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FULL PAPER

The changes in the chemical and microbiological characteristics of lamtoro (*Leucaena leucocephala*) tempe from Pacitan with usar inoculum during continued fermentation

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Abstract

In addition to soybean, *Leucaena leucocephala* is one of the tempe raw materials usually used in Indonesia. These tempe is only made by peoples in a few areas in the southern part of Java such as Wonogiri, Gunungkidul and Pacitan. For some purposes, such as cooking ingredients, tempe is deliberately fermented longer than usual to obtain a savory taste. In a previous study, we had studied leucaena tempe or people known as lamtoro tempe that process in Wonogiri and reported that there had been a change in characteristic during fermentation until over-fermented tempe. Therefore, in this research, we analyzed the change in the chemical and microbiological characteristic of lamtoro tempe from Pacitan during continued fermentation. This research showed that during the fermentation process, mold, yeast, and lactic acid bacteria (LAB) persisted for up to 126 hrs of fermentation time. The growth of yeast and mold tend to decrease while LAB continued to increase until it reached 11 log CFU/mL. In addition, the moisture content, ash content, soluble protein levels, pH and titrated acid increased during continued fermentation time. Glutamic acid and aspartic acid were the highest amino acids by weight percentage. Both amino acids have an important role in the sensory characteristics of lamtoro tempe.

1. Introduction

Tempe is a traditional fermented food from Indonesia. Commonly tempe is made from dehulled cooked soybeans inoculated with certain mold spores or traditional starter culture called *laru* or *usar*. Other beans have been used to produce tempe in several regions, especially in Java. *Koro benguk* is the second choice of beans for tempe. Winged beans, mung beans, jack beans, and cowpeas are also used for tempe (Djaafar *et al*., 2019). This kind of tempe is popular in Central Java. Tempe may be made from *lamtoro* seeds in the southern part of Java. *Lamtoro* tempe producers exist in the Gunungkidul, Wonogiri, and Pacitan regions in Indonesia. Those tempe producers use a traditional starter (*usar)* and different preparation methods to produce tempe. Pacitan tempe producers use the *usar* from *senggani* leaves (*Melastoma candidum*), and Wonogiri tempe producers use the *usar* from teak leaves (*Tectona gandis*). Tempe inoculum can be obtained from the leaves used to wrap tempe that has been covered with tempe fungal mycelia. The fungal mycelia were then taken and dried under the sun (Wipradnyadewi *et al*., 2005).

Instead of the difference in the starter, there is some difference in tempe processing including the step of pretreatment process and packaging after inoculation. The pretreatment process of lamtoro tempe in Pacitan is different from the tempe produced in Wonogiri. In Pacitan. In Pacitan, the boiled seed was washed gently while peeling the hull without removing it. Meanwhile, tempe producers in Wonogiri peel the hull and remove it during washing the seed. So that producers in Pacitan use all parts of the seed unlike in Wonogiri which only uses the cotyledon part of the seeds. Furthermore, the packaging process also varies in each region. The *lamtoro* tempe in Pacitan is wrapped using two pieces of banana stalks joined together, then covered with *senggani* leaves. Meanwhile, tempe in Wonogiri is wrapped using teak leaves. There very few discussions on the making of traditional *lamtoro* tempe using *usar* from Pacitan. Research on *lamtoro* tempe was reported by Nursiwi (2018). Komari (1999) used commercial

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inoculum (Raprima), and Nursiwi (2019) used the traditional inoculum and processing method from Wonogiri.

Tanuwidjaja *et al*. (1991) mentioned that variations in ingredients, mixed materials, and inoculum utilizations, as well as the process of making tempe, greatly affect the quality of tempe. The characteristics of the tempe were influenced by more than the *usar*. The fermentation time caused changes in microbiological, chemical, and sensory characteristics in *lamtoro* tempe. Tempe would be over-fermented if stored for a certain time. The change from fresh to over-fermented tempe happened due to the microbiological change. The numbers of molds, yeasts, and lactic acid bacteria changed during tempe fermentation (Nurdini *et al*., 2015; Nursiwi, 2019).

