International Journal of Agricultural Technology 2025 Vol. 21(2):697-712
DOI: https://doi.org/10.63369/ijat.2025.21.2.697-712
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Application of tempeh and split gill mushroom extracts in herbal fresh sausage: Evolution of antioxidant and antimicrobial activities

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Somsri, A., Yodsenee, K., Suthirawat, S., Pilasombut, K. and Rumjuankiat, K. (2025). Application of tempeh and split gill mushroom extracts in herbal fresh sausage: Evolution of antioxidant and antimicrobial activities. International Journal of Agricultural Technology 21(2):697-712.

Abstract Tempeh, recognized as a high-protein meat alternative, is gaining popularity across Asian countries, while split gill mushroom (*Schizophyllum commune*), rich in essential nutrients, are increasingly consumed in Southeast Asia. The antioxidant activities of all treatments were significantly higher than the control ($p<0.05$). Furthermore, formulations containing split gill mushroom (R3, R4, and R5) exhibited lower initial total plate count (TPC) values compared to the other formulations. Sensory evaluation was then conducted on sausages containing tempeh, split gill mushrooms, and their combinations in ratios of 1:1, 1:3, and 3:1, yielding overall acceptance scores of 3.93, 3.30, 4.27, 5.33, and 3.37, respectively. The combination with a 3:1 tempeh-to-mushroom ratio demonstrated the highest consumer acceptance. Therefore, the study on the application of tempeh and split gill mushroom extracts in herbal fresh sausage is provided valuable information for developing plant-based products and functional foods in the future.

Keywords: Tempeh, Split gill mushroom, Antioxidant, Antimicrobial, Herbal fresh sausage

Introduction

Meat consumption and production have been considered unsustainable in terms of public health and environment as a major contributors to the increasing prevalence of diseases, including obesity, cancer and cardio vascular disorders, (Cofrades *et al*., 2008; Godfray *et al*., 2018; Hygreeva *et al.*, 2014). The growing of consumer demand for healthier meat product has led to a focus

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on non-meat ingredients as potential sources of bioactive compound (Cofrades *et al*., 2008). Various plant-based proteins have been employed as meat extenders and functional ingredient in meat product, contributing to both nutritional enhancement and product quality improvement (Rocchetti *et al*., 2023). Such vegetable protein can be obtained from tempeh, a indigenous food in Indonesia where it has been consumed as a stable of protein more than 300 years. Tempeh is usually made from soybean fermented with *Rhizopus* spp. but it can be made using various nuts, grains, and beans.

Tempeh has been known as a source of significant amounts of protein, vitamin B12, and bioactive compounds (Nout and Kiers, 2005). Tempeh offers numerous health benefits when consumed regularly. According to Rizzo and Baroni (2018) tempeh is highly effective in improving nutritional intake and has been consumed by both vegetarians and non-vegetarians to reduce meat consumption. Similarly, (Kao and Chen, 2006), and (Wang *et al.*, 2015) have revealed that the high protein and amino acid content in tempeh can help reduce levels of harmful cholesterol in the body, thereby lowering the risk of heart disease (Teoh *et al*., 2024). Furthermore, the high isoflavone content in tempeh acts as an antioxidant and exhibits anti-cancer properties (Dubost *et al.*, 2007). Over the past decade, studies have explored the use of tempeh as a meat substitute in various product for example hamburger (Bento *et al*., 2021), sausage (Syamsuri *et al*., 2020), Nugget (Sun and Ruiz-Carrascal, 2023) and Steak (Djalal *et al.*, 2023).

Alongside tempeh, split gill mushroom is also well known as edible mushroom, having significant nutritional values, incredibly high fiber, protein, and low lipid (Hobbs, 2005), including phenolic compounds in its ethanolic extract (Saetang *et al*., 2022; Yelithao *et al*., 2019)). It is a medicinal mushroom with various biological properties (Klaus *et al*., 2011), for instance, antioxidant, anti-inflammatory, immune-enhancing, anticancer (Patel and Goyal, 2012; Saetang *et al*., 2022; Wongaem *et al*., 2021), antiviral and antifungal capacity (Hobbs, 2005). Moreover, the schizophyllan, a polysaccharide derived from split gill mushroom plays a key role in migrating oxidative stress by protecting the human body from free radical-induced damage (Yelithao *et al.*, 2019). Split gill mushroom demonstrates a wide range of industrial application, being utilized in traditional food products (Hobbs, 2005), functional foods (Smirnou *et al*., 2017), pharmaceuticals (Lee and Ki, 2020), vaccine development, and cosmetic formulation (Smirnou *et al*., 2017). Given these versatile properties, the study aimed to access the antioxidant potential and inhibitory activities of tempeh and split gill mushrooms in various herbal sausage formulations, contributing to the development of functional meat alternatives.

