Changes in Chemical and Sensory Characteristics of Gunungkidul’s Lamtoro (*Leucaena Leucocephala*) Tempeh during Extended Fermentation

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**Abstract.** Lamtoro tempeh is a fermented food in Java, made from lamtoro (*Leucaena leucocephala*) seeds. Its extended fermentation creates a unique flavor and taste that can be used as a seasoning. Therefore, this study aims to investigate the chemical (moisture, ash, soluble protein, and amino acids contents) and sensory characteristics (brownish color, ammonia aroma, umami taste, and overall acceptability) of lamtoro tempeh from Gunungkidul, Yogyakarta, during extended fermentation for up to 120 hours. The chemical and sensory characteristics were analyzed every 12 and 24 hours respectively. From multiple comparison tests, tempeh with the longest fermentation had the strongest sensory characteristics. Other results showed that extended fermentation increased moisture content up to the highest amount of 74.6% (wb) at 96 hours and 3.55% ash content (db) at 60 hours, as well as soluble protein from 3.02% to 22.62% (db) at the end. Moreover, glutamic acid decreased during fermentation and aspartic acid had the highest amount afterwards. Investigating microbial changes in the food’s unique characteristics during this process is required.

1. Introduction

Tempeh is very popular in Indonesia and is classified as a fermented food, frequently consumed not only as a side dish but also as a snack. Furthermore, its characteristics are influenced by the microbial activity of mold, yeast, lactic acid bacteria (LAB), and gram-negative bacteria during fermentation. Mold has a major role in tempeh formation, by producing hyphae for binding the seeds to form a compact structure [1]. Hence, this food is rich in nutritional value such as high protein content (37.38%), vitamin B12, and isoflavone compound. Also, during fermentation, the digestibility and protein absorption increase [2,3].

Commonly, it is made from soybeans and is easy to find in the country’s entire markets. However, regions such as Gunungkidul, Trenggalek, Pacitan, and Wonogiri have lamtoro tempeh, made from ripe lamtoro seeds [4,5]. This food uses a traditional inoculum called usar obtained from teak (*Tectona grandis*) leaf, which is initially used to wrap fresh soybeans tempeh and incubated until the molds are sporulated. The leaves’ underneath has a smooth surface that sticks to the mold’s mycelia and is then dried under the sun for utilization. Wonogiri’s lamtoro tempeh fermented for 36 hours has 34.39% (db)
total protein and 7.28% (db) soluble protein [4]. In other studies, 36 hours fermented lamtoro gung tempeh contains 62.1% moisture and 0.65% ash contents [6].

Tempeh fermentation is generally processed for 36 hours with an ambient temperature conditioned at 29°C-32°C or 25°C for 48-72 hours to obtain a product with preferable sensory characteristics and ripeness [3,7]. During that time, there is also a nutritional content change such as carbohydrate and fat decrease but an increase in total and dissolved proteins [8]. However, when this takes longer, the food becomes overripe or over fermented and is classified as semangit (overripe) and bosok (over fermented). The difference between the two lies in the length of fermentation time. Semangit is 2-3 days longer while bosok is 4-5 days [9], and they have a distinctive texture, taste, and odor than fresh tempeh because mold, yeast, and LAB play a role during the process [10]. In over fermented tempeh, Kluyveromyces marxianus is one of the predominant yeasts, while Lactobacillus and Lactococcus are predominant bacteria [11].

Due to microbial activity, the over fermented tempeh have a brown color, less attractive shape, and pungent odor caused by ammonia which leads to an increased pH value [12]. Other chemical characteristics such as carbohydrates and fats decrease due to the activity of lipolytic and carbohydrase enzymes [7]. During fermentation, total and soluble proteins increase [4] as well as the free amino acid content one of which is glutamic acid that gives an umami taste. Furthermore, glutamic acid levels were around 14.5% in soybean tempeh after more than 72 hours [9]. The high intensity of glutamic and aspartic acids indicates the strong umami taste [10].

Tempeh’s texture is changed by extended fermentation, where the fresh one fermented for 2 days has a compact texture, but that of 5 – 8 days more looks tender. Over fermented tempeh, especially that of soybean, is widely used in Javanese cuisine as a food seasoning, like sambal goreng, sambal tum pang, and menyeng/menjeng, because they have distinctive taste and aroma [13]. There are only few discussions on this food, therefore this study aims to investigate the chemical characteristics of Gunungkidul’s over fermented lamtoro tempeh, as well as the sensory characteristics of fresh and over fermented ones in different fermentation times.

