

Changes to the chemical and microbiological characteristics of *Leucaena leucocephala* seeds during tempeh fermentation in Pacitan, East Java

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Abstract

Lamtoro tempeh is a traditional Indonesian fermented food made from *lamtoro* (*Leucaena leucocephala*). In Pacitan, it is usually fermented using *usar* (a traditional tempeh inoculum made from *senggani* leaves). The fermentation process takes 42 hrs and during this period, there are changes in the chemical and microbiological characteristics of the *lamtoro* seeds. To study these changes, samples were collected and tested for chemical characteristics (water content, ash content, dissolved protein, pH, and total acid titration) and microbiological characteristics (the number of fungi, yeast, and lactic acid bacteria) every 6 hrs during the 42 hrs fermentation. During fermentation, the water content increased significantly in the initial 6 hrs of fermentation and then tended to stabilize until the 42-hour. The ash content increased, while the dissolved protein content increased from the 0-hour to the 36-hour of fermentation but dropped at the 42-hour. The pH level of the *lamtoro* tempeh decreased from 0-hour until 30-hour and then increased until the end of the fermentation period. However, the level of total acid titration increased during the *lamtoro* tempeh fermentation. The growth of fungi, yeast and lactic acid bacteria initially declined but then gradually increased until the end of fermentation (42-hour). The fermentation time was found to affect both the number of microbes and the chemical characteristics of the *lamtoro* tempeh from the Pacitan area.

1. Introduction

Tempeh, or tempe in Indonesian, is a traditional Indonesian fermented food (Stephanie *et al.*, 2019). It is generally made from fermented soybeans using *Rhizopus sp.* but can also be produced from other substrates, such as beans, corn, rice, lentils, and barley (Vital *et al.*, 2018). Besides, one of the wide varieties of legumes from which tempeh can be made is *lamtoro* seeds (*Leucaena leucocephala*) (Andriati *et al.*, 2018). Fermenting leguminous seeds has several advantages, including reducing non-nutritional factors, improving nutrient digestibility, reducing allergenicity, activating antioxidant activity, increasing the concentration of phenolic compounds, and decreasing the risk of chronic disease (Vital, 2018). Tempeh fermentation produces nutritious and healthy food products that are easy to digest (Nout and Kiers, 2005); it is known for being rich in fiber, isoflavones, vitamin B-12, protein, calcium, and folic acid, and can be easily digested due to the simpler form of protein following the fermentation process

(Babu *et al.*, 2009). Moreover, tempeh is useful for inhibiting the formation of the blood vessels of cancer cells and improving bone health, and is also antihypertensive and acts as an anti-bacterial agent and active antioxidant. During the fermentation of soybean tempeh, it changes its proximate content; for example, there is an increase in water content and dissolved protein, and a decrease in total fat content and total protein content (Chalid *et al.*, 2019).

Soybean tempeh originated on Java Island and subsequently spread across Indonesia; however, the use of *lamtoro* seed for making tempeh is still limited to a few places in the south of Java Island, such as the Pacitan region in East Java. The producers of *lamtoro* tempeh in Pacitan are generally located at high elevations and mountainous areas; one of these is Mbah Tukiyem, who is a micro-scale producer. Mbah Tukiyem makes *lamtoro* tempeh by boiling ripe *lamtoro* seeds, before grinding, rinsing, soaking, and steaming them. A traditional inoculum, *laru*, is used for the inoculation

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process. Traditional *laru* originated from Pacitan and is made from dry *senggani* leaves, which are mixed with mashed cassava flour. The *lamtoro* seeds are covered in this mixture before being wrapped in banana stalks covered with fresh *senggani* leaves and the inoculated seeds are left to incubate for about 42 hrs.

The characteristics of *lamtoro* tempeh are influenced by the various processes of tempeh production, the inoculum used, and fermentation time. *Waru*, teak, and *senggani* leaves are all used to wrap the tempeh, dried tempeh, *Rhizopus oligosporus* isolate, and commercial tempeh inoculums such as “Raprima” are among the inoculums used in tempeh production (Hidayat *et al.*, 2006). The fermentation time has been reported to affect the total protein, dissolved protein, total phenol, and crude fiber content of *lamtoro* tempeh made using commercial inoculum (Nursiwi *et al.*, 2018). Similarly, the fermentation time also affects trypsin inhibitor activity, mimosine, phytic acid, and the tannin level in *lamtoro* seeds fermented using the traditional inoculums *laru* and *usar* (Slamet and Komari, 1991). Changes have also been noted in microorganisms during soybean fermentation in tempeh production (Nurdini *et al.*, 2015). However, no studies have yet determined what microbiological and chemical changes take place during the fermentation of *lamtoro* tempeh from Pacitan using traditional inoculums; therefore, this research was conducted to determine the effect of fermentation time on the chemical and microbiological characteristics of *lamtoro* seeds during fresh Pacitan *lamtoro* tempeh processing.