Javanese people often used over-fermented tempe (or also called as "*tempe bosok*") as a cuisine or flavor enhancer. Over-fermented tempe is still widely consumed today. However, many people are reluctant to consume over-fermented tempe, so it is necessary to study the nutritional content of the over-fermented tempe. The results of previous studies explained that there were changes in various components during the tempe fermentation process. Research on Pacitan *lamtoro* tempe (*Leucaena leucocephala*) is required to examine the changes in the chemical and microbiological characteristics and the change in amino acid makeup of the Pacitan *lamtoro* tempe (*Leucaena leucocephala*) made with the usar inoculum. These changes need to be observed during advanced fermentation to determine the characteristic changes of the *lamtoro* tempe that had been allowed to ferment over time (0, 42, 54, 66, 78, 90, 102, 114 and 126 hrs).

2. Materials and methods

2.1 Materials

Ripe *lamtoro* seeds (*Leucaena leucocephala*) and traditional *usar* inoculum were obtained from a *lamtoro* tempe producer in Pacitan District, East Java Province, Indonesia. Chemicals used were Lowry A solution, Lowry B solution, CuSO4, NaK tartaric, tartaric acid 0.1%, chloramphenicol, $CaCO_3$, HCl, and distilled water. All chemicals were pro analysis grade. Media for mold enumeration was potato dextrose agar (PDA), for yeast enumeration was malt extract agar (MEA), and for lactic acid bacteria enumeration was de Man Rogosa Sharpe Agar (MRSA). All media were supplied by Merck, Darmstadt, Germany.

2.2 Lamtoro tempe processing

A total of 200 g of riped *lamtoro* seed were boiled for 3 hrs with the addition of 0.29% (w/w) of wooden ash obtained from burning residue. Afterwards, the boiled seeds were washed gently while peeled the hull then soaked for 48 hrs. Every 24 hrs the soaked seeds were washed while changed the water. The process was followed by steaming the seeds for 1 hr and letting the mixture cool to room temperature. *Usar* inoculum (55%) was added to the *lamtoro* seeds, and the mixture was wrapped and incubated at 28±1°C for 42 hrs to produce fresh *lamtoro* tempe. Fermentation continued for 54, 66, 78, 90, 102, 114, and 126 hrs.

2.3 Chemistry analysis

Water and ash content analysis was performed using the AOAC (2005) method. Soluble protein was measured using Lowry *et al*. (1951). The pH was measured using a pH meter (Institute of Medicine, 2003). Acid was titrated using the percentage of lactic acid (Misgiyarta and Widowati, 2002). The amino acid was assessed by UPLC Waters H Class with a photodiode array detector (PDA) (Rohman and Gandjar, 2007; Waters, 2012).

2.4 Microbial analysis

The samples were taken immediately after lamtoro seed has been inoculated with laru (0 hr) and every 12 hrs during continued fermentation begin from 42^{nd} hrs fermentation until 138^{th} hrs. Samples were collected from 3 times lamtoro tempe production. Approximately 1 g of each sample was crushed and homogenized in 9 mL of 0.85% NaCl solution then each sample was serially diluted using sterile 0.85% NaCl solution.

For lactic acid bacteria (LAB) enumeration, 1 mL of suspension from 10^{-5}- 10^{-7} dilution was pour-plated with de Man Ragosa Sharpe Agar (Merck) supplemented with 1% $CaCO_3$ and 0.02 ppm sodium azide then incubated 37°C for 48 hrs (Nudyanto dan Zubaidah, 2015). While for yeast enumeration, 0.1 mL suspension from 10^{-2}-10^{-4} dilution was spread-plated on Malt Extract Agar (Merck) supplemented with 200 ppm chloramphenicol and 0.5% calcium propionate then incubated 28°C for 48 hrs (Ebabhi *et al*., 2013). Whereas mold enumeration was conducted by spread plating 0.1 mL suspension from 10^{-1} - 10^{-3} dilution on Peptone Dextrose Agar (Merck) supplemented with 200 ppm chloramphenicol and 0.1% tartaric acid then incubated 30°C for 48 hrs (Da Silva *et al.,* 2013). All plating for microbial analysis was done in two replicates and the total number of LAB, yeast and mold colonies were expressed as the number of colonies per mL (CFU/mL) sample.

2.5 Statistical analysis

SPSS Version 25 software was used as the tool for statistical analysis. Data obtained were analyzed using one-way ANOVA with a significance level of 5%. A further analysis was carried out using Duncan's multiple range test if there was a significant difference.

