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Microbial growth dynamics during tempe fermentation in two different home industries

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Abstract

Tempe is a traditional fermented food of Indonesia that has been recognised globally. The main starter culture for tempe fermentation is *Rhizopus oligosporus*, however several researchers have detected yeasts and lactic acid bacteria (LAB) from tempe. The present research aimed to evaluate the presence, growth dynamics and interactions of moulds, yeasts and bacteria during tempe fermentation in two different home industries in Bogor. The results revealed that two different preparations of tempe fermentation caused different microbial composition. In process applying second boiling step before inoculation, the number of LAB at initial step was much lower than that of applying one boiling step before soaking. The results indicated that LAB has involved since soaking process and they may be carried over into mould fermentation stage. After inoculation, the number of yeasts and moulds significantly increased. The source of yeasts and moulds was considered being mainly the starter culture. High number of LAB at initial step or the significant growth of LAB during the first 12 h seemed to inhibit the growth of moulds, meanwhile the growth of yeasts was not affected. High number of LAB at initial step also correlated with low pH at initial step and this may also contribute to inhibition of the growth of *Enterobacteriaceae* and bacterial spores. The presence of yeasts and LAB up to 7 to 8 log cfu/g during fermentation indicated that those microorganisms contribute to fermentation process of tempe. Different microbial composition also led to difference composition of tempe especially on protein and carbohydrate.

Introduction

Tempe is an Indonesian traditional food made from soybean with its attractive flavor, mixed of fungi and soybean flavor, texture and superior digestibility. The moulds grow during tempe fermentation knit the bean together into a compact sliceable cake. Tempe has become a daily diet of Indonesian people for hundreds of years and even it has also become an exotic food recognised globally. Beside its excellent nutrition quality, tempe has also known as a source of bioactive compound such as antioxidant. The outstanding characteristic of tempe has made tempe becoming more popular not limited to vegetarians. Tempe is considered to be originated from Java. In general, tempe is produced by small scale at home industries with poorly controlled fermentation process that causes the variation of tempe flavors, for example bitter taste often appears in tempe (Barus *et al.*, 2008). To improve the quality of tempe, quality control must be carried out starting from starter culture production throughout tempe fermentation.

Rhizopus oligosporus is the main microorganism plays important role in tempe fermentation process. Besides fungi, other microorganisms may also participate in tempe fermentation process (Barus *et al*., 2008; Seumahu *et al*., 2013) as the fermentation is not carried out under aseptic condition although starter culture is added.

Currently, the diversity of bacteria during tempe fermentation has been extensively studied. During soaking lactic acid bacteria (LAB) and yeasts have been detected (Nuraida *et al.*, 2008; Efriwati *et al.*, 2013). LAB that present in high numbers during soaking, acidify the bean under natural fermentation process (Mulyowidarso *et al*., 1989). Soaking helps controlling microbial populations in tempe. At the end of the soaking process, *Enterobacteriaceae* could not adapt to the acidic condition due to organic acids produced by LAB and it made their numbers decreased (Mulyowidarso *et al.*, 1989), however Seumahu (2012) has detected *Enterobacteriaceae* in

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tempe. Nuraida *et al.* (2008) reported that the LAB increased during moulds fermentation and reached 10^6 cfu/g. The role of the microorganism other than mould during mould fermentation is still needed to be further elucidated. LAB are considered to have a role in controlling pathogenic bacteria during the tempe production. LAB were reported to suppress the growth of pathogenic bacteria in soybean (Ashenafi and Busse, 1991). Study on soybean that was deliberately inoculated with pathogenic bacteria suggests that the presence of *Lactobacillus plantarum* could inhibit the growth of *Listeria monocytogenes* and inhibits staphylococcal toxin production (Nout and Kiers, 2005). Tempe also contains undesirable microorganisms that can reduce its quality. *Bacillus* grows during tempe fermentation and is considered to cause a bitter taste (Barus *et al.*, 2008). The presence of lactic acid bacteria in tempe has a potential to improve quality and safety of tempe.

