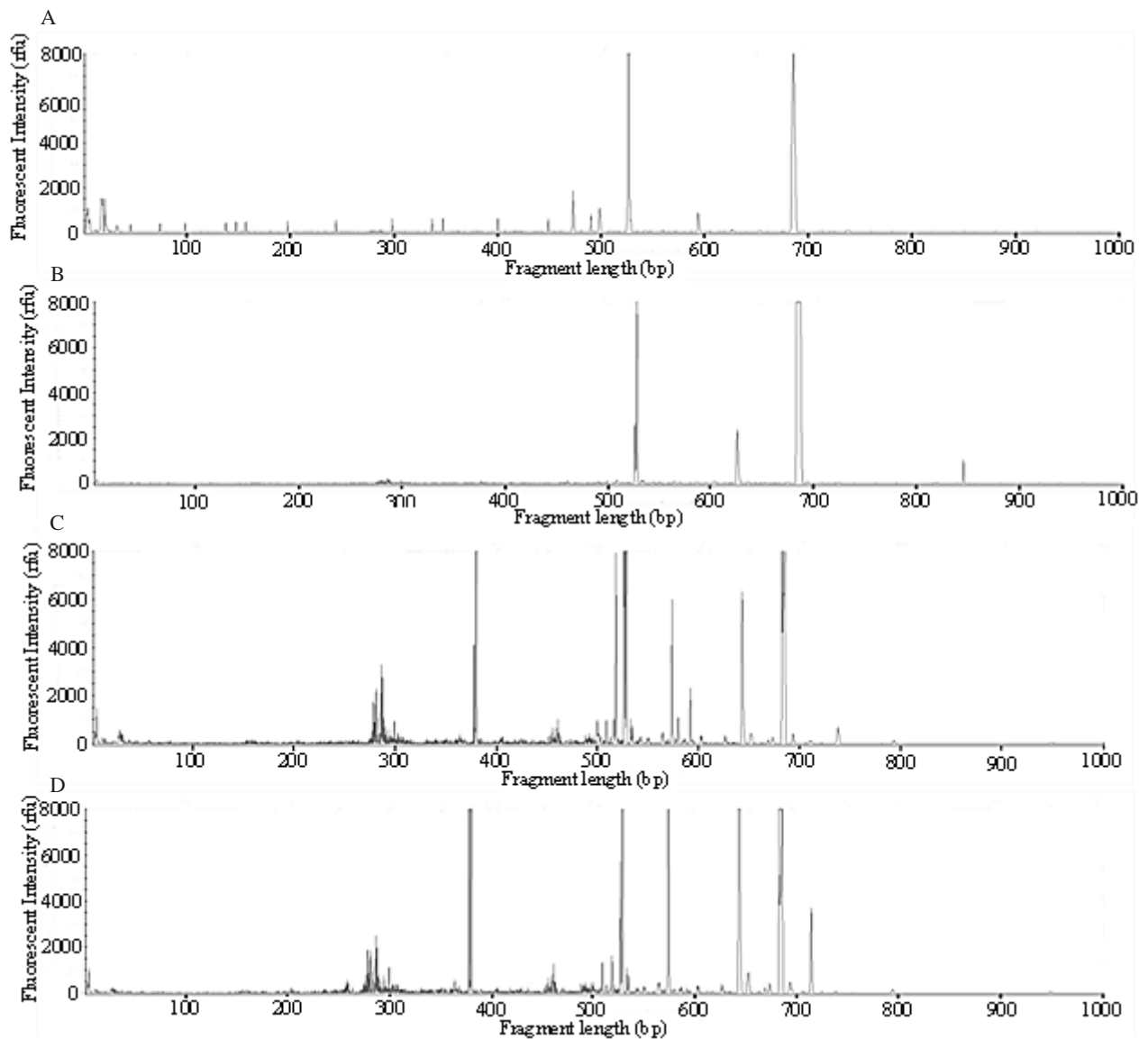
Figure 4. FARISA profile from four tempeh A. HTN (Ambon), B. WHR (Ambon), C. SDJD (Sidoarjo), and D. SDJK (Sidoarjo).