2. Material and Methods

2.1. Materials

The materials were ripe lamtoro seeds obtained from the local market, and the traditional inoculum called laru from tempeh producer in Gunungkidul, Yogyakarta.

2.2. Tempeh Making Process

Tempeh’s preparation followed the traditional method used in Gunungkidul. Lamtoro seeds were boiled for one hour, then it was dehulled manually and washed a few times. They were later soaked for 20 hours and the water was changed every eight hours. The seeds were steamed for one hour and they were allowed to cool to room temperature, then the processed seeds were inoculated with laru (17.5% w/w), stirred well, and packed in brown rice paper to form tempeh. This was subjected to extended fermentation with periods of 0, 48, 60, 72, 84, 96, 108, 120, and 132 h (specific for sensory analysis) at 25-30°C.

2.3. Analysis of Chemical Characteristics

The moisture and ash contents were analysed using the thermogravimetric and dry ashing methods respectively [29]. Total soluble protein was determined using the Lowry method [14], while the amino acid was by UPLC Waters H Class with Photodiode Array Detector (PDA) [15]. Briefly, 0.1-1.0 g sample weight was put in 20 ml vial space to be hydrolyzed by HCl. Afterward, hydrolysate was diluted in 50 ml volumetric flask and filtration was completed using 0.2 μm syringe filter. Furthermore, AccQ.Tag Ultra (Aquabidest) was used in a mobile phase. UPLC determination used analytical column C18 te (set at 49°C), gradient pump system, and a PDA detector. Also, 1 μl volume was injected and monitored at 260 nm wavelength.
2.4. Sensory Analysis of Over fermented Lamtoro Tempeh

Over fermented tempeh’s sensory analysis was conducted using 30 untrained panelists and employing a 9-point multiple comparison test [30]. This item was mashed using mortar to make a paste sample that was served to them for assessment. The total of four paste samples (60, 84, 108, and 132 h of fermentation) were evaluated by comparing each to the control (36 hours fresh tempeh) and scored for four attributes, namely brownish color, ammonia odor, umami taste, and overall acceptability. Previously, 30 untrained panelists were introduced to the standard of ammonia odor using 1% (v/v) ammonia solution and umami taste using 10% (w/v) MSG to identify what these properties look like in over fermented tempeh.

2.5. Statistical Analysis

Moisture, ash, and soluble protein were evaluated statistically in three replicate samples and two replicate analysis. Also, the sensory analysis used ANOVA, followed by Duncan Multiple Range Test (DMRT) with the significance level of p < 0.05. Data were analyzed using IBM SPSS Statistic and presented as mean ± standard deviation.

3. Results and Discussion

3.1. Chemical Characteristic

By fermentation, some of the food’s nutritional content may change because of the microbial activity in it. Also, macromolecules such as carbohydrates, proteins, and fats decrease due to degradation by microbes into low molecular weight like fatty acids, peptides, and monosaccharides [16,17].

Table 1. Chemical Characteristics of Gunungkidul’s Over Fermented Lamtoro Tempeh

| Fermentation time (hours) | Moisture content (% wb) | Ash content (% db) | Soluble protein content (% db) |
|--------------------------|-------------------------|-------------------|-------------------------------|
| 0                        | 67.91 ± 0.99a           | 2.59 ± 0.13a       | 3.02 ± 0.22a                  |
| 48                       | 72.08 ± 0.24b           | 3.20 ± 0.48b       | 14.70 ± 0.25b                 |
| 60                       | 71.94 ± 0.34b           | 3.55 ± 0.16b       | 15.37 ± 0.47bc                |
| 72                       | 72.58 ± 0.50b           | 3.45 ± 0.34b       | 15.77 ± 0.64c                 |
| 84                       | 73.57 ± 0.35c           | 3.10 ± 0.20b       | 16.66 ± 0.49d                 |
| 96                       | 74.67 ± 0.47d           | 3.53 ± 0.11b       | 17.91 ± 0.27c                 |
| 108                      | 74.49 ± 0.32d           | 3.45 ± 0.21b       | 20.19 ± 0.42c                 |
| 120                      | 74.38 ± 0.22d           | 3.39 ± 0.30b       | 22.62 ± 0.61d                 |

