บทความวิจัย

ผลของการเพาะงอกและการหมักด้วย *Rhizopus oligosporus* ต่อฟีนอลิคและเปปไทด์ในถั่ว 4 ชนิด

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บทคัดย่อ

ถั่วอุดมด้วยสารอาหารหลากหลายชนิดซึ่งปริมาณฟีนอลิคและเปปไทด์สามารถเพิ่มขึ้นได้โดยการเพาะงอกและการหมัก อย่างไรก็ตามการศึกษาผลของสภาวะร่วมดังกล่าวยังมีข้อมูลจำกัด ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของการเพาะงอกถั่วเหลือง ถั่วเขียว ถั่วแดง และถั่วลิสงลายเสือที่มีความยากราก 0.2 และ 1.0 เซนติเมตร และการหมักถั่วแต่ละชนิดที่ผ่านการเพาะงอกด้วยเชื้อรา *Rhizopus oligosporus* (เทมเป้ถั่วเพาะงอก) ต่อปริมาณสารออกฤทธิ์ทางชีวภาพและกิจกรรมการต้านอนุมูลอิสระ ผลการศึกษาพบว่าปริมาณฟีนอลิคและฟลาโวนอยด์เพิ่มสูงขึ้นเมื่อระยะเวลาการเพาะงอกนานขึ้น ซึ่งถั่วลิสงลายเสือเพาะงอกที่มีความยาวราก 1.0 เซนติเมตร มีปริมาณฟีนอลิคและฟลาโวนอยด์สูงที่สุดเท่ากับ 3.53 mg GAE/g DW และ 43.75 mg QE/g DW ตามลำดับ ในทางตรงกันข้ามการเพาะงอกส่งผลให้ปริมาณโปรตีนและเปปไทด์ลดลงเมื่อเทียบกับตัวอย่างควบคุม ยกเว้นปริมาณโปรตีนในถั่วแดงและเปปไทด์ในถั่วเหลือง (p-value < 0.05) กิจกรรมการต้านอนุมูลอิสระวิเคราะห์โดยวิธีการทำลายอนุมูลอิสระดีพีพีเอช ($DPPH^{\bullet}$) และวิธีการฟอกสีอนุมูลอิสระเอบีทีเอส ($ABTS^{\bullet+}$) มีแนวโน้มเพิ่มขึ้นระหว่างการเพาะงอกเมื่อเทียบกับตัวอย่างควบคุม ยกเว้นถั่วลิสงลายเสือ ซึ่งกิจกรรมการต้านอนุมูลอิสระวิเคราะห์โดยวิธีการฟอกสีอนุมูลอิสระเอบีทีเอส ($ABTS^{\bullet+}$) มีความสัมพันธ์กับปริมาณสารออกฤทธิ์ทางชีวภาพ โดยเฉพาะเปปไทด์ (r = 0.909, p-value < 0.01) สำหรับเทมเป้ถั่วเพาะงอก ซึ่งผลิตจากถั่วที่มีความยาวราก 1.0 เซนติเมตร พบว่าการหมักช่วยเพิ่มปริมาณฟีนอลิคยกเว้นเทมเป้ถั่วแดงเพาะงอก (p-value < 0.05) โดยเทมเป้ถั่วลิสงลายเสือเพาะงอกมีฟีนอลิคสูงที่สุดเท่ากับ 7.60 mg GAE/g DW (p-value < 0.05) การหมักยังช่วยเพิ่มปริมาณเปปไทด์ โดยเทมเป้ถั่วเหลืองเพาะงอกมีเปปไทด์สูงที่สุดเท่ากับ 281.23 mg BSA/g DW (p-value < 0.05) อย่างไรก็ตามการหมักส่งผลให้เทมเป้ถั่วเพาะงอกมีปริมาณฟลาโวนอยด์และโปรตีนลดลงเมื่อเทียบกับตัวอย่างควบคุม (p-value < 0.05) การหมักส่งผลให้เทมเป้ถั่วเพาะงอกมีกิจกรรมการต้านอนุมูลอิสระวิเคราะห์โดยวิธีการทำลายอนุมูลอิสระดีพีพีเอช ($DPPH^{\bullet}$) และวิธีการฟอกสีอนุมูลอิสระเอบีทีเอส ($ABTS^{\bullet+}$) เพิ่มสูงขึ้นเมื่อเทียบกับตัวอย่างควบคุม ยกเว้นเทมเป้ถั่วแดงเพาะงอก (p-value < 0.05) การศึกษานี้แสดงให้เห็นว่าการเพาะงอกและการหมักมีศักยภาพในการเพิ่มปริมาณฟีนอลิคและเปปไทด์ โดยเฉพาะในถั่วลิสงลายเสือและถั่วเหลือง ซึ่งเพิ่มโอกาสในการประยุกต์ใช้ในการพัฒนาผลิตภัณฑ์อาหารเพื่อสุขภาพ

