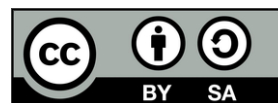




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Research Article

Effect of pasteurization process and fermentation time on physico-chemical properties, antioxidant capacity and total phenolic content of tempeh-based soy sauce

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Abstract

Tempeh can be processed into soy sauce to enhance its functional value. The sauce must go through a pasteurization process that may impact its qualities. Therefore, this study aims to determine the effect of pasteurization (70°C for 15 min) and fermentation time (up to 4 d) on the physico-chemical properties, antioxidant capacity and total phenolic content of tempeh-based soy sauce. The soy sauce was produced through moromi fermentation lasting up to 8 weeks, with tempeh fermentation periods 2, 3 and 4 d tested alongside koji as the control. Results indicated that pasteurization and fermentation time significantly increased the antioxidant capacity (EC_{50}) of tempeh-based soy sauce up to 1.91 ± 0.18 mg soy sauce/mL ($p < 0.05$). Total phenolic content significantly decreased to 897 ± 3 μ g gallic acid equivalent.mL⁻¹ after pasteurization, while the fermentation time significantly increased total phenolic content ($p < 0.05$). The total dissolved solids and total soluble protein increased with the fermentation time ($p < 0.05$). The L*, a*, b* and the total soluble protein decreased after pasteurization ($p < 0.05$). Consequently, fermentation time positively affected the physico-chemical characteristics, total phenolic content and antioxidant capacity of tempeh-based soy sauce, while pasteurization increased the antioxidant capacity and darkened the color of the soy sauce but decreased total phenolic content.

Keywords

food processing, moromi fermentation, antioxidants, soybean, tempeh

Abbreviations

ANOVA – analysis of variance; DPPH – 2,2-diphenyl-1-picrylhydrazyl; EC_{50} – half maximal effective concentration; KCl – potassium chloride; NaCl – sodium chloride; Na_2CO_3 – sodium carbonate; non-GMO – non-genetically modified organism; SA – scavenging activity; SD – standard deviation; TDS – total dissolved solids; UV-Vis – ultraviolet-visible spectroscopy

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Introduction

In recent years, plenty of methods and technologies have been developed to extend the shelf life of food products. Extending the shelf life of food product is essential as it can preserve their functional characteristics. One of the functional foods which are highly perishable is tempeh, an indigenous traditional fermented food from Indonesia. Tempeh has been consumed for a long time because of its health benefits, affordability, and sustainability. Additionally, tempeh is recognized for its presence of phenolic compounds (especially isoflavones) which function as antioxidants to combat oxidative stress (Banjarnahor and Artanti 2014; Atthailah et al. 2019). According to Rizal et al. (2022), fungi will generate protease, lipase, and amylase enzymes in the soybean fermentation, breaking down complex compounds into simpler compounds. Therefore, tempeh can be digested, absorbed, and utilized by the human body more effectively compared to regular soybeans. Regrettably, tempeh has a short shelf life of 36-48 h at room temperature, thus requiring alternative method to be considered for storing tempeh longer (Kustyawati et al. 2020). Prior study has explored methods to extend the shelf life of tempeh by up to 3 years by processing it into soy sauce (Zahidah and Lo 2022).

Soy sauce (*shoyu* in Japanese) is a commonly used seasoning in dishes from East and Southeast Asian regions and is quite popular in Western countries as well. Its distinctive sensory characteristics can enhance the overall savory taste of food because it has an umami and salty taste with a unique aroma (Diez-Simon et al. 2020). Converting tempeh into soy sauce is easily comprehensible for the public. To make soy sauce known as moromi fermentation, tempeh simply requires soaking in a brine solution with 18-20% salt. During fermentation, *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* were found to be the main microorganisms present in the moromi fermentation. Moreover, moromi fermentation is mainly driven by lactic acid bacteria and yeast, which multiply rapidly at the beginning of moromi fermentation. Moromi fermentation naturally takes place for a maximum of one year (Devanti and Gkatzionis 2019; Rosida 2013).

Following the moromi fermentation, soy sauce must go through a pasteurization process in order to

develop its flavor, eliminate all living microorganisms, deactivate enzymes, remove any residue, enhance the color with melanoidin, and dissolve antifungal agents (Rosida 2013). Nonetheless, phenolic compounds may decrease while pasteurized. This impact can be attributed to several reactions, such as the degradation, oxidation, or polymerization of phenolic compounds and the generation of complexes with proteins and carbohydrates during pasteurization. Thus, the antioxidant capacity may decrease because of pasteurization (Magshoudlou et al. 2019).