Mean ± standard deviation n = 3. Values in a column followed by a common letter are not significantly different at the 5% level, as assessed by DMRT

The result (Table 1) showed that the initial moisture content was 67.91 % wb. During fermentation, the value increased significantly up to 74.38% wb. Related to other studies, 36 hours fermented lamtoro gung tempeh already had 62.113% moisture content [6], while that of 48 hours increased by approximately 5 %, possibly because during this period, the Rhizopus sp. was in a log phase to produce hyphae [1]. The microbes’ metabolic activity caused moisture content escalation. During the fermentation in aerobic conditions, microbes’ respiration convert glucose into energy, CO2 and water [31]. Besides, it was also reinforced by bacteria that tend to have a log phase when the process was extended [18].

During fermentation, the ash increased significantly from 2.59 % at 0 hour to 3.20 % at 48 hours, but was not significantly different until 72 hours. It was indicated that over fermented lamtoro tempeh powder contained ash 3.69 – 4.13 % [19] and the total refers to the dominated minerals content (iron, calcium, and cuprum). Most of them were found as a complex with protein or another organic compound. [1]. Whenever protein broke down into a small size or anti-nutrient, compounds degraded and minerals were released [20]. Soluble protein’s level increased significantly from 3.02 % at 0 hour and ended up 22.62 % at 120 hours. Comparing to another result before fermentation, this content increased from 3.92 % to 7.29 %
in Wonogiri’s *lamtoro* tempeh [5]. Similar to other studies, throughout 48 hours fermentation of fava bean tempeh, the soluble protein content increased [21]. During the fermentation process that occured due to protein macromolecules degradation into lower masses like peptides and amino acids that are soluble in water through microbial protease enzyme, the output was used as energy supplies [4] [22]. The predominant proteolytic microbes are generally Rhizopus oligosporus. However, in fermented tempeh, mold’s presence decreases and protein breakdown continues because of proteolysis by several yeast species [23].

3.2. **Amino Acid Composition**
Fermented food was kindly identified as essential and non-essential amino acids’ source. Several free amino acids have different characteristics and are responsible for shaping food’s taste. Each has its properties, such as glutamic and aspartic acids, which gives an umami taste because of being an MSG-like component [24].

**Table 2.** Gunungkidul’s Over Fermented *Lamtoro* Tempeh Amino Acid Composition

| Amino Acid (%) db | 0   | 48  | 60  | 72  | 84  | 96  | 108 | 120 |
|-------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| L - Histidine     | 1.14| 1.03| 1.06| 0.97| 0.90| 1.09| 0.73| 0.85|
| L - Serine        | 1.97| 1.35| 1.47| 1.39| 1.51| 1.36| 1.29| 1.28|
| L - Arginine      | 3.15| 1.21| 1.30| 1.20| 1.21| 0.99| 0.94| 0.97|
| L - Glycine       | 1.74| 1.44| 1.48| 1.60| 1.50| 1.52| 1.45| 1.40|
| L - Aspartic Acid | 3.10| 1.90| 1.97| 2.02| 2.02| 2.14| 2.31| 2.14|
| L - Glutamic Acid | 5.03| 2.43| 2.42| 2.27| 2.16| 2.06| 2.03| 1.99|
| L - Threonine     | 1.28| 0.92| 0.93| 0.92| 0.93| 0.89| 0.79| 0.82|
| L - Alanine       | 1.55| 1.04| 1.08| 1.05| 1.06| 1.09| 1.05| 1.02|
| L - Proline       | 1.41| 0.85| 0.82| 0.81| 0.72| 0.79| 0.77| 0.68|
| L - Lysine        | 1.88| 0.96| 1.17| 1.15| 1.17| 1.05| 1.15| 1.03|
| L - Tyrosine      | 1.35| 1.32| 1.39| 1.07| 1.21| 1.04| 0.81| 1.10|
| L - Valine        | 1.47| 1.35| 1.29| 1.37| 1.29| 1.30| 1.22| 1.09|
| L - Isoleucine    | 1.28| 0.79| 0.73| 0.76| 0.70| 0.73| 0.67| 0.59|
| L - Leucine       | 2.33| 1.48| 1.41| 1.47| 1.35| 1.36| 1.23| 1.06|
| L - Phenylalanine | 2.10| 2.11| 2.14| 1.90| 2.04| 1.73| 1.34| 1.71|