3. Results and discussion

3.1 Microbial change

Figure 1 shows that mold, yeast, and lactic acid bacteria (LAB) increased during the fermentation of fresh *lamtoro* tempe. The mold increased to 5.86 log CFU/mL, and the appearance of hyphae was perfect and very compact. In this period, mold that entered the log phase was able to adapt well to its growth media (Purwoko, 2007). The mold was relatively constant between 54 and 78 hrs of fermentation, began to decline at 90 hrs and was still visible at 126 hrs fermentation time. Mold in this research was more resistant than the mold in the *lamtoro* tempe inoculated using commercial inoculum. Nursiwi (2019) stated that the growth of mold was undetected at 108 hrs fermentation of the *lamtoro* tempe from Wonogiri. This decrease was related to the pH data shown in Figure 1. The pH range was still in the optimum condition for the mold to grow (around pH 5.00 –6.00). According to Pelczar and Chan (2008), the optimum pH for the mold's growth was 3.8–5.6.

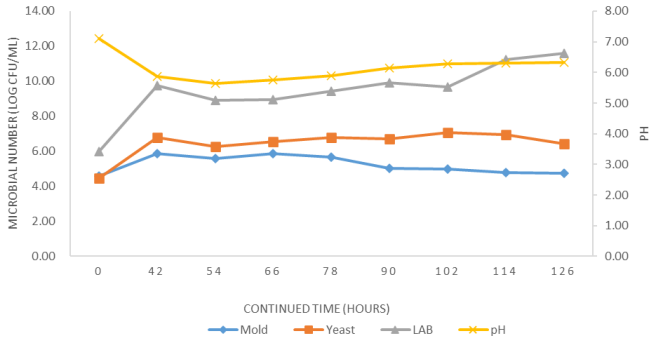

Figure 1. Lactic Acid Bacteria (LAB), mold, yeast, and pH in *lamtoro* tempe from Pacitan during continued fermentation

The yeast growth at 42 hrs was quite high (6.81 log CFU/mL) and higher than the mold, which was only 5.86 log CFU/mL at the same fermentation time. The high yeast growth may be caused by the use of the traditional inoculum *usar,* which already contained yeast. The same results were found in the research conducted by Efriwati *et al*. (2013), which stated that the yeast growth in soybean tempe fermentation increased significantly from 3 log CFU/mL to 6.85 log CFU/mL. The yeast was more abundant than the mold. At 126 hrs fermentation, the yeast still appeared in *lamtoro* tempe from Pacitan, while at 96 h, the yeast disappeared in tempe from Wonogiri (Nursiwi, 2019). This phenomenon was caused by the pH level of the *lamtoro* tempe in this research, which is optimum for yeast growth.

In the ready-to-incubate seed mixture, the LAB was detected at 5.97 log CFU/mL. After being fermented for 42 hrs, the amount of LAB increased significantly to 9.77 log CFU/mL and kept rising until 126 hrs fermentation, when it was found to be 11.59 log CFU/mL. LAB growth during continued fermentation of *lamtoro* tempe was also reported by Nursiwi (2019). In addition, Nurdini (2015) mentioned that such a significant increase was presumed to be caused by the LAB's ability to use nutrients from fungi and yeast metabolites. The high LAB growth could be attributed to the total titrated acid levels, which can be seen in Figure 1. Meanwhile, the titrated acid level continued to increase during continued fermentation. Moreno *et al*. (2002) mentioned that the increase in the concentration of lactic acid in soybean tempe was caused by the growth of LAB that continued to increase to a density of 9 log CFU/mL.

3.2 pH and titrated acid

The acidity level (pH) is an important factor that affected tempe fermentation. The fermentation process is influenced by the pH value, and there is also a correlation between fungal growth and an increase in pH value (Rizal *et al*., 2020). It can be seen in Figure 1 that the pH value of *lamtoro* tempe tends to increase during continued fermentation, even though the increase was relatively small. Mold, LAB, and other bacteria are proteolytic, and both hydrolyze protein into amino acids and can form ammonia (NH_3). The higher the ammonia, the higher the pH level (Muzdalifah *et al*., 2016). A similar result was reported by Tahir *et al*. (2018), which found the pH of soybean tempe after 26 hrs fermentation increased from 4.5 to 6.0. Meanwhile, during the 48 hrs of fermentation, the pH of the *lamtoro* tempe continued to increase to 7.5. Andriati *et al*. (2018) found that the pH of jack bean tempe had the same level as the *lamtoro* tempe (pH 6.18) at the beginning of the fermentation and increased at 60 h fermentation to 7.76.

Table 1 shows that the longer the fermentation time, the higher the value of the titrated acid. This value was closely related to the total number of lactic acid bacteria, which continued to increase. The highest titrated acid value of *lamtoro* tempe was found at the 54 hrs fermentation time (Table 1), similar to the trend of soy tempe mentioned by Steven (2016). LAB growth may have entered the logarithmic phase when glucose breakdown occurred and resulted in an increase of titrated acid.