คำสำคัญ: การเพาะงอก การหมัก เทมเป้ถั่วเพาะงอก โปรตีนทางเลือกจากพืช สารออกฤทธิ์ทางชีวภาพ

การอ้างอิงบทความ: กมลพร สิทธิไตรย์, ดวงใจ โอชัยกุล และ กวินชญา สายแก้ว, "ผลของการเพาะงอกและการหมักด้วย *Rhizopus oligosporus* ต่อฟีนอลิคและเปปไทด์ในถั่ว 4 ชนิด," *วารสารวิชาการพระจอมเกล้าพระนครเหนือ*, ปีที่ 36, ฉบับที่ 2, หน้า 1–20, เลขที่บทความ 262-8090, เม.ย.–มิ.ย. 2569, doi: 10.14416/j.kmutnb.2026.03.003.

Research Article

Effects of Germination and *Rhizopus oligosporus* Fermentation on Phenolics and Peptides in Four Legumes

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Abstract

Legumes are abundant in nutrients, and their phenolic and peptide contents can be enhanced through germination and fermentation. However, research on the effects of these combined conditions is still limited. Thus, this study aims to evaluate the effects of germination of soybean, mung bean, red bean, and tiger-striped peanut at root lengths of 0.2 and 1.0 cm, and the fermentation of each germinated legume with *Rhizopus oligosporus* (germinated-legume tempeh), on their bioactive compounds and antioxidant activities. The results showed that the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) increased as the germination period increased, with tiger-striped peanuts germinated to a root length of 1.0 cm exhibiting the highest TPC and TFC, at 3.53 mg GAE/g DW and 43.75 mg QE/g DW, respectively. In contrast, germination resulted in decreased Total Protein Content (TPRC) and Total Peptide Content (TPEC) compared with the control, except for the TPRC of red bean and the TPEC of soybean (p-value < 0.05). Antioxidant activities measured by DPPH and ABTS assays tended to increase during germination compared with the control, except in tiger-striped peanuts. The antioxidant activity measured by ABTS was correlated with the bioactive compound contents, particularly peptides (r = 0.909, p-value < 0.01). For the germinated-legume tempeh produced from legumes with a root length of 1.0 cm, fermentation increased the TPC, except in germinated red bean tempeh (p-value < 0.05), with germinated tiger-striped peanut tempeh showing the highest TPC at 7.60 mg GAE/g DW (p-value < 0.05). Fermentation also increased the TPEC, with germinated soybean tempeh showing the highest TPEC at 281.23 mg BSA/g DW (p-value < 0.05). However, fermentation caused decreases in the TFC and TPRC compared with the control (p-value < 0.05), while increasing antioxidant activities measured by DPPH and ABTS assays, except in germinated red bean tempeh (p-value < 0.05). This study demonstrates that germination and fermentation can enhance phenolic and peptide contents, particularly in tiger-striped peanuts and soybeans, increasing their potential for application in the development of health-promoting food products.

Keywords: Germination, Fermentation, Germinated-tempeh, Plant-based Protein, Bioactive Compound

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1. Introduction

Legumes and whole grains are widely consumed worldwide, serve as important sources of nutrients, and have essential roles to play in the human diet. They are rich sources of essential nutrients for human daily needs, including carbohydrates, proteins, lipids, vitamins, minerals, and polyphenols. Epidemiological evidence has consistently indicated that eating the recommended intake of whole grains and legumes can reduce the risk of chronic diseases such as cardiovascular disease, diabetes, and obesity [1]. Additionally, legumes are promising sources of sustainable alternative proteins. Legume proteins possess excellent processing properties, including emulsification, gelation, and foaming, leading to their extensive use in the food industry [2]. These properties make legume proteins excellent for the development of healthy and plant-based foods.