Materials and methods

Herbal fresh sausage formulation

Six sausage formulations were prepared, including a control formulation (CT) that are commonly used in fresh sausage product, namely chicken breast, chili paste, shrimp paste, turmeric, sweet kaffir lime, sugar, and salt. The formulation R1, R2, R3, R4, and R5 incorporated tempeh and split gill mushroom in the following ratios: (4:0), (3:1), (2:2), (1:3) and (0:4), respectively, to replace chicken breast. The other ingredients were added at the same conditions as those of the CT formulation as describes in table 1. Subsequently, all herbal fresh sausage were stored at 4ºC for 0, 3, 6 and 9 days for further analysis.

Table 1. Ingredients used in herbal fresh sausage formulations

Ingredients	Herbal fresh sausage formulation					
	CT	**R1**	**R2**	**R3**	**R4**	**R5**
Chicken breast	400	-	-	-	-	-
Tempeh	-	400	300	200	100	-
Split gill mush room	-	-	100	200	300	400
Chili paste	50	50	50	50	50	50
Shrimp paste	5	5	5	5	5	5
Turmeric	20	20	20	20	20	20
Sweet kaffir lime	10	10	10	10	10	10
Sugar	8	8	8	8	8	8
Salt	8	8	8	8	8	8

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom

Determination of physicochemical characteristics

Color and pH determination

The color of herbal fresh sausage was evaluated following the method (Jovanović *et al*., 2017), using the CIE system parameters L* (lightness), a* (redness), and b* (yellowness). The measurements were taken with a Hunter Lab Mini Scan EZ 4000L (Hunter Lab Inc, Reston, VA, USA), with three

readings performed on the sausage surface at three different points. The L* value represents the position along the vertical axis indicating lightness, a* indicates the position on the red (+) to green (-) axis, and b* represents the position on the yellow (+) to blue (-) axis. For pH determination, the sample was mixed with distilled water in the ratio of 1:1 and measured by pH meter (Mettler Toledo Model SG-2, Switzerland(, which was calibrated with standard solution of pH 4.0 and pH 7.0. The measurements were conducted in triplicate to ensure accuracy and reliability of the results.

Protein content determination

Protein content in fresh sausage were determined using the Kjeldahl method (Salo-väänänen and Koivistoinen, 1996) Approximately 3 g of fresh sausages, 8 g catalyst and 25 mL of H_2SO_4 were placed into a digestion tube and digested at 400 °C for 30 minutes using heat mental. After digestion, the tube was allowed to reach the room temperature before protein digestion step, where 50 mL of distilled water, 25 mL of 4% boric acid, and 40% sodium hydroxide solution were added, followed by distillation for 3 minutes. The distillate was then titrated with 0.1 N HCl using a mixed indicator, and the protein content was calculated using the following equation;

$$\text{Protein content (\%)} = [(A-B)\times N\times 0.014\times 100\times F]/W$$

Where A is the Volume of hydrochloric acid used to titrate the sample (mL), B is the volume of hydrochloric acid used to titrate the blank (mL), W is weight of the sample (g), N is concentration of hydrochloric acid (Normality, N) and F is the factor of the sample (soybean products, F=5 and split gill mush room, F=6.25)

Determination of antioxidant activity

Analytical sample preparation

Fresh sausage samples were roasted at 180°C for 10 minutes. Then, 3 g of the roasted sample was placed into a test tube containing 15 mL of distilled water and homogenized at 10,000 rpm for 1 minute. After homogenization, 10 mL of chloroform was added, and the mixture was centrifuged at 5,000 rpm for 15 minutes. The supernatant was filtered through filter paper No.1 and diluted to 3% with distilled water for further analysis of 2,2-diphenyl-1-picryhydrazla hydrate (DPPH) radical scavenging activity and 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization.

2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) radical scavenging activity

The antioxidant activity of herbal fresh sausage was investigated using DPPH assay as mentioned in (Jung *et al*., 2010)with slightly modifications. Firstly, 2 ml of sausage supernatant was individually put into test tube which contained 2 ml of 0.2 mM (DPPH) in absolute ethanol. The mixture of ethanol (2ml) and sausage supernatant (2ml) were prepared as a blank. The control solution was prepared by mixing ethanol (2ml) and DPPH radical solution (2ml). The mixture was kept for 30 minutes in dark condition at room temperature then the reaction of DPPH radicals was measured at 517 nm. by a spectrophotometer (GENESYS 20, Thermo scientific, USA). The antioxidant activity of DPPH assay of herbal fresh sausage was calculated as following equation;

$$\text{DPPH activity (\%)} = \left[1 - \frac{\text{A sample} - \text{A blank}}{\text{A control}}\right] \text{x } 100$$

Where A sample is the absorbance of the sample mixed with DPPH, A blank is the absorbance of ethanol and the sample without DPPH, and A control is the absorbance of ethanol mixed with DPPH without the sample. All experiments were performed in triplicate.