The presence of yeasts during tempe fermentation has been reported by Efriwati *et al*., (2013) who showed that different methods of tempe fermentation could affect the diversity of yeasts. The interaction between yeasts and LAB can produce stimulatory or inhibitory effects depending on the combination of the species (Alvarez-Martin, 2007). However, the role of yeast in tempe fermentation has to be elaborated.

The different diversity of microorganisms in traditional tempe fermentation will lead to inconsistency of tempe quality. This will cause a problem in the standard compliance and large-scale production of tempe. Study on microbial dynamics during traditional tempe fermentation will help mapping desirable microorganism other than moulds during tempe fermentation. Previously, Mulyowidarso *et al.* (1990) have reported the microbial dynamics during tempe fermentation, however, the fermentation was done under laboratory conditon. This study aimed to evaluate the presence, growth of moulds, yeasts, lactic acid bacteria, bacterial spores and *Enterobacteriaceae*; and their interactions during tempe fermentation in two home industries in Bogor with different production methods. The profile of these microorganisms during tempe fermentation would give idea whether yeasts and LAB have a significant role in tempe fermentation. This information is necessary to develop a multicultural tempe starter that is expected to improve the quality and safety of tempe consistently. During traditional microbial fermentation process, the microbes that appear are possibly inconsistent, causing several data with a high standard deviation.

Materials and Methods

Samples collection

Samples collected included soybeans before being mixed with the starter culture (called *laru*), and soybeans/tempe after the addition of starter culture at 0 (immediately after starter culture inoculation), 12, 24, 48 and 72 hours, commercial starter culture, subcultured starter culture and cassava solid waste. Samples were obtained from two different tempe home industries designed as SDBR and WJB. The selection of tempe home industries was based on the differences in the method of tempe production, i.e. SDBR applied only one boiling step before soaking, while WJB applied two boiling steps, i.e. before and after soaking process. Sampling was conducted in two replicates in the adjacent time.

Microbiological analysis during tempe fermentation

Fifty gram sample taken from different fermentation steps as described above was mixed with 450 ml of 0.1% peptone water and homogenised with a stomacher (BagMixer, Interscience) then the sample mixture was diluted in serial. Microbiological analyses were conducted similarly to analyses of tempe starter culture. One ml sample from appropriate dilution was pipetted into a sterile petri dishes (duplo) and the media was poured to correspond with the microbes that would be enumerated, as follows: Violet Red-Bile Agar (VRBA, Oxoid) for *Enterobacteriaceae*; Man Rogosa and Sharpe Agar (MRSA, Oxoid) supplemented with 0.02% sodium azide (Reina *et al*., 2005) to prevent yeasts growth (Plengvidhya *et al.*, 2007) for LAB and Potato Dextrose Agar (PDA, Oxoid) supplemented with 0.6 g/L of tartaric acid for moulds and yeasts. To calculate bacterial spores, prior to serial dilution, 150 ml of the sample was heated at 80°C for 10 minutes using an autoclave to kill vegetative cells (Harrigan, 1998), and then the sample was enumerated in Nutrient Agar (NA, Merck). Moulds, yeasts and bacterial spores were incubated at 30°C for 48 hours, LAB for 5-7 days and *Enterobacteriaceae* for 24 h.

Microbiological analysis of tempe starter culture and cassava solid waste were also done. Fifty gram samples were diluted with 450 ml of 0.1% peptone water, then followed by serial dilution.

Acidity and chemical analyses

Tempe and soybeans samples that have been homogenised were centrifuged at 3100 rpm for 10 minutes at 4°C. The supernatant pH was measured with a pH meter instrument (Eutech instrument, Singapore). Total titratable acid (TTA) was measured

using a titration method with 0.1 N NaOH and phenolphthalein as indicator (Ko *et al*., 2012). Determinations of moisture, carbohydrate, crude fat, crude protein and total ash of tempe were done using AOAC (1995) method. Total soluble protein was carried out with Lowry method (Lowry *et al*., 1951). Data were analysed using statistical software, Minitab version 16.0 for windows (Minitab, State College, PA, USA). T-test was used to determine the differences for proximate analysis. Level of significance was set at $p < 0.05$.