2. Materials and methods

2.1 Materials

Ripe *lamtoro* seeds (*Leucaena leucocephala*) and a *usar* inoculum which utilized *senggani* leaves were obtained from the micro-scale tempeh producer “Mbah Tukiyem”, located in Pacitan, East Java. The materials used for the chemical and microbiological analysis were Lowry solution A, Lowry solution B, CuSO₄, NaK tartrate, potato dextrose agar, tartaric acid, malt extract agar, chloramphenicol, de Man Rogosa, and Sharpe agar, and CaCO₃. All materials were supplied by Merck, Darmstadt, Germany.

2.2 Lamtoro tempeh processing

Lamtoro tempeh processing was conducted according to the standard operating procedure of the micro-scale tempeh producer Mbah Tukiyem, from Pacitan, East Java. Approximately 200 g of ripe *lamtoro* seeds were boiled in water containing wood ash for 3 hrs. The seeds were then ground, rinsed, and soaked for

24 hrs, before being ground again and rinsed three times. This process was repeated once. Next, the seeds were steamed for 1 hr and left to stand until they reached room temperature. Finally, the *lamtoro* seeds were mixed with 55% *usar* inoculum, wrapped in paper-plastic laminate, and incubated at 28±1°C. The inoculated *lamtoro* seeds were observed at 0, 6, 12, 18, 24, 30, 36, and 42 hrs.

2.3 Chemical analysis

The chemical characteristic under observation were water and ash content (AOAC, 2005), soluble protein content (Lowry *et al.*, 1951), pH (Institute of Medicine, 2003), and total acid titration counted as lactic acid (Aristya *et al.*, 2013).

2.4 Microbial analysis

Every 6-hour of fermentation, the inoculated *lamtoro* seed was sampled for lactic acid bacteria, yeast, and mold enumeration. 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 sample was pour plating on *de Man Rogosa Sharpe Agar* (Merck) supplemented with 1 % CaCO₃ and 0.02 ppm sodium azide then incubated 37°C for 48 hrs (Nudyanto dan Zubaidah, 2015). For yeast enumeration, 0.1 mL sample was spread plating 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). For mold enumeration, 0.1 mL sample was spread plating 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). The total number of LAB, yeast and mold colonies was expressed as the number of colonies per mL (CFU/mL) sample.

2.5 Statistical analysis

Statistical analysis was performed using SPSS Version 25 software. The chemical and microbial data were analyzed using one-way ANOVA with a significance level of 5%, followed by Duncan’s multiple range test if a significant difference had been identified.

3. Results and discussion

3.1 Chemical changes

3.1.1 Water content

Water content tended to increase during the fermentation of *lamtoro* tempeh, as shown in Table 1. The water content of the *lamtoro* seeds at the 0-hour was

Table 1. Chemical characteristics of Pacitan *Lamtoro* (*Leucaena leucocephala*) tempeh during fermentation

Sample	Fermentation Time (hour)	Water Content (% wb)	Ash Content (% db)	Dissolved Protein Content (% db)
<i>Lamtoro Tempeh</i>	0	62.33±2.76 ^a	2.25±0.13 ^a	0.04±0.01 ^a
	6	65.40±2.08 ^b	2.44±0.12 ^{ab}	0.20±0.01 ^d
	12	63.85±1.79 ^{ab}	2.33±0.16 ^{ab}	0.14±0.01 ^b
	18	64.05±1.23 ^{ab}	2.52±0.16 ^b	0.22±0.01
	24	64.06±2.39 ^{ab}	2.42±0.17 ^{ab}	0.22±0.01 ^c
	30	64.74±1.89 ^{ab}	2.41±0.17 ^{ab}	0.31±0.01 ^e
	36	64.55±1.49 ^{ab}	2.47±0.21 ^b	0.26±0.01 ^f
	42	64.48±0.96 ^{ab}	2.44±0.18 ^{ab}	0.19±0.01 ^c

Values are expressed as mean±SD. Values with different superscript in the same column are significantly different at 0.05 significance level. db, dry basis; wb, wet basis

62.33% (wet basis) and increased to 64.48% (wet basis) by the end of the fermentation period. An increase in water content during tempeh fermentation has been previously reported by Chalid *et al.* (2019) and Tahir *et al.* (2018) in soybean tempeh. Fermentation time is one of the most important factors in the increase in water content (Qomariyah and Deny, 2016); the longer the fermentation time, the higher the water content of the tempeh. This occurs due to the metabolic process and breakdown of macromolecular compounds into simpler compounds during fermentation. According to Steinkraus (1995), during the tempeh fermentation process, water synthesis occurs as a result of the breakdown of carbohydrates by microorganisms (molds).