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization

The stock solution of ABTS (2,2-Azion-Bis-3-Ethylbenzothiazoline-6-Sulphonic Acid) was prepared using 2.45 mM of $ABTS^{+,}$ mixed with 7 mM potassium persulphate solution and left the mixture in dark with room temperature for 12-16 hrs. Afterward, the concentration of blue-green ABTS radical solution was adjusted to an absorbance of 0.7± 0.02 at 734 nm with the ratio 1: 80 of $ABTS^{+}$ stock solution and ethanol before use. The determination of ABTS radical scavenging activity was started adding 3 mL of $ABTS^{+}$, prepared as described above to the test tube, and contained 0.3 ml of diluted sausage extract. After mixing rigorously, the mixture was kept at room temperature and measured the absorbance at 734 nm after 6 minutes of incubation.

$$\text{ABTS activity (\%)} = \left[1 - \frac{\text{A sample} - \text{A blank}}{\text{A control}}\right] \text{x } 100$$

Where A sample is the absorbance of the sample mixed with $ABTS^{+,}$, A blank is the absorbance of ethanol and the sample without $ABTS^{+,}$, and A control is the absorbance of ethanol mixed with $ABTS^{+,}$ without the sample. All experiments were performed in triplicate.

Determination of antimicrobial ability using total plate count

The total plate count (TPC) of the samples was determined following standard procedures (Marshall, 1992). Briefly, 25 g of fresh sausage sample was homogenized in 0.85% normal saline using a stomacher (Model, company, country) at 230 rpm for 30 seconds. Then, 1 mL of the suspension was transferred to a test tube containing 9 mL of saline solution. Serial dilutions were prepared by transferring 1 mL from each dilution into subsequent tubes. The diluted samples were then poured onto nutrient agar (NA) plates and incubated at 37°C for 24–48 hours. Colony counts were recorded and expressed as log CFU/g.

Experimental design and statistical analysis

The experimental design was conducted as a completely randomized design with three replications and was repeated three times using one-way analysis of variance. Analysis of variance was performed using raw data with mean values and standard deviation was calculated using SPSS version 23 software (IBM).

Sensory analysis

Sensory evaluation was conducted to assess the overall appearance, odor, texture, and overall acceptability of each fresh sausage sample, following the descriptive hedonic scale method of (Beinner *et al*., 2010). The sensory test involved 30 consumer panelists, who were trained in sensory evaluation procedures. Prior to evaluation, fresh sausage samples were prepared by roasting at 180°C for 10 minutes. Panelists were asked to evaluate the sensory properties using a 7-point descriptive hedonic scale, ranging from 1 (extremely dislike) to 7 (extremely like), with the scale defined as follows: 1 = extremely dislike, 2 = very dislike, 3 = dislike, 4 = neither like nor dislike, 5 = like, 6 = very like, and 7 = extremely like.

Results

Protein content determination

The analysis of the protein content in herbal fresh sausage productsfound that the control (CT) formulation which mainly used chicken breast as the main ingredient, had the highest protein content at 16.77% (Table 2), and followed by formulation R1 (tempeh), with a protein content of 15.25%, and R2 (tempeh: split gill mushroom ratio 3:1), R3 (tempeh: split gill mushroom ratio

2:2), R4 (tempeh: split gill mushroom ratio of 1:3), and R5 (split gill mushroom), with protein contents of 14.12%, 13.53%, 12.57%, and 11.75%, respectively. According to Thai Industrial Standards Institute for fresh sausage (TISI 294/2547), the protein content should not be less than 13%. The finding revealed that formulations R1, R2, and R3 had protein contents exceeding this standard, indicating that formulations containing tempeh had significantly higher protein content than those with split gill mushroom.