Results and Discussion

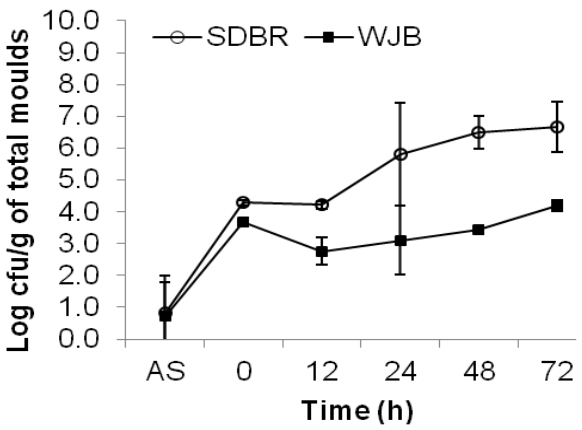
Total moulds and yeasts

Total moulds and yeasts during tempe fermentation in WJB were lower than that of SDBR (Figure 1). Total moulds in this study were lower than the result of Mulyowidarso *et al.* (1990) reported 10^7-10^8 cfu/g moulds in fresh tempe (48 h). Meanwhile, the highest numbers of moulds were at SDBR and WJB tempes of 6.7 log cfu/g and 4.2 log cfu/g respectively at 72 h fermentation. Total moulds at WJB process slightly decreased at the first 12 h.

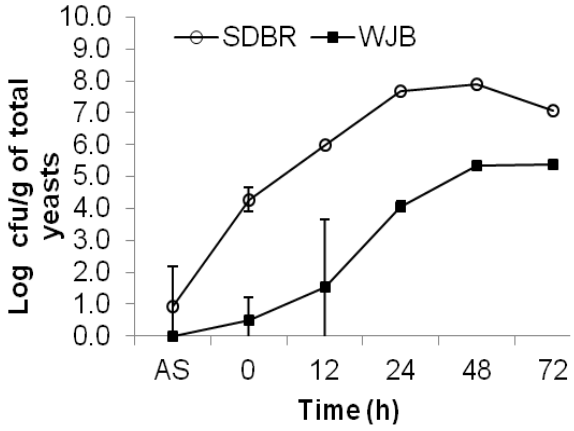
In this study, two tempe producers used different types of starter culture. SDBR used cassava solid waste starter culture obtained by subculturing commercial starter culture in cassava solid waste. Meanwhile WJB used commercial starter culture (the brand name Raprima) without subculturing process. Tempe starter culture with the brand name Raprima was produced commercially by PT Aneka Fermentasi Industri (AFI) (Bandung, Indonesia) and commonly used for tempe fermentation in Indonesia (Seumahu 2012). According to Purwijatiningsih *et al*. (2005), type of mould that present in starter culture Raprima was *R. oligosporus.* Other moulds also present in local starter cultures such as *Aspergillus niger, R. oryzae, Rhizomucor pusillus* and *Mucor rouxii*.

Interaction between moulds and another microorganism in tempe had been mentioned in several studies. The growth of *R. oligosporus* was not influenced by LAB (Feng *et al*., 2005) and yeast (Feng *et al*., 2007). However, the recent research showed that during the first 12 h, the growth of moulds was inhibited. This is probably due to the presence of LAB at high numbers at initial step of SDBR process and the significant growth of LAB during the first 12 h at WJB process. High numbers and significant growth of LAB (Figure 2) seemed not affecting the growth of yeast both at WJB and SDBR (Figure 1b).

At WJB process, during the first 12 h, the number of bacterial spores increased (Figure 3a). This may



(a)



(b)

Figure 1. Changes in total moulds (a) and yeasts (b) during SDBR's and WJB's tempe fermentation. AS: after soaking

also contribute to the inhibition of moulds at the first 12 h. At individual level, spore-forming bacteria could inhibit *R. oligosporus* growth (Ashenafi and Busse, 1991).