3.1.2 Ash content

Table 1 shows that the ash content slightly increased during the *lamtoro* tempeh fermentation process; at the 0-hour of fermentation, the *lamtoro* seeds contained 2.25% (dry basis) ash, which increased to 2.44% (dry basis) by the 42-hour. This trend of an ash increase during fermentation was also observed in soybean and white bean tempeh, fermented using a stock culture of *Rhizopus oligosporus* in a rice-flour matrix and incubated for 30 hrs (Vital *et al.*, 2018). However, Chalid *et al.* (2019) found that the ash content in fermented soybean tempeh incubated for 24 hrs decreased from 4.95% (dry basis) to 1.64% (dry basis).

3.1.3 Dissolved protein

Table 1 shows a change in the dissolved protein content during the *lamtoro* tempeh fermentation, increasing from 0.04% (dry basis) at the 0-hour to 0.26% at the 36-hour before falling to 0.19% (dry basis) at the 42-hour. The rising dissolved protein content in the tempeh was caused by the *Rhizopus* sp., which produced protease (Hsiao *et al.*, 2014), an enzyme that catalyzes the hydrolysis of peptide bonds into amino acids (López-Otín and Bond, 2008). *Rhizopus oryzae* triggered proteolytic activity, breaking down protein into amino

acids and short-chain polypeptide (Cempaka *et al.*, 2018; Nout and Kiers, 2005). The increased dissolved protein content during the fermentation of jack bean tempeh was accompanied by a decrease in peptide bonds by protease microbes (Andriati *et al.*, 2018). A study by Chalid *et al.* (2019) also reported an increase in dissolved protein from 75.19 part per million (ppm) to 236.31 ppm during the fermentation of soybean tempeh using the commercial inoculum "Raprima" with 48 hrs' incubation. An increase in the dissolved protein content was also found during the fermentation of *lamtoro* seeds using a stock culture of *Rhizopus oryzae* for 48 hrs (Komari, 1999). In addition, Nursiwi *et al.* (2018) found that dissolved protein rose during *lamtoro* tempeh fermentation using the commercial inoculum "Raprima" as a starter. However, protein content could also decrease, as *Rhizopus* sp. mold uses some amino acids to gain nitrogen, triggering their growth in the fermentation process (Astuti *et al.*, 2000).

3.1.4 pH and total acid titration changes

The pH value fluctuated during the *lamtoro* tempeh fermentation but generally decreased (Table 2); the pH at the 0-hour was 7.11 and dropped to 5.91 by the 42-hour. The pH was also found to decrease during the fermentation process of soybean tempeh, from 6.73 at the 0-hour to 6.65 at the 48-hour (Nurdini *et al.*, 2015), and jack bean tempeh, from 6.34 at the 0-hour to 6.16 at the 36-hour (Andriati *et al.*, 2018). The pH drop was due to the growth of LAB and mold during the tempeh fermentation. LAB produces lactic acid, which decreases the pH of the tempeh and inhibits the growth of rotting microorganisms (Feng, 2005). In contrast, the increase in pH during the fermentation of soybean tempeh fermented using the traditional starter culture *laru* is presumed to be caused by the proteolytic activity of the molds, which can produce amino acids, contributing to a rise in pH (Nurdini *et al.*, 2015). Komari (1999) reported an increase in the pH level from 5.25 to 6.03 during the 48-hour fermentation of *lamtoro* tempeh, using a stock

Table 2. pH and total acid titration of Pacitan *Lamtoro* (*Leucaena leucocephala*) tempeh during fermentation

Sample	Fermentation Time (hour)	pH	Total Acid Titration (% as lactic acid)
<i>Lamtoro Tempeh</i>	0	7.11±0.04 ^f	0.10±0.01 ^a
	6	6.35±0.03 ^c	0.13±0.01 ^b
	12	5.70±0.04 ^c	0.15±0.03 ^{ab}
	18	5.58±0.03 ^a	0.17±0.03 ^{ab}
	24	5.70±0.07 ^c	0.15±0.03 ^{ab}
	30	5.64±0.08 ^{ab}	0.19±0.06 ^b
	36	5.67±0.05 ^{bc}	0.18±0.06 ^b
	42	5.91±0.05 ^d	0.18±0.03 ^b

Values are expressed as mean±SD. Values with different superscript in the same column are significantly different at 0.05 significance level.

culture of *Rhizopus oryzae* as the starter inoculum. Fermentation is also known to cause changes in several organic acids, such as acetic acid, oxalic acid, citric acid, and succinic acid, which affect pH, as observed in soybean tempeh fermentation using a *Rhizopus* starter (Mo et al., 2013).