Table 2. Determination of protein content in herbal fresh sausage formulation

Treatment	Protein content (%)
CT	16.77±0.13[a]
R1	15.25±0.35[b]
R2	14.12±0.22[c]
R3	13.53±0.13[d]
R4	12.57±0.13[e]
R5	11.75±0.18[f]

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom
[a-f] represents different letters in the same column that have statistically significant differences

pH and color determination

Fresh sausage samples were investigated for pH value as presented in Table 3, with all formulations exhibiting pH levels approximately 5. The CT formulation had a pH of 5.86, which was higher than the formulations containing tempeh and split gill mushrooms. Specifically, R1, R2, R3, R4 and R5 showed pH values of 5.63, 5.52, 5.46, 5.13, and 5.25, respectively. No statistically significant differences in pH were observed during storage at 4°C for 3 and 6 days. However, at day 9, a significant increase in pH was recorded in all formulations (Table 3).

The lightness (L*) of each sausage formulation (Table 4) revealed significant differences across all samples. The control (CT) had a lightness value of 50.72, whereas formulations R1 and R2 recorded values of 44.80 and 47.83, respectively. Formulations R3, R4, and R5 exhibited lightness values of 43.25, 41.76, and 40.29, respectively. These variations are attributed to the higher tempeh content in R1 and R2, as tempeh has a yellowish hue, while the split gill mushroom presents a darker brown color. However, no significant changes in lightness were observed during storage at 4°C over 3, 6, and 9 days.

In case of redness (a*) values, there were no statistically significant differences among the formulations. The CT had a redness value of 8.84, while R1, R2, R3, R4, and R5 recorded values of 9.58, 10.54, 10.75, 11.89, and 12.67, respectively. Similarly, no significant changes in redness were noted over the storage period at 4°C. In contrast, the yellowness (b*) values showed statistically significant differences among the formulations. The CT recorded a yellowness value of 38.46, while R1, R2, and R3 exhibited higher yellowness values of 47.30, 50.00, and 48.91, respectively. These values were greater than those of R4 and R5, which had yellowness values of 45.50 and 41.99, respectively. The increased yellowness in R1 and R2 is likely due to the higher proportion of tempeh. No significant changes in yellowness were observed during storage at 4°C over 3, 6, and 9 days.

Table 3. Determination of pH values in herbal fresh sausage after 0, 3, 6 and 9 days of storage

Storage day	pH value					
	CT	R1	R2	R3	R4	R5
0	5.86 ± 0.02^{aD}	5.63 ± 0.01^{bC}	5.52 ± 0.02^{cC}	5.46 ± 0.01^{dC}	5.13 ± 0.01^{eD}	5.25 ± 0.01^{eC}
3	6.05 ± 0.01^{aC}	5.67 ± 0.01^{bBC}	5.57 ± 0.01^{cB}	5.57 ± 0.02^{cB}	5.47 ± 0.01^{dC}	5.33 ± 0.02^{eB}
6	6.15 ± 0.01^{aB}	5.75 ± 0.08^{bB}	5.60 ± 0.21^{bB}	5.69 ± 0.01^{cA}	5.51 ± 0.01^{dB}	5.45 ± 0.02^{dA}
9	6.26 ± 0.02^{aA}	6.03 ± 0.02^{bA}	5.72 ± 0.03^{cA}	5.72 ± 0.02^{cA}	5.59 ± 0.01^{dA}	5.48 ± 0.03^{eA}

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom

$^{a-f}$ indicates different letters in the same row that have statistically significant differences.

$^{A-D}$ indicates different letters in the same column that have statistically significant differences.

± represents the standard deviation of the samples when the experiment is repeated three times.

DPPH radical scavenging activity

We compared the antioxidant capacity of herbal fresh sausage 5 formulations, consisting of CT, R1, R2, R3, R4 and R5 using DPPH assay (Table 5). All formulations were singnificantly differed, especially R5 which utilized split gill mushroom as the main ingredient, demonstrated the highest DPPH radical scavenging activity of 70.27%, and followed by R4 R3, R2 and R, with percentage of inhibition of 67.60, 65.14, 64.26, and 51.31, respectively. The control formulation (CT), containing chicken breast showed a DPPH radical scavenging activity of 39.51%. The results indicated that formulations containing split gill mushroom as the main ingredient exhibited significantly higher antioxidant activity as compared to other formulations. However, after storage at 4°C for 3, 6 and 9 days, a significant decrease in antioxidant activity was observed in all formulations, with longer storage durations resulting in further reductions in antioxidant capacity. The results indicated that

formulations containing split gill mushroom as the primary component exhibited significantly higher antioxidant activity compared to other formulations. However, after storage at 4°C for 3, 6, and 9 days, a significant decrease in antioxidant activity was observed to all formulations, with longer storage durations leading to further reductions in antioxidant capacity.