Table 2. Ecology characters of BARISA and FARISA profile from eight tempeh analyzed

Sample	Sample resouces	BARISA			FARISA		
		Shannon-Wienner Index (H)	Simpson Index (D)	Number of "BARISA type"	Shannon-Wienner Index (H)	Simpson Index (D)	Number of "FARISA type"
WJB	Bogor	1.34	0.11	83	0.88	0.22	36
EMP	Bogor	1.17	0.09	46	1.46	0.11	131
HTN	Ambon	1.09	0.11	37	0.71	0.30	29
WHR	Ambon	1.22	0.11	66	1.09	0.21	46
MLGS	Malang	1.61	0.04	104	1.62	0.05	145
MLGA	Malang	1.53	0.05	93	1.57	0.06	145
SDJD	Sidoarjo	1.57	0.04	90	1.39	0.08	104
SDJK	Sidoarjo	1.62	0.04	91	1.50	0.07	124

community in tempeh could be used as barcoding system to characterized tempeh from different producers. The 'BARISA type' from tempeh was not the same to any of eight tempeh samples while some OTU from 'FARISA type' were found in all of samples analyzed. This result suggested that even

the H' diversity index were almost the same for bacterial than fungal communities, the D value for bacterial communities was lower for bacterial communities. This result indicated that the probability to find the same OTU in bacterial community was small and could be used as barcoding system for
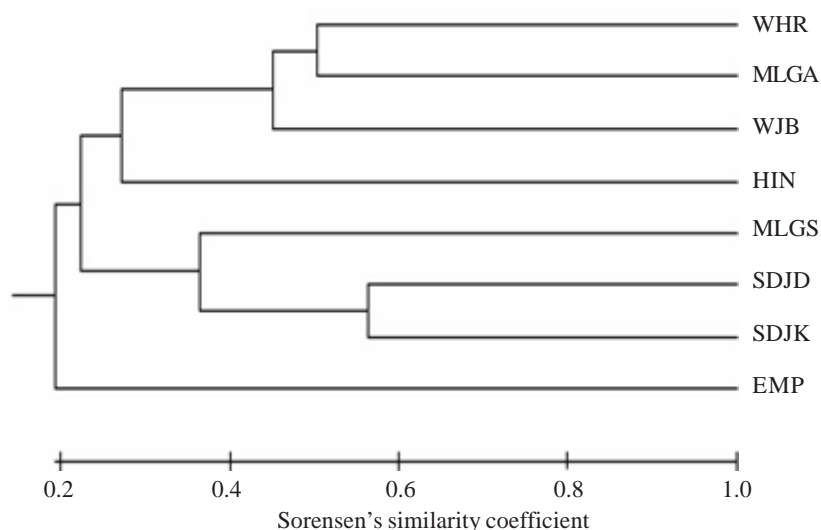
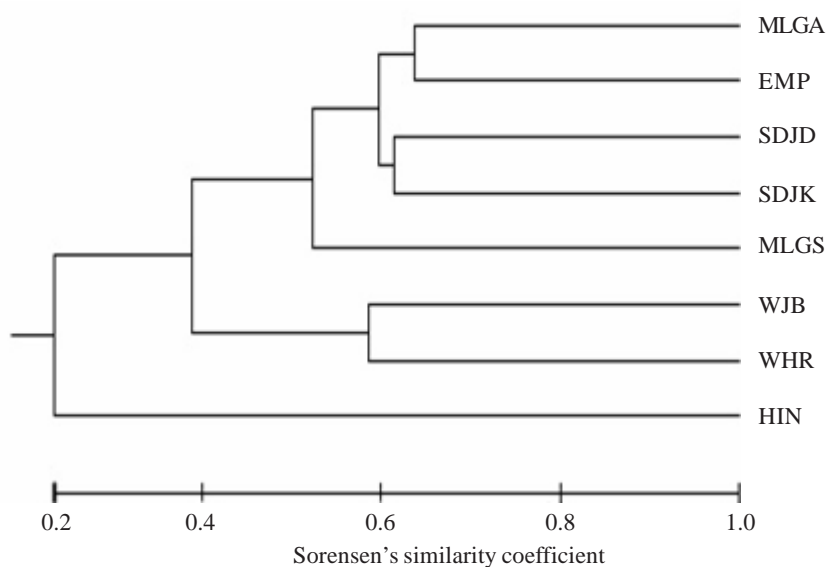Figure 5. Phylogenetic tree showing clustering of bacteria communities in eight tempeh.

Figure 6. Phylogenetic tree showing clustering of fungal communities in eight tempeh.

tempeh than OTU in fungal community. This could happen because fungi were used as a starter while bacteria could regularly and naturally be obtained from fermentation environment. Different 'BARISA type' and 'FARISA type' were also found but clustering analysis showed that bacterial and fungal community from Sidoarjo tempeh samples were grouped into the same cluster. Tempeh from Sidoarjo were collected from producers in the same area. These results could indicate that the variation in microbial composition of tempeh depends on the process and the environment, or material used by tempeh producers (Scheirlink *et al.* 2007; Jung *et al.* 2012). Sorensen's similarity coefficient also showed that bacterial community has the low similarity among BARISA type than fungal community in FARISA type. Even producers were grouped in the same cluster, the similarity between

OTU in bacterial communities were small. This could happen because bacterial could regularly naturally be obtained from fermentation environment while fungal was add as starter. This result indicated that BARISA type from tempeh could be more discriminative and more appropriate as tempeh barcoding system than FARISA type.

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