After soaking process, yeasts were almost undetectable in SDBR tempe fermentation (0.9 log cfu/g) and undetectable in WJB process. This result was in contrast with the study of Efriwati *et al.* (2013) who found 4 log cfu/g yeasts after soybeans soaking process. Addition of subcultured starter culture containing yeasts of 5.6 log cfu/g in SDBR tempe process increased the yeasts count by 3.4 log cfu/g, however addition of commercial starter culture into WJB process did not increase the yeasts which correlate with the results of microbiological analyses of commercial starter culture in which yeast being undetected. Subculturing of commercial starter culture in cassava solid waste contributed to the presence of yeast at initial fermentation of SDBR. Growth of yeasts in SDBR process ceased after 24 h, while in WJB process continued up to 48 h. Overall, the number of yeasts during SDBR tempe fermentation was higher than WJB yeasts.

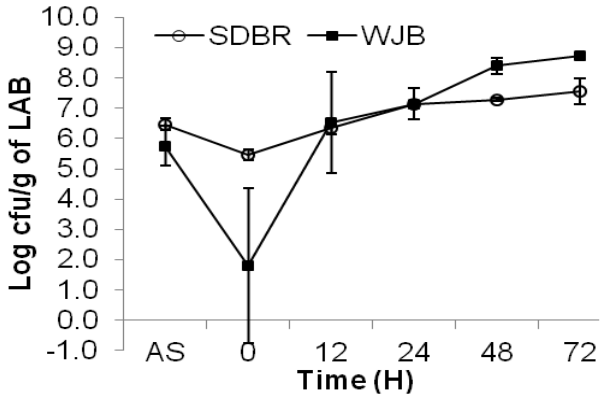


Figure 2. Changes in lactic acid bacteria during SDBR's and WJB's tempe fermentation. AS: after soaking

Lactic acid bacteria

Dehulling and washing process after soaking reduced total LAB in SDBR process by 1 log as shown in the change of LAB after soaking to 0 h (Figure 2). Higher decreased was observed in the WJB process after second boiling step before inoculation as observed at 0 h fermentation. High number of LAB count after soaking indicated that significant growth of LAB occured during soaking process. Previous studies also showed that the number of LAB after soaking amounted to 6 log cfu/g (Efriwati *et al.*, 2013) to 9 log cfu/g (Nuraida *et al*., 2008).

At initial fermentation (0 h), LAB at SDBR process was much higher than WJB process as shown in Figure 2, because there is no heating process before inoculation as explained above. In addition, LAB were present in subcultured starter culture that was used by SDBR process, amounting to LAB 3.9 log cfu/g. Meanwhile, they were absent in commercial starter culture. Subculturing of commercial starter culture to cassava solid waste contributed to the presence of LAB. Cassava solid waste contained LAB at 2.2 log cfu/g. This LAB continued to grow during subculturing tempe starter culture.

During tempe fermentation, the LAB grew and reached the maximum number of more than 7 log cfu/g at 72 h in both processes (Figure 2). Significant increase of LAB was observed in the first 12 h during WJB process. At 72 h fermentation, the number of LAB in WJB's tempe was higher than in SDBR's tempe. The profile of LAB growth during tempe fermentation, suggested that when no boiling process applied before inoculation, the LAB developed during soaking may be carried over and continue to grow during mould fermentation and contribute to the fermentation process. Meanwhile, when boiling step applied, the LAB present during moulds fermentation may come from the environment.

The maximum number of LAB was observed at 72 h fermentation (8.7 log cfu/g in WJB process and