Germination or sprouting is a complex biological process driven by phytohormones and enzyme activation, during which the seed absorbs water to transition into an active stage. Consequently, the starch, fat, and protein contained in the seed are hydrolyzed into sugars, fatty acids, and free amino acids and peptide chains during germination [3], [4]. A recent study demonstrated that the germination of lentils and fava beans enhances the release of amino acids and bioactive peptides, which is associated with increased protease activity [5]. Chickpea germination up to 3 days (0.1–0.8 cm root length) increases the peptide content by denaturing chickpea storage proteins, and His-Ala-Lys peptide significantly contributes to the increase in antioxidant activity [6]. The germination of cereal seeds can significantly enhance the levels of bioactive compounds, including free and bound phenolics [7], total flavonoids [8], folate [9], γ-aminobutyric acid, and vitamins [10], by activating the Phenylalanine Ammonia-Lyase (PAL) enzyme, which contributes to increased antioxidant capacity. Meanwhile, levels of antinutritional compounds such as tannins, phytic acid, and oxalate are reduced through germination [4], [11]. James *et. al.* [12] found that germination for 2, 3, and 4 days of cowpea, bambaranut, red bean, pigeonpea, African breadfruit seeds, African yam bean seeds, African oil bean seeds, and groundnuts increased the total phenolics in all samples; however, the levels of carotenoids and anthocyanins decreased as the germination time increased (p-value < 0.05). After three days of germination, there was a significant increase in antioxidant capacities, ranging from 15–54%.

Cereal tempeh is a nutritious fermented legume food resulting from solid-state fermentation. It is originally made from soybeans and commonly consumed in Indonesia and Malaysia. The fermentation process involves the growth of *Rhizopus* spp. especially the *R. oligosporus* strains. The preparatory state of this process involves hydrothermal treatment before the inoculation of the legume with mold spores [13]. The inoculation transforms the legume into a white firm cake-like product as enzymes break down complex nutrients in the seeds into simpler forms, which makes the nutrient-rich food more bioavailable [14]. Fermentation is conducted at a temperature range of 30–34°C for a duration of 24–48 hours [15]. *R. oligosporus* produces five key enzymes, including proteases, lipases, amylases, and cellulases/hemicellulases during the fermentation process, resulting in peptides and amino acids,

glycerol and free fatty acids, simpler sugars, and softening the texture, respectively. These enzymatic actions not only improve the nutritional profile but also enhance its sensory qualities, making it a flavorful and nutrient-rich food [16], [17]. Starzyńska-Janiszewska *et. al.* [13] reported that the soluble antioxidant potential of cooked spelt wheat was enhanced after fermentation with *R. oligosporus* ATCC 64063, on average by 100% (buffer extracts) and 70% (acetone/water 1:1 v/v extracts). Spelt tempeh also contained 25% more soluble phenolic acids, including a 300% higher ferulic acid level. Consumption of tempeh has been linked to various health advantages including antidiabetic effects, cholesterol-lowering properties, improved cognitive function, antitumor and anticancer properties, anti-aging effects, and improved gut health as well as reduced risk of cardiovascular disease [14], [18].

The reviews indicated that germination and fermentation significantly enhance legume nutrition and bioactive compounds. However, studies of the combination of sprouting and biotreatment of legumes are still limited. As a result, four selected famous legumes consumed in Thailand, with contrasting composition profiles, were sprouted and then used to produce tempeh: soybeans (rich in protein, carbohydrates, and fats), mung beans (high in protein and carbohydrates), red beans (high in carbohydrates), and tiger-striped peanuts (abundant in protein and fats). Therefore, this study aimed to examine the influence of germination (root length of 0.2 or 1.0 cm) and fermentation of the legume sprouts by *R. oligosprous* (germinated legume tempeh) on the phenolic, peptide, and antioxidant activity to assess whether germination or the combination of germination and fermentation could produce superior nutritional and functional characteristics. Findings from this study provide guidelines for the development of effective food processing techniques based on partially germinated legume fermentation that offer higher health benefits.