Table 4. Color variables (L*, a*, b*) in herbal fresh sausage formulation after 0, 3, 6 and 9 days of storage

Parameters	Storage day	Fresh sausage formulations					
		CT	R1	R2	R3	R4	R5
Lightness (L*)	0	$50.72\pm1.52^{a}_{A}$	$44.80\pm0.70^{c}_{A}$	$47.83\pm1.17^{b}_{A}$	$43.25\pm1.56^{cd}_{A}$	$41.76\pm1.11^{de}_{A}$	$40.29\pm1.72^{e}_{A}$
	3	$51.12\pm0.22^{a}_{A}$	$45.92\pm0.81^{b}_{A}$	$45.85\pm0.95^{b}_{A}$	$43.90\pm0.99^{c}_{A}$	$41.73\pm1.37^{c}_{A}$	$41.59\pm0.55^{c}_{A}$
	6	$50.12\pm1.61^{a}_{A}$	$46.57\pm1.15^{b}_{A}$	$45.25\pm0.84^{b}_{A}$	$40.29\pm0.99^{c}_{B}$	$40.53\pm0.67^{c}_{A}$	$40.65\pm0.50^{c}_{A}$
	9	$51.42\pm4.39^{a}_{A}$	$45.88\pm3.39^{bc}_{A}$	$47.01\pm2.72^{ab}_{A}$	$40.52\pm1.99^{cd}_{B}$	$41.95\pm0.50^{cd}_{A}$	$39.22\pm1.74^{d}_{A}$
Redness (a*)	0	8.84 ± 1.52^{aA}	9.58 ± 2.16^{aA}	$10.54\pm2.44^{a}_{A}$	$10.75\pm3.16^{a}_{A}$	$11.89\pm3.40^{a}_{A}$	$12.67\pm1.15^{a}_{A}$
	3	8.81 ± 0.89^{aA}	9.37 ± 2.55^{aA}	$11.76\pm0.76^{a}_{A}$	$12.39\pm3.51^{a}_{A}$	$12.24\pm0.53^{a}_{A}$	$11.44\pm1.14^{a}_{A}$
	6	8.02 ± 1.26^{cA}	8.01 ± 0.69^{cA}	$10.09\pm2.13^{bc}_{A}$	$11.39\pm1.24^{ab}_{A}$	$13.54\pm1.35^{a}_{A}$	$11.31\pm0.40^{ab}_{A}$
	9	7.65 ± 0.42^{cA}	8.07 ± 0.73^{cA}	9.47 ± 2.27^{bcA}	$12.87\pm1.46^{a}_{A}$	$11.37\pm1.33^{ab}_{A}$	$11.23\pm0.82^{ac}_{A}$
Yellowness (b*)	0	$38.46\pm3.08^{d}_{A}$	$47.30\pm1.13^{ab}_{A}$	$50.00\pm2.20^{a}_{A}$	$48.91\pm0.18^{ab}_{A}$	$45.50\pm2.60^{bc}_{A}$	$41.99\pm1.28^{cd}_{A}$
	3	$37.18\pm2.25^{b}_{A}$	$46.02\pm2.15^{a}_{A}$	$48.94\pm1.22^{a}_{A}$	$47.38\pm6.63^{a}_{A}$	$46.46\pm1.10^{a}_{A}$	$39.01\pm0.94^{b}_{A}$
	6	$37.17\pm1.92^{b}_{A}$	$47.45\pm5.04^{a}_{A}$	$47.48\pm1.25^{a}_{A}$	$47.87\pm2.54^{a}_{A}$	$45.51\pm5.82^{a}_{A}$	$39.98\pm6.84^{ab}_{A}$
	9	$37.29\pm0.59^{c}_{A}$	$46.50\pm2.58^{a}_{A}$	$48.83\pm2.30^{a}_{A}$	$49.01\pm1.58^{a}_{A}$	$46.88\pm1.01^{a}_{A}$	$41.91\pm2.57^{b}_{A}$

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom
$^{a-d}$ indicates different letters in the same row that have statistically significant differences.
^{A}indicates different letters in the same column that have statistically significant differences.
± represents the standard deviation of the samples when the experiment is repeated three times.

ABTS radical scavenging activity

The antioxidant activity of fresh herbal sausage products containing tempeh and split gill mushroom were evaluated using the ABTS assay. As presented in Table 6, formulation 5, containing split gill mushroom as the main ingredient, exhibited the highest ABTS radical scavenging activity, with a value of 62.28%. This was followed by formulation 4 (tempeh and split gill mushroom ratio of 1:3), formulation 3 (tempeh and split gill mushroom ratio of 1:1), formulation 2 (tempeh and split gill mushroom ratio of 3:1), and formulation 1 (tempeh only), with ABTS antioxidant activities of 61.94%,

57.15%, 53.46%, and 48.73%, respectively. In case of control formulation displayed an ABTS antioxidant activity of 37.25%. All formulations showed statistically significant differences (Table 6). Furthermore, during storage at 4°C for 3, 6, and 9 days, a significant reduction in antioxidant activity was observed in all formulations as storage time increased.