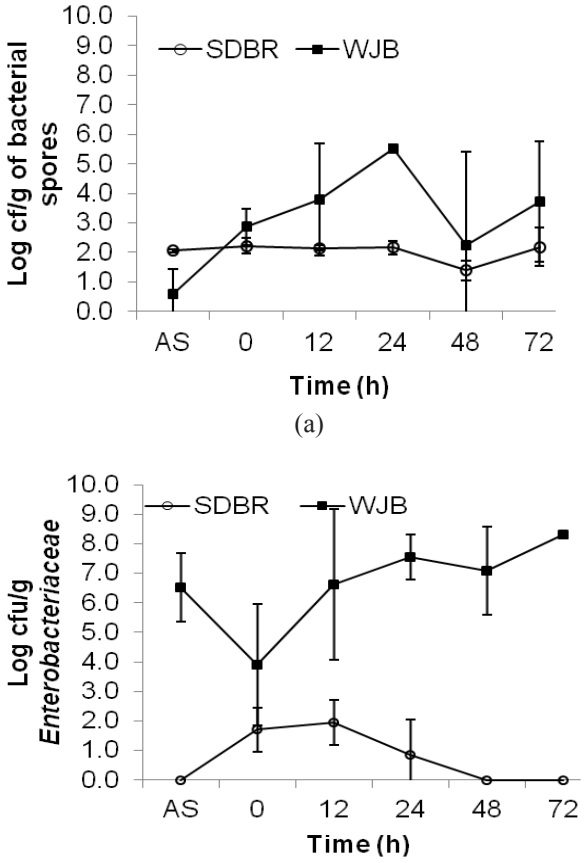


Figure 3. Changes in (a) bacterial spores and (b) *Enterobacteriaceae* during SDBR's and WJB's tempe fermentation. AS: after soaking

7.6 log cfu/g in SDBR process). The data is consistent with those reported by Efriwati *et al.* (2013) stated that the number of LAB in fresh tempe was about 8 log cfu/g.

The presence of high number of LAB during tempe fermentation suggested that there was interaction between mould as main organism for tempe fermentation and yeast that also presence at high number. LAB may obtain nutrients from moulds or yeasts metabolism. LAB could hydrolyse soy protein, but the yield was much less than mould and yeast (Roubos-van den Hil *et al.* (2010). In food fermentation, yeast is known to grow in synergism with LAB, i.e. able to synthesize vitamins, amino acids and purines, also breaks down carbohydrates complex which is essential for the LAB growth (Arroyo-Lopez *et al*., 2008). Meanwhile, yeast could consume lactic acid that produced by LAB (Cheirsilp *et al*., 2003).

On the other hand, high number of LAB at initial fermentation process (0 h) as occurred at SDBR process possibly affected the growth of moulds that was stable during the first 12 h (Figure 1a). Meanwhile, the growth of yeasts was not affected (Figure 1b). Although, at initial step the LAB present

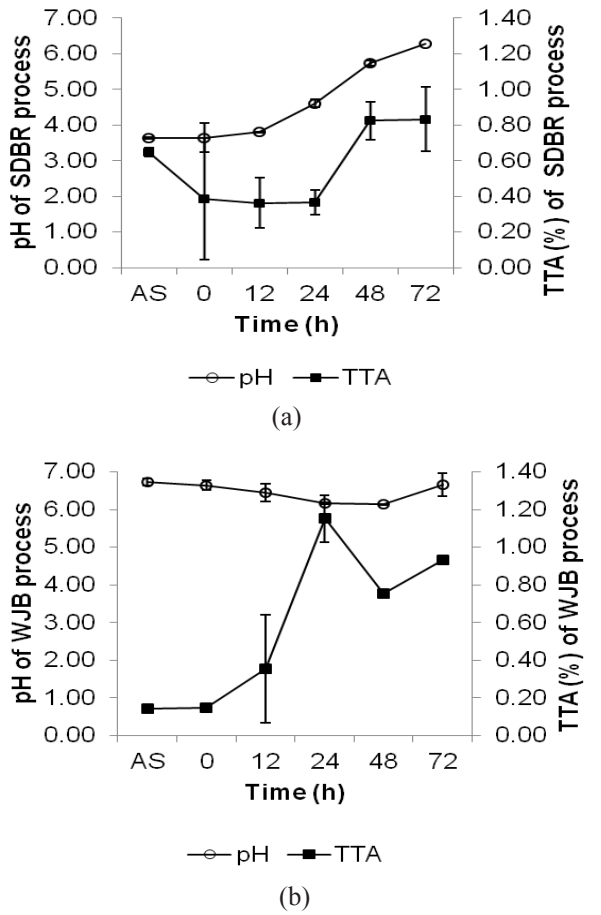


Figure 4. Changes in pH and TTA during (a) SDBR's and (b) WJB's tempe fermentation. AS: after soaking

in low number in WJB process that applied second boiling step before inoculation, but they grew fast during the first 12 h. This negatively correlated with the growth of moulds (Figure 1a). At the first 12 h, the growth of yeasts also slow (Figure 1b). Although there is indication of interaction between yeasts, moulds and LAB during tempe fermentation, however, it should be further studied at individual level.