2. Materials and Methods

2.1 Materials

Folin-Ciocalteu's reagent was purchased from LOBA Chemie Ltd. (Maharashtra, India). Gallic acid, quercetin, 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picryl hydrazyl (DPPH), and 2,2′-azino-bis (3-ethyl benzthiaz-oline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Bovine Serum Albumin (BSA) was obtained from Sisco Research Laboratories (Maharashtra, India). Other chemicals used were analytical grade. *R. oligosporus* pure culture code no. 3138 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). Soybeans (*Glycine max* L.), mung beans (*Vigna radiata* L.), and red beans (*Phaseolus vulgaris* L.) were purchased at a local supermarket in Bangkok. Tiger-striped peanuts (*Arachis hypogaea* L.) were purchased from a local farmer in Mae Hong Son province, Thailand.

2.2 Germination of Legumes

Germination followed the method described by James *et. al.* [12] and Hsieh *et. al.* [19] with modification. Legumes were washed using tap water and sterilized using 1.25% sodium hypochlorite with a ratio of 1:5 (w/v) for 5 min and

then washed twice with distilled water. The legumes were soaked in distilled water at an ambient temperature (35 ± 2°C) for 12 h. The soaked seeds were rinsed thoroughly with distilled water. The seeds were spaced 2 cm apart on two layers of food-grade kitchen paper towels and then covered. They were germinated in a plastic airflow basket at 35 ± 2°C in dark conditions. The papers were kept moist by spraying with distilled water every 12 hours. The germination was monitored at 6-hour intervals until the grains reached a root length of 0.2–1.0 cm. After germination, the samples were washed with distilled water. All samples were then oven-dried at 45 ± 2°C, ground, and passed through a 36-mesh sieve. They were kept in a PE Ziplock bag and stored at 4 ± 2°C before extraction for phenolic, flavonoid, protein, and peptide content determination, and antioxidant activity analysis (Figure 1).

2.3 Starter Preparation

The *R. oligosporus* pure culture was inoculated on potato-dextrose agar slants and incubated at 30 ± 2°C for 5 days. The spore suspension, containing approximately 6×10^6 spores/mL, was prepared by washing the PDA slants with 5 mL of sterile 0.1% Tween 80 solution. A 2 mL spore suspension was inoculated into 20 g of sterile rice and incubated at 30 ± 2°C for 3 days. After incubation, the spores and sterile rice were dried at 45 ± 2°C, ground into fine powder, and stored at 4 ± 2°C. This prepared mixture served as the commercial form of the *R. oligosporus* starter.

2.4 Production of Germinated Legume Tempeh

To produce germinated legume tempeh, seeds with root lengths of 0.2 ± 0.1 or 1.0 ± 0.1 cm, which yielded higher levels of bioactive compounds were utilized. The sprouted seeds were washed and then soaked in drinking water for 12 hours. Each seed type was boiled in water for different times to be fully gelatinized: mung beans for 10 min soybeans or red beans for 25 min and tiger-striped peanuts for 40 min. After removing the water and then cooling to room temperature, 100 g of gelatinized seeds were inoculated with 0.2, 0.2, 0.6, and 0.4 g of the commercial form of *R. oligosporus* starter for soybeans, mung beans, red beans, and tiger-striped peanut, respectively, following on from our preliminary study. After thorough mixing, the mixtures were tightly placed in perforated plastic bags to ensure the development of the mycelium. Fermentation was performed at 30 ± 2°C in an incubator, which followed the method of Erkan *et. al.* [15] with a slight modification. In this study, fermentation was conducted at the stage of full mycelium growth, where the mycelium completely covered the legumes and firmly tied them together. This procedure followed our preliminary study regarding inoculation conditions. The fermentation times of the soybean tempeh, mung bean tempeh, red bean tempeh, and tiger-striped peanut tempeh were 27, 27, 30, and 30 hours, respectively. The obtained tempeh was cut into a thin sheet of approximately 3 mm., then dried at 45 ± 2°C before being pulverized and sieved though 36 mesh screen. The samples were stored in a PE Ziplock bag at 4 ± 2°C prior to extraction for phenolics, flavonoids, proteins, peptides, and antioxidant activity analysis (Figure 1).