Tempeh fermentation time and moromi fermentation time are thought to affect the antioxidant capacity of soy sauce. Lo et al. (2022) have reported that tempeh fermentation time could impact the phenolic compounds and antioxidant capacity. Another study indicated that water-soluble phenolic compounds in tempeh rose as fermentation time increased from 0 to 60 h (Atthailah et al. 2019). Additionally, Zahidah and Lo (2022) have discussed the physico-chemical properties of soy sauce made from tempeh. Nevertheless, studies reporting the effects of pasteurization and fermentation time on the antioxidant capacity of soy sauce are limited, especially studies of soy sauce made from tempeh. This study used tempeh soy sauce from 3 different tempeh fermentation times (2, 3, and 4 days) to compare its antioxidant capacity and physico-chemical properties. Moreover, the analysis of antioxidant capacity, total phenolic, and total flavonoid content were done for up to 8 weeks of moromi fermentation. Therefore, this study aims to determine the effect of pasteurization and fermentation time on the physico-chemical properties, antioxidant capacity and phenolic compounds of soy sauce made from tempeh.

Materials and Methods

Sample preparation. Sample preparation is segmented into two parts: tempeh fermentation and moromi fermentation. Tempeh fermentation was done according to Surya et al. Kyai H. Syahdan No. 9

(2021) with slight modifications. Non-GMO (USA) soybeans were acquired from a reliable online shop in Jakarta, Indonesia. The process of preparing tempeh started with cleaning and soaking soybeans

for 24 h using water at room temperature (25°C), boiling for 30 min using water with the addition of 1% lactic acid, cooling, adding *Rhizopus oryzae* starter from a local store (2 g.kg⁻¹ soybeans), then wrapped in a perforated plastic bag and left fermented in room temperature (25°C) for 2, 3, and 4 days. Koji fermentation on soybean was carried out as a control treatment. Soybeans that had been soaked were cooked in water for 30 minutes, and then a koji starter (*Aspergillus oryzae*) was added (2 g.kg⁻¹ soybeans) to the sterilized tray. Koji fermentation was done at room temperature (25°C) for six days and stirred daily to lower the temperature. Next, the moromi fermentation process involved soaking 500 g of tempeh in a brine solution (15% NaCl and 5% KCl in 1 L water), then allowing it to spontaneously ferment for two months at room temperature (25°C). Finally, the jar opening was covered with cheesecloth to prevent contamination. The solution of moromi fermentation was taken by filtering using cheesecloth and then pasteurized at 70°C for 15 min.

Physico-chemical properties. Color analysis was performed using mobile phones' "Color Meter" application with CIELab matrices. The measurement was done by bringing the sample close to the "Color Meter" camera. Color measurements were carried out periodically every week during the soy sauce fermentation period. Total dissolved solids (TDS) analysis was done using an ATAGO Digital Pocket Refractometer PAL-COFFEE (BS/TDS). Each sample is dripped using a pipette on a refractometer prism then the instrument will display the TDS of each sample. Total soluble protein was estimated by measuring absorbance at 280 nm and calculated based on L-tyrosine (0-200 µg.mL⁻¹) standard curve (Zambari et al. 2021). Total soluble protein analysis was done using a Thermo Fisher Genesys 10-S UV-Vis spectrophotometer (China).

Total phenolic content. Total phenolic content was determined using a method described by Damayanti et al. (2020) with slight modifications. The amount of 1 mL sample was taken and then put in a test tube, added with 5 mL Folin-Ciocalteu 50% reagent and reacted for 5 min. Folin-Ciocalteu reagent (50%) was prepared by diluting it in 80% methanol. After that, the mixture was added with 4 mL Na₂CO₃ 7.5% and allowed to stand for 2 h. Then, the absorbance was measured at a wavelength of 765

nm using a Thermo Fisher Genesys 10-S UV-Vis spectrophotometer (China). The amount of total phenolic content was calculated based on a gallic acid (0-250 µg.mL⁻¹) standard curve.