3.3 Chemical change

Table 1. Chemical change of *lamtoro* tempe in Pacitan during continued fermentation

Sample	Fermentation Time (Hrs)	Moisture Content (%wb)*	Ash Content (%db)*	Soluble Protein levels (%db)*	Titratable Acidity (%)*
Ready to Incubate Seeds	0	62.33±2.76^{a}	2.25±0.13^{a}	0.04±0.01^{a}	0.10±0.00^{a}
Tempeh	42	64.48±0.96^{ab}	2.44±0.18^{a}	0.19±0.01^{b}	0.18±0.02^{b}
	54	66.01±1.63^{b}	2.42±0.57^{a}	0.41±0.40^{c}	0.57±0.05^{e}
	66	66.39±1.92^{b}	2.48±0.82^{ab}	0.51±0.02^{d}	0.37±0.06^{c}
	78	66.70±2.20^{b}	2.68±0.72^{ab}	0.58±0.02^{e}	0.42±0.07^{dc}
	90	70.25±2.98^{c}	2.76±0.41^{ab}	0.61±0.04^{e}	0.51±0.07^{de}
	102	69.96±0.65^{c}	2.89±0.41^{ab}	0.66±0.03^{f}	0.71±0.14^{fg}
	114	70.00±2.53^{c}	3.12±0.36^{b}	0.69±0.05^{f}	0.62±0.11^{ef}
	126	71.72±0.82^{c}	3.78±0.52^{c}	0.69±0.05^{f}	0.81±0.02^{g}

Values are expressed as mean±SD. Values with different superscripts within the column are significantly different ($\alpha < 0.05$).

Fermentation time was one of the factors causing the microbiological and chemical changes in *lamtoro* tempe. As with soy tempe, fresh *lamtoro* tempe also has a short shelf life and would immediately over-ferment during storage. The fermentation process, including continued fermentation during storage, caused changes in chemical components from complex forms to simpler compound forms.

Water is one of the results of the metabolic process that greatly affected other components, including the growth of microorganisms that have an essential role in tempe fermentation. The water content of the *lamtoro* seeds just before incubation and during fermentation could be seen in Table 1. The *lamtoro* seeds ready for incubation contained 62.33% moisture, which increased significantly to 71.72% at the end of fermentation. Fresh *lamtoro* tempe fermented for 42 hrs contained 64.48% water, similar to fresh soybean tempe that has been fermented for 24 hrs (62.38%) as reported by (Tahir *et al*., 2018). During tempe fermentation, microbes produced water, carbon dioxide and ATP during their metabolic process. The *Rhizopus* mold breaks the matrix between the bacterial cells on the third day of storage when the tempe became soft, and then the cells in the tempe were destroyed by the water from the breakdown of carbohydrates. This physical change is one of the factors that makes the over-fermented tempe soft and juicy.

The ash content of *lamtoro* continued to increase along with the increasing fermentation time (Table 1). The increase in ash content was thought to be caused by the vitamins formed by bacteria that continued to grow during the tempe fermentation, especially vitamin B12 (Omosebi and Otunola, 2013). Astuti *et al*. (2000) mentioned that the amount of vitamin B complex increased during the fermentation process, except for thiamine. Vitamin B12 contained nitrogen and a cobalt (Co) atom. Cobalt is similar to the iron-bound in hemoglobin or the magnesium bound in chlorophyll.

Table 1 shows that soluble protein levels continued to increase along with the increasing fermentation time from 0.04% in seeds ready for incubation to 0.69% in *lamtoro* tempe incubated for 126 hrs. Such an increase was attributed to the activity of the protease enzyme produced by the mold during its growth. This enzyme breaks down complex proteins into simpler protein compounds by breaking the peptide bonds. The chain-breaking caused the protein to dissolve easily, so that soluble protein levels increased (Arianingrum *et al*., 2007)

3.4 Amino acids composition

Lamtoro seeds contained several essential amino acids, such as arginine, cysteine, alanine glutamic acid, isoleucine, leucine, and lysine (Benjakul *et al*., 2014). In this research, other amino acids, such as serine, valine, glycine, threonine, aspartic acid, tyrosine, proline, and histidine, were found in the *lamtoro* tempe produced in Pacitan District (Table 2). Amino acid levels of *lamtoro* tempe changed during the fermentation process and continued to change during the fermentation of fresh tempe to over-fermented *lamtoro* tempe.