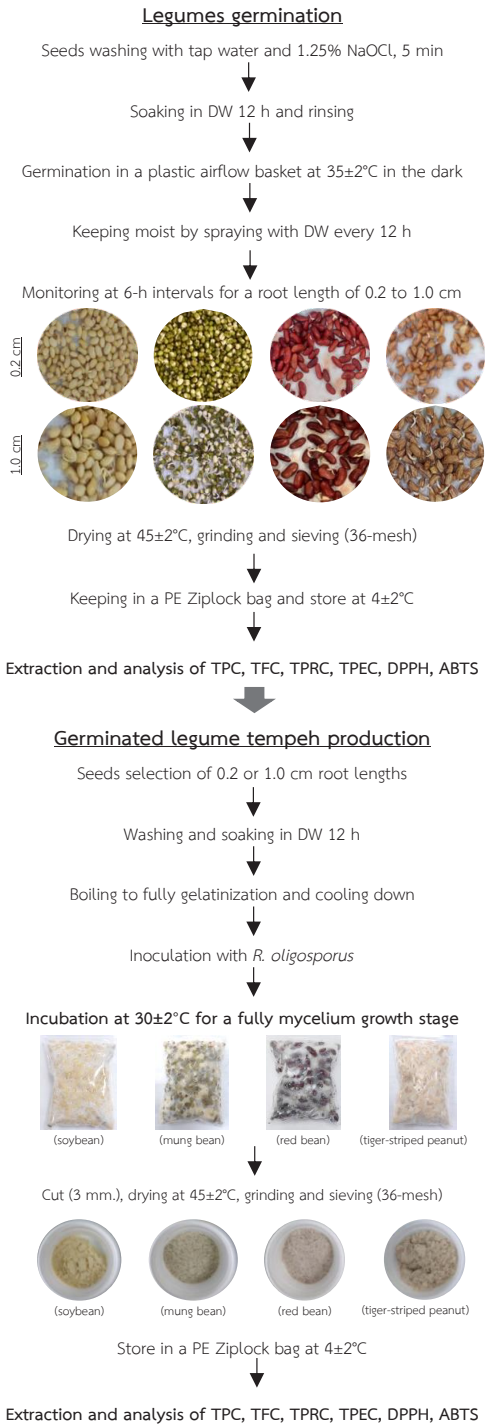

Figure 1 Flow process illustrates the legume germination and the production of germinated legume tempeh in this study.

2.5 Sample Extraction

2.5.1 Extraction for Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity analysis was performed as follows:

A 1 g pulverized sample was mixed with 10 mL of 80% ethanol, shaken at 150 rpm and 30 ± 2°C for 30 min (LT-X incubator shaker, Kuhner, Switzerland) and centrifuged at 4,600×g and 4°C for 10 min (Z383K, HERMLE, Germany). To ensure maximum extraction, the supernatant was collected, and the residues were re-extracted using the same procedure. The extract was adjusted to a volume of 25 mL using the extracting solvent and then stored at 4 ± 2°C.

To remove lipids in the tiger-striped peanut tempeh, 2 g of pulverized sample was twice extracted with 10 mL of acetone by shaking at 150 rpm and 30 ± 2°C for 30 min. After centrifugation at 4,600×g and 4°C for 10 min, the sample was dried in the fume cupboard to evaporate the residual solvent for 30 min before extraction for analysis as mentioned above.

2.5.2 Extraction for Total Protein Content (TPRC) and Total Peptide Content (TPEC) analysis

A 1 g sample was extracted with 10 mL of 10% ethanol (v/v) at 150 rpm and 4°C for 2 h. After centrifugation (4,600×g, 10 min, 4°C) supernatants were collected, adjusted for final volume, stored at 4 ± 2°C, and used for further analysis [20].

2.6 Bioactive Compound and Antioxidant Activity Analysis

2.6.1 Determination of TPC

TPC was determined following the method described by Saikaew *et. al.* [21]. A 50 µL sample (or standard solution) was mixed with 500 µL of distilled

water and 50 µL of Folin Ciocalteu's phenol reagent, and 3 min later, 500 µL of Na_2CO_3 (10% v/v) was added. After 30 min in the dark, the absorbance at 725 nm was measured using a spectrophotometer (UV-1900i, Shimadzu, Japan). The blank was the extracting solvent. The results were expressed as Gallic Acid Equivalents (GAE) in mg/g Dry Weight (DW).