Total bacterial spores and Enterobacteriaceae

After soaking, the number of bacterial spores of SDBR process was higher than WJB (Figure 3a). The presence of spore-forming bacteria in tempe fermentation could be due to the resistance of these bacteria toward soybeans boiling step (Mulyowidarso *et al.*, 1990). Other source of bacterial spores at present research was also the starter cultures that contain 4.8 log cfu/g. During fermentation, the number of spores remained stable at SDBR process. However, its number was increased after inoculation at WJB process (Figure 3a) with the maximum level of 5.5 log cfu/g at the 24 h. At 48 h, its number decreased to 2.2 log cfu/g, probably caused by the spore germination process, and re-increased by 1.5 log at 72 h which indicates the occurrence of sporulation. The present results indicated the cycle of germination and sporulation may occur during tempe fermentation in WJB.

Bacillus, which is a spore-forming bacteria and commonly found in beans and legumes (Tilak *et al*., 2005), is known to have high proteolytic and saccharolytic activities toward soybeans (Roubos-van den Hil *et al.,* 2010). They were also considered as spoilage microorganisms in tempe since long ago (Ashenafi and Busse, 1991). *Bacillus* sp. has a high protease activity to convert protein into amino acids. These bacteria are considered to cause a bitter taste in tempe. Tempe with higher number of *Bacillus* was considered to have bitter taste (Barus *et al*., 2008).

The number of *Enterobacteriaceae* during WJB tempe fermentation process was higher than SDBR tempe fermentation process (Figure 3b). After soaking, *Enterobacteriaceae* was present in much higher amount at WJB process than at SDBR process. High number of *Enterobacteriaceae* (6.5 log cfu/g) after soaking process indicated that this bacteria was present during soaking of WJB process. Natural acidic fermentation that occurs during soaking has a risk of overgrown by spoilage bacteria. At initial soaking step, when desirable bacteria such as LAB are on the lag phase, spoilage microorganisms grow quickly and compete for nutrients (Botta and Cocolin, 2012). Second boiling step applied at WJB process significantly reduced the number of *Enterobacteriaceae*, however it continued to growth during tempe fermentation and reach maximum at 72 h. Significant growth of *Enterobacteriaceae* observed at the first 12 h of WJB process. Different profile was observed at SDBR process in which increased of Enterobacteriaceae observed after inoculation, however it remained constant from 0 h to 12 h and then decreased to undetected level. Cassava solid waste based starter cultures used by SDBR process contained *Enterobacteriaceae* at 1.6 log cfu/g.

The present results indicated that initial number of LAB affected the growth of undesirable bacteria in the fermentation process. The difference between initial number of LAB (after inoculation, 0 h) between SDBR and WJB process might affect the growth of bacterial spore (Figure 3a) and *Enterobacteriaceae* (Figure 3b). At 0 h, SDBR process had a higher number of LAB (5.5 log cfu/g) and also higher acidity (TTA 0.39%, pH 3.64) than those of WJB process (TTA 0.15%, pH 6.65). This condition was considered to suppress the growth of *Enterobacteriaceae* and bacterial spore at SDBR process (Figure 3a and 3b) during fermentation. In contrast, those undesirable bacteria grew well during WJB process having lower

initial number of LAB and higher initial pH (Figure 4 b). Seumahu (2012) found that *Enterobacteriaceae* was dominant in tempe when no domination of LAB. In tempe dominated by LAB, tempe was also dominated by *Acetobacter* and other non-pathogenic bacteria.