**Fig. 1** Glutamic and Aspartic Acids Content
Table 2 showed that in Gunungkidul's over fermented lamtoro tempeh, the amount of glutamic and aspartic acids is higher than other amino acids during fermentation. Moreover, Fig 1. showed that the glutamic acid before fermentation (0 hour) compared to that of fresh tempeh (at 48 hours) was highly decreased from 5.03 to 2.43 %. Thereafter, it slightly reduced from 0.01 – 0.15 % db in each period during extended fermentation. The soybean tempeh cake study similarly indicated that this component decreased from 12.8 in fresh tempeh to 12.57% in semangit [14]. Conversely, another study reported that glutamic acid in soybean tempeh increased from 140.0 mg/100 g after 48 hours to 147.0 mg/100 g after 72 hours fermentation [9]. The reduction was caused by the hydrolyzed protein that is probably used immediately to build microbes biomass, especially yeast and LAB metabolism during the extended period. But, the protease production by mold was slower because it entered the mature phase. The result was unbalanced between protein hydrolysis and available amino acids [9,11]. Also, aspartic acid increased from 48 hours fermentation and reached approximately 2.31% at 108 hours. Meanwhile, at 96 hours, it had 2.14% compared to glutamic acid of 2.06% (Fig. 1). The inequality in each amino acid number is induced by the difference in the free amino acid balance produced by autolysis and microbial activity [25]. Several studies reported that glutamic and aspartic acids are the highest in over fermented tempeh [19]. The reduction in amino acids allows degradation into ammonia, and it was reported that the decrease after 24 hours of fermentation lasted until there was an extension [26]. Moreover, the bitter taste also urged in a high level as umami taste often found in over fermented soybean tempeh [10], and it was influenced by some amino acids such as arginine, histidine, isoleucine, leucine, methionine, phenylalanine, valine, and tyrosine [24].

3.3. Sensory Analysis of Over fermented Lamtoro Tempeh
The sensory analysis fermentation time was completed at 60, 84, 108, and 132 hours. Fresh lamtoro tempeh purchased from a traditional market in Solo was used as a control against which the panelists compared and scored these samples.

Table 3 Sensory Characteristics Scores of Gunungkidul’s Lamtoro Tempeh during Extended Fermentation

| Fermentation Time (hours) | Brownish Colour | Ammonia aroma | Umami taste | Overall |
|--------------------------|-----------------|---------------|-------------|---------|
| 60                       | 5.37a           | 5.27a         | 5.33a       | 5.13a   |
| 84                       | 2.97b           | 3.00b         | 4.20b       | 3.57b   |
| 108                      | 2.23c           | 2.07c         | 2.97c       | 2.47c   |
| 132                      | 1.43d           | 1.63c         | 2.30d       | 1.90d   |

Different letter in the same column shows a significant difference at α = 0.05.
Score: 1= very strong; 2= strong; 3= rather strong; 4= slightly strong; 5= neither strong nor weak; 6= slightly weak; 7= rather weak; 8= weak; 9= very weak (all scores compared with control) [30]

Table 3 showed that the extended fermentation process affected tempeh’s stronger properties and perhaps its flavor increased. From the score, the 132 hours fermented sample had more brownish color, stink aroma, and umami taste. Due to the highest intensity of aspartic and glutamic acids as umami taste’s contributor, Gunungkidul’s lamtoro tempeh had the potential to be processed as a seasoning. Furthermore, lamtoro’s over fermented tempeh is more preferable to be used as a seasoning rather than that of soybean [19]. The darker color was associated with a longer fermentation period while matured mold impacted lower enzyme production [27]. Moreover, ammonia aroma was determined by amino acid and ammonia compound’s released. The over fermented items are no longer acceptable due to the most non-desirable properties (aroma) that could affect consumer acceptance for further processed products [19].
4. Conclusion

It can be concluded that during extended fermentation, the moisture content increased significantly as well as the soluble protein, while the ash did not change. However, glutamic and aspartic acids were dominated in the entire fermentation time. Lamtoro tempeh produced from extended fermentation had stronger intensity of brownish color, ammonia, umami taste, and overall sensory acceptability.

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