2.6.2 Determination of TFC

TFC was determined using the method described by Saikaew *et. al.* [21] with minor modifications. 700 µL of distilled water and 50 µL of 5% $NaNO_2$ were mixed with the extracts (300 µL), and 5 min later, 50 µL of 10% $AlCl_3$ solution was added. The mixture was allowed to stand for another 5 min, and 50 µL of 1 M NaOH was then added. The reaction solution was well mixed and maintained in the dark for 15 min, and the absorbance at 415 nm was determined. The TFC was calculated using the standard quercetin curve and was expressed as Quercetin Equivalents (QE) in mg/g DW.

2.6.3 Determination of TPRC

Protein concentration was determined using the dye-binding method Bradford (1976) [22]. The sample (25 µL) and Bradford reagent (1.2 mL) were mixed and left in the dark for 15 min before the absorbance was read at 595 nm. A standard curve was constructed using Bovine Serum Albumin. Protein content was expressed as mg BSA/g DW.

2.6.4 Determination of TPEC

The peptide concentration in the extracts was determined with the Folin phenol protein quantitative assay following the method of Wang *et. al.* [23] with a slight modification. A 0.2 mL sample of each extract was mixed with 1 mL of the alkaline copper reagent. After incubation for 10 min, 0.1 mL of Folin-Ciocalteu reagent was added, and the mixture was shaken immediately. The mixture was left to stand at room temperature for 20 min, and the absorbance was measured at 680 nm. The peptide content was calculated using a BSA standard. The results were expressed in mg BSA/g DW.

2.6.5 Measurement of antioxidant activity

The DPPH free-radical scavenging assay was carried out using the method described by Saikaew *et. al.* [21] with minor modification. A DPPH radical solution was prepared by mixing 24 mg of DPPH with 100 mL of ethanol. The mixture was then left in the dark for 12–16 hours. The solution was adjusted to an absorbance of 0.80 ± 0.02 at 515 nm and left for 1 hour before use. Next, a 1.2 mL aliquot of the DPPH working solution was mixed with 30 µL of each sample. After incubation in the dark for 30 min, the absorbance was measured at 515 nm. The antioxidant capacity was calculated using the standard Trolox curve, and the results were expressed as micromoles of Trolox equivalents per gram of dry weight (µmol TE/g DW).

The ABTS free-radical scavenging assay was slightly modified from that described by Re *et. al.* [24]. A 7 mM ABTS was combined with 2.45 mM potassium persulfate in a 2:1 ratio to generate the ABTS radical cation ($ABTS^{\bullet+}$). The working solution was diluted to achieve an absorbance of 0.70 ± 0.02 at 734 nm using phosphate-buffered saline (1x PBS) and left to equilibrate for 1 h before use. For the assay, 50 µL of the sample extract was mixed with 1 mL of the working solution, and the reaction was left in the dark for 6 min before measurement. The results were expressed as µmol TE/g DW.

2.7 Statistical Analysis

The results were expressed as means ± standard deviations. The data were analyzed using SPSS software (version 29, SPSS Inc., Armonk, NY, USA). The means were compared by analysis of variance and Duncan's multiple range test. Paired-sample t-tests were performed to evaluate the significant differences between two samples. Comparisons with p-values less than 0.05 were considered to indicate that the means were significantly different. Pearson's correlation analysis was performed to analyze the correlations among variables.