Antioxidant capacity. The method used is 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity following the method of Barus et al. (2019) with slight modifications. The amount of 1 mL of soy sauce was taken, then put in a test tube, added with 2 mL DPPH 0.2 mM solution and 10 mL methanol. The sample was allowed to stand for 30 min at room temperature, and then the absorbance was measured at a wavelength of 517 nm using a Thermo Fisher Genesys 10-S UV-Vis spectrophotometer (China). The EC₅₀ value (half maximal effective concentration) was determined for DPPH radical scavenging activity, which represents the effective concentration to cause a 50% decrease in the radical absorbance. The percentage of DPPH decolorization of the sample was calculated according to equation 1, where SA is scavenging activity.

$$SA (\%) = \left[1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \right] \times 100\% \quad (1)$$

Statistical analysis. Each sample analysis was performed in triplicate (n=3). All data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison between treatments. When ANOVA showed a statistically significant effect (p<0.05), a comparison among data was made using Tukey's HSD post hoc test.

Results and Discussion

Different treatments were used in this study. Two factors are suspected to affect physico-chemical properties, antioxidant capacity and phenolic compounds. The first factor was tempeh fermentation time (2 days, 3 days, 4 days, and koji as control), and the second factor was soy sauce fermentation time before pasteurization (up to 8 weeks).

Color. Fermentation time, filtration, and pasteurization significantly affect the color properties of tempeh-based soy sauce and koji-based soy sauce. According to Table 1, the L* value of tempeh-based soy sauce decreased with the fermentation time. Additionally, filtration and pasteurization significantly decreased the L* value.

Compared between the sample treatments, tempeh 4 days had the lowest L^* value, whereas koji was the highest. The fermentation time of tempeh significantly decreased the L^* value of soy sauce.

After filtration, koji had the lowest L^* value, while tempeh 2 days had the highest value, even after pasteurization.

Table 1. The L^* value of soy sauce produced with different fermentation times (up to 8 weeks) and after pasteurization process.

Week	L^*			
	2 days	3 days	4 days	Koji control
0	36.50 ± 0.24 ^{aA}	33.99 ± 1.07 ^{aB}	34.36 ± 0.35 ^{aB}	35.01 ± 0.73 ^{aAB}
1	31.96 ± 1.13 ^{bB}	30.41 ± 0.42 ^{bB}	28.23 ± 0.19 ^{bC}	34.97 ± 0.02 ^{aA}
2	28.49 ± 0.37 ^{cB}	26.84 ± 0.42 ^{cC}	25.89 ± 0.44 ^{cC}	35.07 ± 0.93 ^{aA}
3	26.59 ± 0.48 ^{dB}	24.60 ± 0.42 ^{dBC}	24.08 ± 0.75 ^{dC}	35.09 ± 1.37 ^{aA}
4	25.14 ± 0.23 ^{dB}	23.35 ± 0.18 ^{dC}	23.56 ± 0.43 ^{dC}	30.29 ± 0.95 ^{bA}
5	21.30 ± 1.04 ^{eB}	20.79 ± 0.11 ^{eB}	20.08 ± 0.49 ^{eB}	28.19 ± 0.28 ^{cA}
6	20.97 ± 0.57 ^{eB}	18.78 ± 0.27 ^{fC}	17.81 ± 0.84 ^{fC}	26.98 ± 0.14 ^{cdA}
7	19.28 ± 0.21 ^{fB}	17.67 ± 0.42 ^{fgC}	16.99 ± 0.41 ^{fgC}	25.17 ± 0.03 ^{deA}
8	18.80 ± 0.36 ^{fB}	17.36 ± 0.18 ^{gC}	16.33 ± 0.40 ^{ghD}	24.95 ± 0.07 ^{eA}
Filtrated	16.71 ± 0.15 ^{gA}	15.84 ± 0.15 ^{hB}	14.97 ± 0.53 ^{hC}	11.08 ± 0.18 ^{fd}
Pasteurized	13.62 ± 0.32 ^{hA}	12.09 ± 0.16 ^{iB}	11.60 ± 0.17 ^{iB}	8.18 ± 0.05 ^{gC}

¹Lowercase superscripts within the rows indicate the significant differences at $p < 0.05$ (amongst soy sauce fermentation times).

²Uppercase superscripts within the columns indicate the significant differences at $p < 0.05$ (amongst samples).