Low acid at 0 h (TTA 0.15%, pH 6.65) at WJB soybean was the ideal condition for *Enterobacteriaceae* and bacterial spore in WJB process. This was not the case in SDBR soybean with lower pH (3.64) and higher TTA (0.39%). The increase of TTA at 24-48 h (Figure 4a) at SDBR process was considered causing slight reduction of bacterial spore (Figure 3a) and significant reduction of *Enterobacteriaceae* (Figure 3b). The increase of TTA at 12-48 h at WJB process was also considered causing significant reduction of bacterial spore and slight reduction of *Enterobacteriaceae* at 48 h (Figure 3a and 3b).

Acidity and chemical composition

Different pattern of pH and TTA (total titrated acid) changes were observed during WJB and SDBR fermentation process (Figure 4). After soaking process, the bean of SDBR process was more acid (TTA 0.65%, pH 3.63) than that of WJB (TTA 0.14%, pH 6.73). During SDBR fermentation process from after soaking stage to 72 h, pH gradually increased from 3.63 to 6.27, while its TTA remained increased from 0.65% to 0.82% at 48 h. After 48 h, the TTA remained constant. In contrast, during fermentation process of WJB, the pH slightly decreased from 6.73 to 6.65 and the TTA gradually increased from 0.14% to 0.93%. The high number of LAB at initial fermentation process of SDBR process and low number of LAB at WJB process may contribute to the different pattern of pH and TTA changes.

The increase in pH during tempe fermentation could be generated by proteolytic activity of moulds and *Bacillus* that able to produce amino acids and contribute to the increase in tempe pH (Roubos-van den Hil *et al*., 2010). The present results suggested that different process of soybean preparation for tempe fermentation affected the type of microorganisms present and their growth dynamics and lead to different chemical composition of tempe such as pH, moisture, ash, fat and carbohydrate which significantly different between SDBR's tempe and WJB's tempe (Table 1). However, different process did not affect significantly ($p < 0.05$) protein and soluble protein content.

Higher number of moulds in SDBR tempe (Figure 1a) might cause higher moisture content (68.69%) and lower carbohydrate (16.38%) of SDBR tempe.

Table 1. Chemical composition of SDBR's and WJB's tempe

Parameters	Wet basis		Dry basis	
	SDBR	WJB	SDBR	WJB
Proximate (%)				
Moisture	68.69±3.74[a]	61.31±1.18[b]		
Ash	0.62±0.07	1.02±0.05	1.98±0.07[a]	2.64±0.09[b]
Protein	21.00±3.38	24.33±0.10	66.81±2.81[a]	62.92±1.73[a]
Fat	4.58±0.11	4.57±0.34	14.83±2.11[a]	11.8±0.55[b]
Carbohydrate	5.11±0.42	8.77±0.73	16.38±0.81[a]	22.64±1.21[b]
Soluble protein (%)	0.02±0.00[a]	0.02±0.00[a]		
pH	5.74±0.08[a]	6.14±0.01[b]		
TTA	0.82±0.11[a]	0.76±0.01[a]		

*Mean ± standard deviation n = 4. Values in a column followed by a common letter are not significantly different at 5% level, as assessed by t-test

Mould respiration could contribute to the production of H_2O during fermentation. Carbohydrases of *R. oligosporus* help the mould metabolising carbohydrates in soybean (Nout and Kiers, 2005).

Conclusions

During fermentation process, LAB and yeast gradually grew up to 72 h fermentation, while moulds reach maximum after 48 h. The presence of LAB and yeasts were quite high at 7-8 log cfu/g which showed that both groups of microorganisms may play a significant role in tempe fermentation process. Environment and different preparation of tempe fermentation affected the microbial profile and load. At the begining of fermentation process, the number of LAB in SDBR process was higher than in WJB process. High numbers of LAB correlates to the growth of *Enterobacteriaceae* and bacterial spores. The presence of high number of LAB at initial tempe fermentation or significant growth of LAB during the first 12 h may correlate with the inhibition of moulds at the first 12 h, but not affected the growth of yeasts. There is also indication that the presence of undesirable bacteria such as spores forming bacteria and *Enterobacteriaceae* may inhibit the growth of moulds especially at initial step of fermentation. The difference in diversity of microorganisms and their count between the two home industries has caused different changes in pH and total acid during fermentation and affected chemical composition of tempe.

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