3. Results and Discussion

3.1 Effects of Germination on the Bioactive Compounds and Antioxidant Activities of Four Legumes

It was found that mung beans needed the shortest time to achieve 0.2- and 1.0-cm root lengths, which were 6 and 12 hours, respectively. Meanwhile, tiger-striped peanuts, red beans, and soybeans required 12, 24, and 30 hours to achieve 0.2 cm root length, respectively, and 36 hours for 1.0 cm root length (Table 1). The germination rate of legumes varies based on seed type and is influenced mainly by genetic and physical characteristics under the same conditions of temperature, moisture, oxygen, and light. The different seed species contain varying fat, protein, and carbohydrate levels. During germination, these macromolecules break down into fatty acids, peptides, free amino acids, nitrogen, carbon, and glucose, which are essential for seed growth. The mobilization efficiency of these reserves during germination depends on hormone signaling pathways, including gibberellin, abscisic acid, and ethylene [25]. Additionally, a hard seed coat can impede water absorption, which has a significant impact on germination. Due to these factors, the germination time of mung beans was shorter than that of tiger-striped peanuts, red beans, and soybeans. To enhance the germination rate of legumes, Chen *et. al.* [26] found that ultrasound pretreatment shortened the soybean sprouting time by 24 hours after a dual-frequency treatment of 20/60 kHz compared to the control group, which could have been due to the formation of significant cracks and holes in the seed coat after ultrasonication, leading to accelerated water absorption. Furthermore, He *et. al.* [27] reported that a 10 mT static magnetic field applied during the final hydration stage enhanced mung bean seed germination, and that 4 mg/L selenium and 20 mg/L chromium concentration also promoted germination. A mixture of selenium and chromium at a volume ratio of 1:2 resulted in a 1.30-fold increase in germination potential and a 1.25-fold increase in the germination index compared to the control.

Table 1 Root length and germination time

Legumes	Root Length (cm)	Germination Time (hour)
Soybean	0.2 ± 0.1	30
	1.0 ± 0.0	36
Mung bean	0.2 ± 0.0	6
	1.0 ± 0.0	12
Red bean	0.2 ± 0.1	24
	1.0 ± 0.1	36
Tiger-striped peanut	0.2 ± 0.0	12
	1.0 ± 0.0	36

Note: Root length presented as means ± SD (n=20)

The results indicated that both bean variety and germination had a significant effect on the levels of bioactive compounds and antioxidant activity. As shown in Figure 2(a), longer root lengths yielded more TPC, except in the case of soybeans (p-value < 0.05). The 1.0 cm root length mung beans and tiger-striped peanuts had significantly higher TPC than did the control (p-value < 0.05). Tiger-striped peanuts and soybeans at 1.0 cm root length provided high TPC values of 3.53 and 3.36 mg GAE/g DW, respectively. Meanwhile, overall, legumes with 1.0 cm roots had significantly higher TFC than did the 0.2 cm and control, except in the case of mung beans (p-value < 0.05). Tiger-striped peanuts and soybeans at 1.0 cm root length provided high TFC levels of 43.75 and 43.06 mg QE/g DW, respectively (Figure 2(b)). Similarly, in earlier work, germination increased the phenolic or flavonoid content of chickpeas, red lentils, mung beans [8], and soybean [28]. The increment of TPC and TFC during germination probably occurred due to an increase in the endogenous enzymes Phenylalanine Ammonia Lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate coenzyme A ligase (4CL), which led to the biosynthesis of different phenolic components [28].

TPRC tended to decrease during germination compared to the control samples, except in the case of red beans, but for soybean and mung bean, the samples with 1.0 cm root length had higher levels of TPRC than those with 0.2 cm root length. Tiger-striped peanuts contained the highest TPRC level for all root lengths (Figure 2(c), p-value < 0.05). Similarly, TPEC tended to decrease during germination compared to the control samples, except in the case of soybean, but for soybean and red bean, the samples with 1.0 cm root length had higher levels of peptide than those with 0.2 cm root length (Figure 2(d), p-value < 0.05). Changes in nutritional composition associated with metabolic activities within the seed that produced simpler compounds from storage proteins and carbohydrates that were needed to support the growing embryo during the germination process may well have led to the decline in TPRC and TPEC [8]. In contrast, Wintersohle *et. al.* [29] observed fluctuations in protein content during mung bean germination. They observed that protein increased to 24.95% after 32 hours and then decreased to 21.02% after 48 hours. These changes were likely attributable to the hydrolysis of stored proteins by proteolytic enzymes during germination, which resulted in the release of amino acids that could be used to synthesize new proteins or remain free. Therefore, an increase in peptides during germination could be related to germination time and legume variety.

As shown in Figure 3(a), the DPPH activity during germination increased in comparison with the control samples, except in the case of the tiger-striped peanuts at a root length of 1.0 cm. A lower DPPH activity of the 1.0 cm root length legume sprouts compared to the 0.2 cm was observed in soybeans, red beans, and tiger-striped peanuts (p-value < 0.05), while a slight increase was observed in mung beans (p-value ≥ 0.05). Similarly, the ABTS activity of germinated soybean, mung bean, and red bean legumes increased slightly when compared to the control samples (p-value ≥ 0.05). However, the activity significantly declined at a root length of 1.0 cm in tiger-striped peanuts (Figure 3(b), p-value < 0.05).