Table 5. The percentage of DPPH in herbal fresh sausages formulation after 0, 3, 6 and 9 days of storage

Storage day	Percentage of inhibition (%)					
	CT	**R1**	**R2**	**R3**	**R4**	**R5**
0	39.51 ± 0.25^{fA}	$51.31\pm0.33^{e}_{A}$	$64.26\pm0.16^{d}_{A}$	$65.14\pm0.25^{c}_{A}$	67.60 ± 0.41^{bA}	$70.27\pm0.25^{a}_{A}$
3	37.70 ± 0.43^{fB}	$51.69\pm0.25^{e}_{A}$	58.08 ± 0.39^{dB}	59.18 ± 0.16^{cB}	60.16 ± 0.75^{bB}	66.56 ± 0.16^{aB}
6	32.79 ± 0.16^{eC}	$49.51\pm0.59^{d}_{B}$	56.61 ± 0.25^{cC}	56.83 ± 0.19^{cC}	$58.42\pm1.19^{bB}_{C}$	62.30 ± 0.16^{aC}
9	$30.98\pm0.10^{e}_{D}$	$48.14\pm0.50^{d}_{C}$	55.41 ± 0.71^{cD}	$55.63\pm0.19^{c}_{D}$	57.54 ± 1.18^{bC}	$61.42\pm0.25^{a}_{D}$

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom.
$^{a-f}$ indicates different letters in the same row that have statistically significant differences.
$^{A-D}$ indicates different letters in the same column that have statistically significant differences.
± represents the standard deviation of the samples when the experiment is repeated three times.

Table 6. The percentage of ABTS radical cation decolorization in herbal fresh sausages after 0, 3, 6 and 9 days of storage

Storage days	Percentage of inhibition (%)					
	CT	**R1**	**R2**	**R3**	**R4**	**R5**
0	$37.25\pm0.66^{e}_{A}$	48.73 ± 0.10^{dA}	$53.46\pm0.17^{c}_{A}$	$57.15\pm1.55^{b}_{A}$	$61.94\pm0.17^{a}_{A}$	62.28 ± 0.92^{aA}
3	$34.31\pm1.55^{d}_{B}$	45.56 ± 1.85^{cB}	$52.25\pm0.17^{b}_{B}$	$53.17\pm0.10^{b}_{B}$	$59.69\pm0.35^{a}_{B}$	61.01 ± 0.44^{aB}
6	31.03 ± 0.66^{fC}	$44.64\pm0.17^{eB}_{C}$	$48.85\pm0.20^{d}_{C}$	$54.61\pm0.26^{c}_{B}$	$59.05\pm0.10^{b}_{C}$	$60.78\pm0.36^{aB}_{C}$
9	29.87 ± 0.56^{fC}	43.43 ± 0.62^{eC}	$47.12\pm0.26^{d}_{D}$	$49.13\pm0.17^{c}_{C}$	$57.09\pm0.30^{b}_{D}$	59.86 ± 0.35^{aC}

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom
$^{a-f}$ indicates different letters in the same row that have statistically significant differences.
$^{A-D}$ indicates different letters in the same column that have statistically significant differences.
± represents the standard deviation of the samples when the experiment is repeated three times.

Determination of antimicrobial ability using total plate count

To evaluate the antimicrobial ability of gill mushroom and tempeh, we conducted the total plate count (TPC) of fresh herbal sausage samples. Our

results revealed that all formulations increased steadily over the 9-day storage period at 4°C (Table 7). Initially, the control (CT) had the highest microbial count, while formulations with higher proportions of split gill mushroom (R3, R4, R5) exhibited lower initial TPC values. At the end of storage period, microbial growth had enhanced in all formulations, with the control consistently showing the highest TPC. Formulations containing more split gill mushroom demonstrated slower microbial growth, indicating enhanced preservation compared to the control and tempeh-dominant formulations. Notably, none of the formulations exceeded 4 log CFU/g, complying with the standards of TISI 294/2547. Our result indicated that the herbs and raw materials in these fresh herbal sausages can effectively slow microbial growth and extend their shelf life.