The results of a^* value is presented in Table 2. Fermentation time significantly affects the a^* value of soy sauce. Week 0 shows the lowest a^* value for tempeh-based soy sauce. The a^* value significantly increased after week 1 fermentation for all treatments. Fermentation time contributed to the significant decrease in a^* value. Nonetheless, filtration and pasteurization did not have a significant impact on the a^* value of most tempeh-

based soy sauce, while the a^* value was significantly decreased on koji-based soy sauce. The a^* value of each treatment showed significant differences following filtration and pasteurization. Koji had the lowest amount, while tempeh at 2 days had the highest. Furthermore, the a^* value of soy sauce decreased as the tempeh fermentation time increased.

Table 2. The a^* value of soy sauce produced with fermentation times (up to 8 weeks) and after pasteurization process.

Week	a^*			
	2 days	3 days	4 days	Koji control
0	0.91 ± 0.32 ^{fB}	0.73 ± 0.26 ^{gB}	0.63 ± 0.35 ^{gB}	9.46 ± 0.52 ^{efA}
1	26.51 ± 0.55 ^{aA}	24.86 ± 0.27 ^{aAB}	24.37 ± 0.23 ^{aB}	16.18 ± 1.23 ^{aC}
2	25.35 ± 0.42 ^{aA}	23.99 ± 0.32 ^{aB}	22.90 ± 0.35 ^{bC}	13.45 ± 0.27 ^{bD}
3	25.26 ± 0.16 ^{aA}	22.39 ± 0.44 ^{bB}	22.44 ± 0.52 ^{bB}	12.29 ± 1.08 ^{bcC}
4	22.84 ± 0.22 ^{bA}	20.53 ± 0.69 ^{cB}	20.27 ± 0.22 ^{cB}	11.22 ± 0.13 ^{cdC}
5	20.70 ± 0.97 ^{cA}	19.38 ± 0.33 ^{cA}	19.96 ± 0.13 ^{cA}	10.84 ± 0.07 ^{cdeB}
6	16.89 ± 0.84 ^{dA}	16.49 ± 0.28 ^{dA}	16.57 ± 0.20 ^{dA}	10.38 ± 0.17 ^{deB}
7	16.63 ± 0.44 ^{dA}	15.90 ± 0.51 ^{deAB}	15.19 ± 0.25 ^{eB}	9.60 ± 0.34 ^{efC}
8	16.24 ± 0.14 ^{deA}	15.92 ± 0.42 ^{deAB}	15.18 ± 0.58 ^{eB}	9.33 ± 0.21 ^{efC}
Filtrated	15.57 ± 0.20 ^{deA}	14.93 ± 0.28 ^{efB}	14.07 ± 0.18 ^{fC}	8.35 ± 0.19 ^{fd}
Pasteurized	15.12 ± 0.03 ^{eA}	14.17 ± 0.32 ^{fB}	13.32 ± 0.54 ^{fC}	6.14 ± 0.04 ^{gD}

¹Lowercase superscripts within the rows indicate the significant differences at $p < 0.05$ (amongst soy sauce fermentation times).

²Uppercase superscripts within the columns indicate the significant differences at $p < 0.05$ (amongst samples).

The result of b^* value in Table 3 showed that the b^* value significantly increased after 1 week of fermentation. The b^* value is significantly increased by fermentation time, filtration, and pasteurization. Additionally, koji has the lowest b^* value, however it boasts the highest b^* value before filtration and pasteurization. Koji exhibited the slightest decrease in b^* value compared to tempeh-based soy sauce. The b^* value of tempeh-based soy sauce decreased as the tempeh fermentation time increased. The fermentation time can impact the characteristics of soy sauce. During moromi fermentation, an aging process will occur, which causes the color change in the soy sauce to become darker, which is dominated by the role of *Bacillus* via amylase and protease activities (Tan et al. 2022). In addition, the dark color is formed during moromi fermentation due to the formation of melanoidin compounds produced from the Maillard reaction (Rosida 2013; Al-Abbasy et al. 2024). Melanoidin contributed to the deep brown hue of soy sauce by lowering the L^* , a^* and b^* measurements.