The pattern in pH change during the fermentation of Pacitan *lamtoro* tempeh slightly differed from that of the total acid titration change, which tended to increase throughout the fermentation period, from 0.10% at the 0-hour to 0.18% at the 42-hour (Table 2). A similar pattern was observed by Komari (1999) during *lamtoro* tempeh fermentation using a stock culture of *Rhizopus oryzae* and soybean tempeh fermentation using a traditional inoculum, *laru* (Nurdini et al., 2015). The rise in total acid titration occurred as the LAB produced lactic acid, which is LAB's main metabolite produced in the carbohydrate metabolism; lactic acid could lower the pH of the substrate, increasing the total acidity as well as being antibacterial (Feng et al., 2005).

3.2 Microbiological changes

Many factors, such as substrate, pH value, and temperature, can affect microbe activity (Dewi et al., 2004). Table 3 shows the mold, yeast, and LAB growth during the *lamtoro* tempeh fermentation. Mold and yeast number dropped in the first 6 hrs before increasing, from

hour 30 for mold and hour 12 for yeast, until the end of the fermentation period. A study by Chalid et al. (2019) on the fermentation of soybean tempeh reported that *Rhizopus sp.* number increased initially at the 18-hour and continued to increase the next hrs, reaching optimum growth at the 24-hour of fermentation. Nurdini et al. (2015) reported a similar trend in mold growth in soybean tempeh produced by two home industries; following an initial decrease, there was an increase at hrs 24 and 48 of fermentation. In contrast, yeast numbers increased constantly until hour 48 of fermentation. *Rhizopus oryzae* can grow in environments with a pH ranging from 4–9, but its optimal growth is at a pH of 3.4–5.5 (Hernandez et al., 2017). Compared to bacteria and mold, yeast can grow better at a range of pH levels and generally demonstrates optimal growth in conditions with a pH of 4–4.5 (Kurtzman and Jack, 1998). The pH level of the *lamtoro* tempeh in this research was intended to be ideal for both mold and yeast growth (Table 2). Generally, the longer the fermentation, the better the mold growth; this was characterized by mycelia growth that covered the surface of the tempeh, creating a compact texture.

Meanwhile, the LAB number increased until the 18-hour, decreased at the 24-hour, and then remained stable until the 42-hour of fermentation (Table 3). According to Bagenda et al. (2008), LAB *Pediococcus pentosaceus*

Table 2. pH and total acid titration of Pacitan *Lamtoro* (*Leucaena leucocephala*) tempeh during fermentation

Sample	Fermentation Time (hour)	Mold (log CFU/mL)	Yeast (log CFU/mL)	LAB (log CFU/mL)
<i>Lamtoro Tempeh</i>	0	4.60±0.17 ^b	4.48±0.05 ^b	5.97±0.22 ^a
	6	3.53±0.09 ^a	3.85±0.17 ^a	6.18±0.17 ^a
	12	3.79±0.16 ^a	4.59±0.09 ^b	8.84±0.02 ^b
	18	3.69±0.07 ^a	5.44±0.01 ^c	10.00±0.18 ^c
	24	3.70±0.07 ^a	5.90±0.18 ^d	9.80±0.06 ^c
	30	4.75±0.07 ^b	6.04±0.01 ^d	9.82±0.19 ^c
	36	5.79±0.05 ^c	6.46±0.03 ^c	9.91±0.05 ^c
	42	5.86±0.14 ^c	6.81±0.34 ^c	9.77±0.01 ^c

Values are expressed as mean±SD. Values with different superscript in the same column are significantly different at 0.05 significance level. LAB, lactic acid bacteria.

grows optimally in an environment with a pH ranging from 5–6 and can tolerate a pH of 4.5–7.5. This kind of environment was found in the *lamtoro* tempeh fermentation (Table 2). LAB in tempeh can grow with little or even no oxygen. Therefore, the relatively few pores on the tempeh wrapper would trigger the increase of microbes, especially LAB. LAB and yeast played an essential role in improving the quality of the tempeh produced (Kustyawati, 2009).

4. Conclusion

In this study, fermentation time was found to affect the microbiological and chemical characteristics of *lamtoro* seeds during the fermentation of *lamtoro* tempeh inoculated with a traditional inoculum, *usar*, from Pacitan. During the 42-hour fermentation of *lamtoro* tempeh, an increase was observed in water, ash, dissolved protein, total acid titration, mold, yeast, and LAB, while pH was found to decline. Further research on the effect of fermentation time for *lamtoro* tempeh on the level of mimosine, a toxic compound present in *lamtoro* seeds, is necessary to ensure the right fermentation time to produce *lamtoro* tempeh which is safe for consumption.

Conflict of interest

The authors declare no conflict of interest.

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