Table 7. Evaluation of total plate count of herbal fresh sausage during refrigerated storage at 4 °C for 0, 3, 6 and 9 days

Storage day	log CFU/g					
	CT	R1	R2	R3	R4	R5
0	3.22 ± 0.02^{aC}	3.23 ± 0.02^{aC}	3.20 ± 0.03^{aC}	3.02 ± 0.02^{bC}	3.01 ± 0.17^{bC}	2.94 ± 0.03^{bC}
3	3.57 ± 0.03^{aB}	3.31 ± 0.01^{bBC}	3.29 ± 0.01^{bcBC}	3.25 ± 0.01^{cB}	3.15 ± 0.03^{dBC}	3.13 ± 0.05^{dB}
6	3.60 ± 0.05^{aB}	3.37 ± 0.02^{bB}	3.32 ± 0.03^{bcB}	3.27 ± 0.03^{cdB}	3.21 ± 0.01^{deB}	3.15 ± 0.07^{eB}
9	4.10 ± 0.04^{aA}	3.75 ± 0.08^{bA}	3.75 ± 0.10^{bA}	3.73 ± 0.09^{bA}	3.64 ± 0.06^{bcA}	3.58 ± 0.07^{cA}

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom
$^{a-e}$ indicates different letters in the same row that have statistically significant differences.
$^{A-C}$ indicates different letters in the same column that have statistically significant differences.
± represents the standard deviation of the samples when the experiment is repeated three times.

Table 8. The sensory evolution of herbal fresh sausage after 0, 3, 6 and 9 days of storage

Treatment	Sensory characteristics			
	Appearance	Odor	Texture	Overall acceptability
CT	5.37 ± 0.93^{a}	5.97 ± 0.96^{a}	6.00 ± 0.83^{a}	5.67 ± 0.66^{a}
R1	3.67 ± 0.61^{c}	4.40 ± 0.56^{c}	3.10 ± 0.31^{c}	3.93 ± 0.69^{d}
R2	5.10 ± 0.71^{a}	5.47 ± 0.78^{b}	4.73 ± 0.78^{b}	5.33 ± 0.55^{b}
R3	4.30 ± 0.79^{b}	5.37 ± 0.61^{b}	4.63 ± 0.67^{b}	4.27 ± 0.45^{c}
R4	3.40 ± 0.77^{c}	4.17 ± 0.70^{cd}	3.33 ± 9.48^{cd}	3.37 ± 0.49^{e}
R5	3.00 ± 0.69^{d}	3.97 ± 0.76^{d}	2.97 ± 0.61^{d}	3.30 ± 0.53^{e}

CT= Chicken breast, R1 = Tempeh, R2 = Tempeh: split gill mushroom (3:1), R3 = Tempeh: split gill mushroom (2:2), R4 = Tempeh: split gill mushroom (1:3), R5 = Split gill mushroom
$^{a-e}$ indicates different letters in the same column that have statistically significant differences.
± represents the standard deviation of the samples when the experiment is repeated three times.

Acceptance test

The results of consumer acceptance for herbal fresh sausage indicated that the control formulation (CT), which contained chicken breast as the main ingredients, was the most preferred across all sensory attributes, including appearance, flavor, texture, and overall acceptability. Formulation R2, with a 3:1 ratio of tempeh to split gill mushrooms, also demonstrated moderate levels of consumer satisfaction. In contrast, formulations R1 (tempeh), R4 (1:3 of tempeh: mushroom ratio), and R5 (split gill mushroom) were rated lower due to unfavorable texture and taste, with R5 receiving the lowest scores overall (Table 8).

Discussion

The increasing concern about health and nutrition has led to the development of food products aimed at enhancing dietary quality. In particular, meat product consumption has garnered negative perceptions due to its association with heightened risks of cardiovascular diseases, obesity, and cancer. These risks are linked to high fat content, especially saturated fats, as well as the use of synthetic additives like antioxidants and antimicrobials (Hygreeva *et al*., 2014). Split gill mushrooms have emerged as both a nutritional and therapeutic superfood, owing to their rich protein content and bioactive compound schizophyllan (Saetang *et al.*, 2023). Additionally, they contain potent antioxidants such as hydroxybenzoic acid, protocatechuic acid, and tocopherol which contribute to their health-promoting properties (Saetang *et al*., 2022, 2023). Alongside split gill mushrooms, tempeh is recognized as an excellent plant-based protein source. It is nutritionally dense, containing high levels of protein, fiber, vitamins, and minerals while being low in saturated fats. Compared to meat, tempeh is nutritionally favorable due to its superior profile in protein, fat, fiber, and essential micronutrients (Ahnan-Winarno *et al*., 2021; Bavia *et al*., 2012). In our study, split gill mushrooms and tempeh were combined to develop plant-based fresh sausage. Five formulations were prepared, with the control (CT) formulation, using chicken breast as a main ingredient. The experimental formulations replaced chicken breast with varying ratios of tempeh and split gill mushroom to evaluate their potential as healthier plant-based substitutes while maintaining sensory and nutritional quality. Regarding protein content, formulations R1, R2, and R3 contained more than 13% of protein, indicating that tempeh-based formulations had significantly higher protein levels than those containing split gill mushrooms. This difference is attributed to the soybean-based nature of tempeh, which contains