The effectiveness of the Maillard reaction is greatly influenced by the makeup of food; for example, sugars, like xylose are much more reactive than others, such as glucose. Amino acids were also involved in the Maillard reaction (Del-Udom et al. 2019). Hence, the variation in color traits of tempeh and koji is likely affected by the amounts of amino acids, xylose, and glucose generated. Furthermore, the duration of fermentation has an impact on the amount of amino acid present. Amino acids content increased with fermentation time due to the *R. oligosporus* fungi metabolizing during its growth and producing protease enzymes that can break down proteins into free amino acids (Rizal et al. 2022). The study by Langner and Rzeski (2014) showed that melanoidin is the final product of the Maillard reaction, which takes place during the heat processing of food containing reducing sugars and compounds possessing free amino groups. Therefore, when heat processing, such as pasteurization, melanoidin is formed as a result of the Maillard reaction.

Table 3. The b^* value of soy sauce produced with different fermentation times (up to 8 weeks) and after pasteurization process.

Week	b^*			
	2 days	3 days	4 days	Koji control
0	11.36 ± 0.62 ^{hB}	11.58 ± 0.26 ^{gB}	12.23 ± 0.65 ^{hB}	31.45 ± 1.49 ^{abA}
1	33.33 ± 0.91 ^{aA}	32.32 ± 0.53 ^{aAB}	31.76 ± 0.49 ^{aB}	31.23 ± 0.21 ^{abB}
2	30.76 ± 1.11 ^{bA}	31.31 ± 0.65 ^{aA}	31.47 ± 0.42 ^{aA}	32.44 ± 0.10 ^{aA}
3	30.22 ± 0.97 ^{bcAB}	28.29 ± 1.46 ^{bB}	28.78 ± 0.17 ^{bB}	32.65 ± 1.12 ^{aA}
4	28.77 ± 0.51 ^{cB}	26.48 ± 0.10 ^{bC}	26.23 ± 0.70 ^{cC}	32.58 ± 0.40 ^{aA}
5	25.41 ± 0.33 ^{dB}	24.58 ± 0.34 ^{cB}	24.43 ± 0.77 ^{dB}	31.62 ± 0.07 ^{abA}
6	21.57 ± 0.44 ^{eB}	21.46 ± 0.41 ^{dB}	20.56 ± 0.50 ^{eB}	30.63 ± 0.16 ^{bA}
7	20.13 ± 0.39 ^{eBC}	20.80 ± 0.27 ^{deB}	19.56 ± 0.38 ^{efC}	28.71 ± 0.32 ^{cA}
8	20.43 ± 0.26 ^{eB}	19.70 ± 0.27 ^{eB}	18.38 ± 0.55 ^{fC}	27.57 ± 0.38 ^{cA}
Filtrated	17.66 ± 0.23 ^{fA}	16.32 ± 0.19 ^{fB}	15.54 ± 0.19 ^{gC}	10.73 ± 0.36 ^{dD}
Pasteurized	15.57 ± 0.39 ^{gA}	14.82 ± 0.36 ^{fAB}	14.52 ± 0.28 ^{gB}	8.44 ± 0.11 ^{eC}

¹Lowercase superscripts within the rows indicate the significant differences at $p < 0.05$ (amongst soy sauce fermentation times).

²Uppercase superscripts within the columns indicate the significant differences at $p < 0.05$ (amongst samples).

Total dissolved solids. Soy sauce fermentation time contributed to the significant increase in total dissolved solids, as demonstrated in Table 4. Filtration had no impact on the total dissolved solids in tempeh based-soy sauce, but resulted in a significant decrease in koji-based soy sauce. Additionally, pasteurization significantly increased the total dissolved solids. Koji had the highest total dissolved solids, while tempeh 2 days had the

lowest. Total dissolved solids of tempeh-based soy sauce increase with increasing the tempeh fermentation time. Lubis (2019) studied the total dissolved solid of soy sauce. The study demonstrated that total dissolved solids contributed to the deep hue of soy sauce. The total dissolved solids increase along with the fermentation time, so this study aligns with Lubis (2019). This is due to hydrolysis into simpler substances by anaerobic

bacteria, which requires longer time. Moreover, salt possesses hygroscopic traits that can draw in water and dissolved substances, leading to a rise in osmotic pressure within the substrate. Therefore, during the moromi fermentation process, simple compounds dissolve in the filtrate (Iskandar and Darusalam 2020). Filtration contributed to a significant decrease in koji-based soy sauce, but not tempeh-based soy sauce. While the koji in tempeh does not bind together during fermentation, the soybeans stay interconnected, leading to the absorption of most of the water by the soybeans as they undergo koji fermentation. Furthermore, during the fermentation of koji, the soybeans lose moisture and thus decrease in size, resulting in a reduced surface area. However, during the process of moromi fermentation, the soybeans once again