During further fermentation, there was a change in the content of 15 amino acids. The amount of L-valine, L -alanine, glycine, L-lysine, and L-threonine was fluctuated and tend to increase, otherwise, the amount of L-serine, L-glutamic acid, L-phenylalanine, L-isoleucine, L-arginine, L-aspartic acid, L-leucine, L-tyrosine, L-proline, and L-histidine tend to decrease. During continued fermentation, L-glutamic acid had the highest reduction (29%). However, at the end of continued fermentation (126 hrs), its content was the highest among other amino acids, followed by L-aspartic acid. A similar result was reported by Handoyo and Morita (2007), in which the amount of glycine increased gradually during the over-fermentation of soybean tempe. Meanwhile, the levels of non-essential amino acids, especially alanine, aspartic acid, cysteine, proline,

Table 2. Amino acid Composition (% dw) of *lamtoro* tempe in Pacitan during continued fermentation

Amino acid	0 hrs (Ready to Incubate)	42 hrs (Fresh Tempe)	54 hrs	66 hrs	78 hrs	90 hrs	102 hrs	114 hrs	126 hrs
L-Serine	1.33	1.13	1.07	1.15	1.05	0.98	1.00	0.92	1.14
L- Glutamic acid	2.81	2.29	2.46	2.35	2.21	2.03	2.43	2.11	1.98
L-Phenylalanine	1.73	1.57	1.21	1.44	1.08	1.25	1.26	1.19	1.48
L-Isoleucine	0.80	0.84	0.79	0.88	0.72	0.74	0.87	0.67	0.71
L-Valine	0.99	1.16	1.05	1.12	0.99	1.11	1.18	1.03	1.12
L-Alanine	1.04	1.28	1.44	1.38	1.42	1.57	1.68	1.54	1.64
L-Arginine	2.61	1.53	1.53	1.40	1.26	0.87	1.00	0.94	1.22
Glycine	1.33	1.31	1.36	1.34	1.35	1.33	1.33	1.23	1.61
L-Lysine	0.88	0.89	1.05	0.97	1.05	0.78	1.03	1.01	0.97
L-Aspartic acid	1.96	1.71	1.97	1.93	1.95	1.94	2.02	1.84	1.93
L-Leucine	1.51	1.47	1.37	1.50	1.18	1.22	1.43	1.12	1.20
L-Tyrosine	1.05	1.05	0.89	0.98	0.78	0.86	0.90	0.92	1.04
L-Proline	0.88	0.79	0.75	0.78	0.68	0.69	0.76	0.64	0.69
L-Threonine	0.90	0.93	0.91	1.01	0.84	0.87	0.94	0.84	1.02
L-Histidine	0.91	0.82	0.67	0.76	0.58	0.51	0.76	0.54	0.64

and serine, increased during fermentation. The levels of other amino acids, such as glutamic acid and asparagine fluctuated during fermentation but showed the highest amounts at 72 hrs of fermentation. The decrease in the amount of glutamic acid after 102nd hrs fermentation can be affected by the decrease in the number of mold colonies. In addition, glutamic acid and other amino acids can be utilized by lactic acid bacteria, which show an increase in the number of colonies until the end of fermentation (Figure 1). Kawai *et al*. (2007) and Tseng *et al*. (2005) explained that glutamic acid was important for the umami (savory) taste. The use of over-fermented *lamtoro* tempe from Pacitan as a flavoring agent was supported by this amino acid profile.

4. Conclusion

In this research, the continued fermentation of the *lamtoro* tempe from Pacitan affected its microbiological and chemical characteristics. Continued fermentation up to 126 hrs triggered the increase of pH, mold, yeast, and LAB. Changes in chemical characteristics such as water, ash, and dissolved protein content also increased every 12 hrs during continued fermentation. Of the 15 types of amino acids in the over fermented *lamtoro* tempe, the levels of L-valine, L-alanine, glycine, L-lysine, L-threonine acids increased. Meanwhile, the levels of L-serin, L-glutamic acid, L-phenylalanine, L-isoleucine, L-arginine, L-aspartic acid, L-leucine, L-tyrosine, L-proline, and L-histidine decreased. The amino acids L-glutamic acid and L-aspartic acids were the highest by weight percentage in both fresh and over-fermented *lamtoro* tempe.

Conflict of interest

The authors declare no conflict of interest.

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