up to 52% protein (Bavia *et al*., 2012) compared to the 20% protein content found in split gill mushrooms (Wongaem *et al*., 2021). When the fresh herbal sausage was stored at 4°C for 3 and 6 days, no statistically significant differences in pH values were observed. However, after 9 days of storage, a significant increase in pH was recorded across all formulations. This finding aligns with the study by (Xiong *et al*., 2022) which reported that extended storage time leads to elevated pH levels due to microbial growth. The initial pH reduction (Table 3), maybe because of the accumulation of acid-producing microorganisms, for instance lactic acid bacteria and the subsequent enhancement in pH maybe related to the accumulation of nitrogenous compounds such as a result of autolysis and microbial action as meat deteriorates (Holmer *et al*., 2009; Xiong *et al*., 2022). Moreover, we determined the color which is a critical quality attribute, as it is one of the primary factors influencing consumer perception (Purlis, 2010). Therefore, its evaluation during storage is crucial, given the oxygenation and oxidation of myoglobin, which directly affect the final color of herbal fresh sausage products. The color of the sausages was assessed using the CIELAB system (International Commission on Illumination) and expressed as L* (lightness), a* (redness), and b* (yellowness) parameters, with results presented in Table 4. Significant differences in lightness, redness, and yellowness were observed between the CT formulation and the formulations containing split gill mushrooms and tempeh. However, no significant differences were detected across the 0, 3, 6, and 9-day storage periods. In the case of antioxidant capacity, the formulation containing split gill mushrooms exhibited significantly higher antioxidant activity compared to other formulations for both the DPPH and ABTS radical scavenging assays (Table 6, 7). This finding aligns with previous studies, which have demonstrated that the antioxidant capacity of mushrooms is strongly correlated with their phenolic and ergothioneine content (Kalaras *et al*., 2017). For instance, Vamanu and Nita (2013) reported that mushrooms contain phenolic compounds (14.07 mg gallic acid equivalent [GAE]/g of dry extract) and ergothioneine (6.22 mg GAE/g of dry extract) (Saetang *et al*., 2022), both of which contribute to their antioxidant properties. Moreover, a significant reduction in antioxidant activity was observed across all formulations after 3, 6, and 9 days. The decline in antioxidant capacity after prolonged storage could be attributed to the degradation of phenolic compounds and other bioactive components due to prolonged exposure to oxygen and enzymatic activities during storage. This observation is consistent with related studies, which have reported that extended storage periods often lead to the deterioration of antioxidant potential in food products as oxidative processes are amplified (Amorati *et al.*, 2013). In acceptance test, we discovered that among all

treatment which contained split gill mushroom and tempeh, R2 formulation with 3:1 ratio of tempeh to split gill mushroom was the most preferred. Meat substitutes are relatively novel to most consumers. Tempeh, a soy-based meat alternative, is rich in nutrients but lacks a distinctive flavor, which can hinder consumer acceptance. Split gill mushrooms, with their texture resembling shredded chicken, are suitable for enhancing the textural properties of products; however, their use in large quantities may impart a slight bitterness. Therefore, in the development of fresh herbal sausage products, it is crucial not only to consider nutritional benefits but also to ensure that various sensory attributes and flavors are acceptable to consumers. In this study, sensory evaluation demonstrated that the formulation using tempeh and split gill mushrooms as a meat substitute specifically, the formulation with a tempeh-to-split gill mushroom ratio of 3:1 was significantly more preferred by consumers compared to other formulations. Furthermore, this product met the protein content and microbial quality standards established by TISI 294/2547. This study represents the first attempt to combine tempeh and split gill mushrooms in the development of a meat alternative, thereby adding value to fresh herbal sausage products.

Acknowledgments

The author would like to express sincere gratitude to the College of Agricultural Innovation and Food Technology, Rangsit University, and the Excellence Center of Meat and Protein Innovation Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand, for their valuable support.

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(Received: 30 September 2024, Revised: 5 March 2025, Accepted: 10 March 2025)