increase in size to their initial dimensions. Hence, soaking 500 g of tempeh and 500 g of koji in water yields varying outcomes. Furthermore, total dissolved solids increase along with the tempeh fermentation time due to the higher amino acid content and simpler water-soluble compounds (Bowden et al. 2018; Nursiwi et al. 2021). Pasteurization also significantly increased total dissolved solids, which related to conductivity. This is because pasteurized samples had higher conductivity. The soy sauce was treated at a higher temperature, which influenced the solubility of salt and minerals. Fermented soybeans were mixed with a brine solution, and the temperature affected conductivity, leading to increased ion mobility and enhanced salt solubility (Techakanon and Sirimuangmoon 2020).

Table 4. Total dissolved solids of soy sauce produced with different fermentation times (up to 8 weeks) and after pasteurization process.

Week	Total dissolved solids, %			
	2 days	3 days	4 days	Koji control
0	13.16 ± 0.15 ^{eC}	13.46 ± 0.13 ^{dC}	14.30 ± 0.30 ^{eB}	17.94 ± 0.14 ^{gA}
1	15.52 ± 0.21 ^{dB}	15.94 ± 0.39 ^{cB}	15.96 ± 0.33 ^{dB}	20.55 ± 0.28 ^{fA}
2	15.89 ± 0.07 ^{dC}	16.20 ± 0.17 ^{cBC}	16.88 ± 0.35 ^{cdB}	25.00 ± 0.48 ^{dA}
3	16.59 ± 0.12 ^{cC}	16.85 ± 0.12 ^{bC}	17.94 ± 0.02 ^{bcB}	29.40 ± 0.49 ^{cA}
4	17.08 ± 0.10 ^{bC}	16.98 ± 0.17 ^{bC}	18.72 ± 0.34 ^{bB}	33.65 ± 0.35 ^{bA}
5	17.09 ± 0.24 ^{bC}	17.13 ± 0.07 ^{bC}	18.95 ± 1.13 ^{bB}	35.57 ± 0.10 ^{aA}
6	17.18 ± 0.15 ^{bC}	17.16 ± 0.10 ^{bC}	18.70 ± 0.49 ^{bB}	35.93 ± 0.13 ^{aA}
7	17.13 ± 0.03 ^{bC}	17.30 ± 0.33 ^{bC}	18.72 ± 0.22 ^{bB}	36.18 ± 0.06 ^{aA}
8	17.37 ± 0.02 ^{bC}	17.30 ± 0.03 ^{bC}	18.86 ± 0.21 ^{bB}	36.32 ± 0.08 ^{aA}
Filtrated	17.27 ± 0.07 ^{bC}	17.14 ± 0.12 ^{bC}	18.22 ± 0.15 ^{bB}	22.37 ± 0.05 ^{eA}
Pasteurized	18.79 ± 0.02 ^{aD}	20.22 ± 0.01 ^{aC}	23.35 ± 0.02 ^{aB}	25.16 ± 0.04 ^{dA}

¹Lowercase superscripts within the rows indicate the significant differences at $p < 0.05$ (amongst soy sauce fermentation times).

²Uppercase superscripts within the columns indicate the significant differences at $p < 0.05$ (amongst samples).

Total soluble protein. Table 5 demonstrates the impact of fermentation time and pasteurization on total soluble protein levels. Fermentation time significantly increased the soluble protein, whereas pasteurizations showed a significant decrease. Based on sample treatments, koji has the highest soluble protein. Tempeh fermentation time was significantly increasing the soluble protein. Hence, tempeh 2 days has the lowest soluble protein. The total dissolved solids increased along with total soluble protein, which can be seen in Table 4 and Table 5, but not for the pasteurized soy sauce. The fermentation time of tempeh contributed to the increase of soluble protein. This is because the

longer the fermentation time of tempeh, the higher the amino acid content, which resulted from protein hydrolysis by the activity of *R. oligosporus* (Nursiwi et al. 2021). Hydrolyzing protein transforms non-soluble protein into soluble protein by reducing the protein's molecular weight. Moreover, enzymatic hydrolysis provides better interaction of hydrophilic groups with the water molecules due to the decreased size of peptides, which increases protein solubility. Thus, soy sauce's total soluble protein content could result from several facts, such as forming small peptides, protein structural changes, and hydrophilic amino acids (Opazo-Navarette et al. 2022). At the same

time, koji has the highest amount of total dissolved protein, because it undergoes a longer fermentation time process compared to tempeh. This longer

fermentation time results in slower enzymatic hydrolysis and shorter peptide sizes.

Table 5. Total soluble protein of soy sauce produced with different fermentation times (up to 8 weeks) and after pasteurization process.

Sample	Total soluble protein, mg.mL ⁻¹		
	4 Weeks	8 Weeks	Pasteurized
2 days	12.18 ± 0.01 ^{dC}	12.37 ± 0.02 ^{dA}	12.25 ± 0.01 ^{dB}
3 days	12.30 ± 0.01 ^{cC}	12.96 ± 0.02 ^{cA}	12.54 ± 0.03 ^{cB}
4 days	12.63 ± 0.01 ^{bC}	13.29 ± 0.01 ^{bA}	12.70 ± 0.01 ^{bB}
Koji control	53.85 ± 0.03 ^{aC}	60.83 ± 0.12 ^{aA}	56.18 ± 0.05 ^{aB}

¹Lowercase superscripts within the rows indicate the significant differences at p<0.05 (amongst samples).

²Uppercase superscripts within the columns indicate the significant differences at p<0.05 (amongst soy sauce fermentation times).

Pasteurization significantly decreased the total soluble protein of soy sauce. This occurs since protein solubility is influenced by protein-water interactions, causing protein molecules to unfold at denaturation temperature. In addition, protein solubility is impacted by the binding of the polypeptide chain's secondary and tertiary structures, which results in hydration (Lee et al. 2019). Pasteurization leads to the denaturation of proteins, potentially leading to the loss of their biological function (Boghawaththa and Vasiljevic 2020). Protein denaturation causes the precipitation of denatured proteins by adding small amounts of neutral salts, which causes a decrease in the solubility of proteins (Acharya and Chaudhuri 2021). Another study conducted by Pelegri (2008) showed that the protein solubility is

increased with a temperature between 40-50°C. However, if the temperature of the solution is raised high enough for a given time, the protein is denatured. This could impact the stability of secondary and tertiary structures through the disruption of hydrogen, hydrophobic, and electrostatic bonds, leading to decreased water binding and protein solubility.

Total phenolic content. According to Table 6, phenolic compounds of soy sauce were significantly increased with increasing the fermentation time. Nevertheless, pasteurization significantly decreases the phenolic compounds. Koji showed the highest levels of total phenolic compounds, while tempeh that has been fermented for 2 days had the lowest amounts. Tempeh fermentation time contributed to the increase of phenolic compounds of soy sauce.

Table 6. Total phenolic content of soy sauce produced with different fermentation times (up to 8 weeks) and after pasteurization process.

Sample	Total phenolic content, µg gallic acid equivalent.mL ⁻¹		
	4 Weeks	8 Weeks	Pasteurized
2 days	651.26 ± 13.85 ^{dB}	746.84 ± 3.58 ^{dA}	586.30 ± 12.24 ^{dC}
3 days	697.86 ± 4.25 ^{cB}	858.06 ± 3.58 ^{cA}	633.91 ± 14.33 ^{cC}
4 days	932.55 ± 16.27 ^{bB}	1090.38 ± 11.77 ^{bA}	897.86 ± 3.28 ^{bC}
Koji control	1472.01 ± 3.68 ^{aB}	1563.51 ± 4.82 ^{aA}	1247.52 ± 16.86 ^{aC}

¹Lowercase superscripts within the rows indicate the significant differences at p<0.05 (amongst samples).

²Uppercase superscripts within the columns indicate the significant differences at p<0.05 (amongst soy sauce fermentation times).

The phenolic compounds increase along with tempeh and soy sauce fermentation times. The amount of total phenolic content in soy sauce is connected to the water-soluble phenolic compounds found in tempeh. Prior research (Attahailah et al. 2019) has indicated that soybeans contain various phenolic compounds and water-soluble phenols,

comprising approximately 68-81% of the total phenolic compounds. Overall, the present study demonstrated that tempeh's total water-soluble phenolic compounds increased along with the fermentation time (Attahailah et al. 2019). Table 6 indicated that soy sauce produced from a 4 day fermentation period of tempeh contain higher total

phenolic compounds than soy sauce made from 2 and 3 days of tempeh. This aligns with the findings of [Suharto et al. \(2017\)](#) who found that the highest amount of total phenolic compounds during incubation was obtained on 4 days of tempeh fermentation time. Nevertheless, pasteurization significantly decreased the phenolic compounds.

This effect may result from processes like the degradation, oxidation, or polymerization of phenolic compounds ([Maghsoudlou et al. 2019](#)).

Antioxidant capacity. Table 7 shows the analysis of antioxidant capacity using DPPH radical scavenging ability methods. Both have similar value conditions for each soy sauce treatment.

Table 7. EC₅₀ of antioxidant capacity of soy sauce produced with different fermentation times (up to 8 weeks) and after pasteurization process.

Sample	EC ₅₀ of DPPH radical scavenging ability, mg.mL ⁻¹		
	4 Weeks	8 Weeks	Pasteurized
2 days	7.03 ± 0.66 ^{aA}	4.14 ± 0.11 ^{aB}	2.85 ± 0.20 ^{aC}
3 days	6.35 ± 1.16 ^{aA}	3.91 ± 0.14 ^{aB}	2.60 ± 0.18 ^{aB}
4 days	4.52 ± 0.07 ^{bA}	3.38 ± 0.14 ^{bB}	1.91 ± 0.18 ^{bC}
Koji control	2.24 ± 0.10 ^{cA}	1.82 ± 0.14 ^{cB}	1.09 ± 0.04 ^{cC}

¹Lowercase superscripts within the rows indicate the significant differences at p<0.05 (amongst samples).

²Uppercase superscripts within the columns indicate the significant differences at p<0.05 (amongst soy sauce fermentation times).

Fermentation time and pasteurization contributed to significant decreases in EC₅₀ value, which indicates increases in antioxidant capacities. Koji had the lowest EC₅₀ value, while tempeh at 2 days had the highest. Furthermore, tempeh fermentation time contributed to a significant decrease in EC₅₀ value. The antioxidant capacity of soy sauce is likely impacted by the antioxidant capacity of tempeh. [Lo et al. \(2022\)](#) examined how various fermentation conditions impact the antioxidant capacity of soy tempeh. The study indicated that EC₅₀ value decreased along with the increase in tempeh fermentation time. Consistently with the aforementioned study, this study found that EC₅₀ of tempeh-based soy sauce is increased along with the increase of tempeh fermentation time. The antioxidant capacity of soy sauce is directly linked to its phenolic compounds. During fermentation time, the amount of phenolic compounds increases. Moreover, fermentation causes the structural breakdown of plant cell walls, leading to the liberation or synthesis of various antioxidant compounds ([Hur et al. 2014](#)). Moreover, the bioavailability of several bioactive compounds responsible for antioxidant activity increases during the fermentation period due to proteolysis activity by the mold. Nonetheless, koji-based soy sauce has a significantly higher EC₅₀ value than tempeh-based soy sauce. This is likely due to koji having a longer fermentation period, allowing for the production of antioxidant compounds to continue for a longer duration compared to tempeh. After pasteurization,

the increase in EC₅₀ value of soy sauce was not aligned with the total phenolic content. Although the phenolic compounds decrease after pasteurization, the antioxidant capacity of soy sauce increases. There is a study with similar cases from this research conducted by [Maghsoudlou et al. \(2019\)](#). That study demonstrated that the resulting data could be clarified by generating stable intermediate products and/or non-phenolic compounds deriving from pyrolysis, Maillard reaction, and/or carbohydrate caramelization when the thermal process is applied. Furthermore, [Sun et al. \(2022\)](#) reported that Maillard reaction products (MRPs) (e.g., furans, ketones, pyrazine) could significantly increase antioxidant capacity.

Conclusions

It was observed that pasteurization process and fermentation time significantly affected characteristics of tempeh-based soy sauce. Pasteurization on tempeh-based soy sauce significantly decreased the color value, total soluble protein, and total phenolic content. Nevertheless, pasteurization significantly increased total dissolved solids and antioxidant capacity (EC₅₀). However, physico-chemical characteristics, total phenolic content, and antioxidant capacity tend to be increased along with the increase of fermentation time, except for color value. Therefore, the longer fermentation time of tempeh-based soy sauce resulted in the darker color and better antioxidant of soy sauce produced. The pasteurization process on

soy sauce also has positive results: antioxidant capacity was significantly increased, and the darkened color was produced.

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