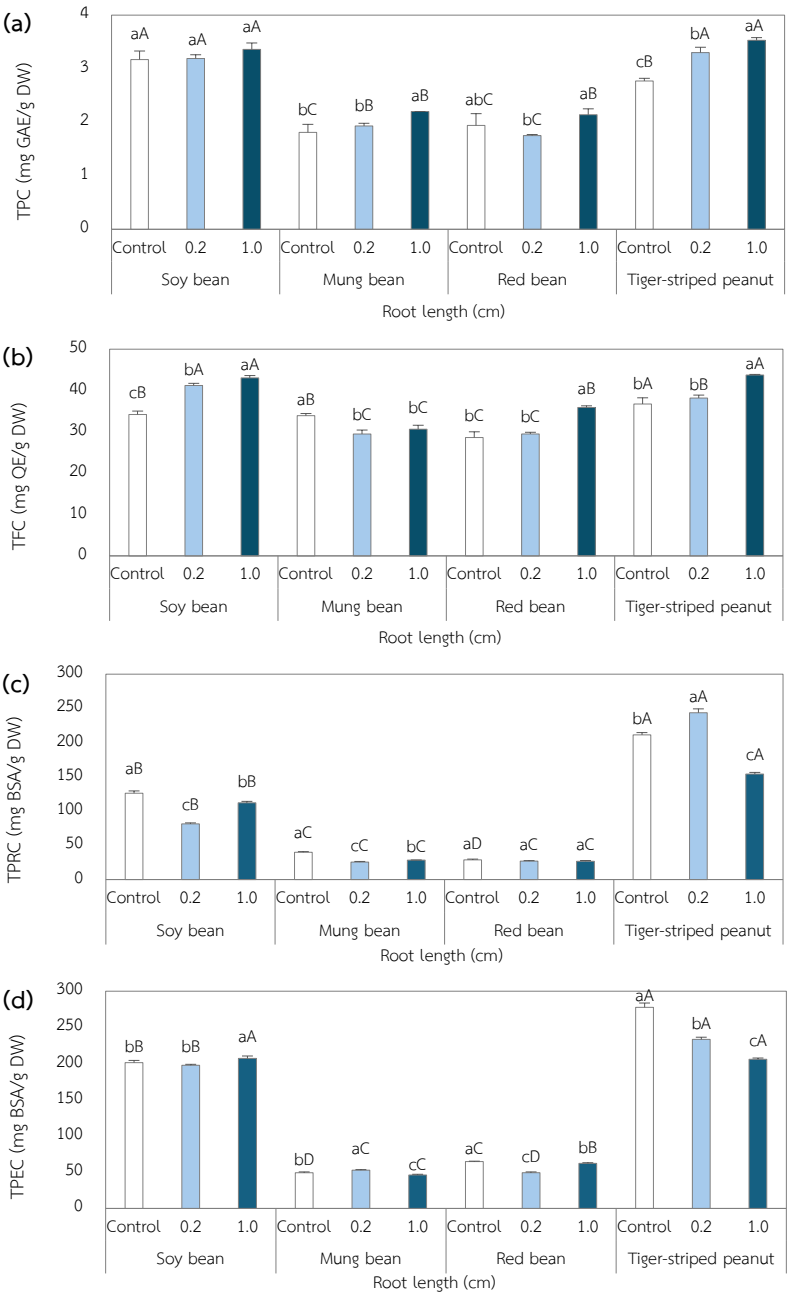

Figure 2 Total Phenolic Content (TPC), (a) Total Flavonoid Content (TFC), (b) Total Protein Content (TPRC), (c) Total Peptide Content (TPEC), (d) of the germinated legume extracts. Data are presented as means ± standard deviations (n=3). Different lowercase letters indicate significant differences among root length within the same bean, and the different capital letters indicate significant differences among beans within the same root length (p-value < 0.05). Control represents the initial root length of the sample (0 cm) at 0-hour germination time.

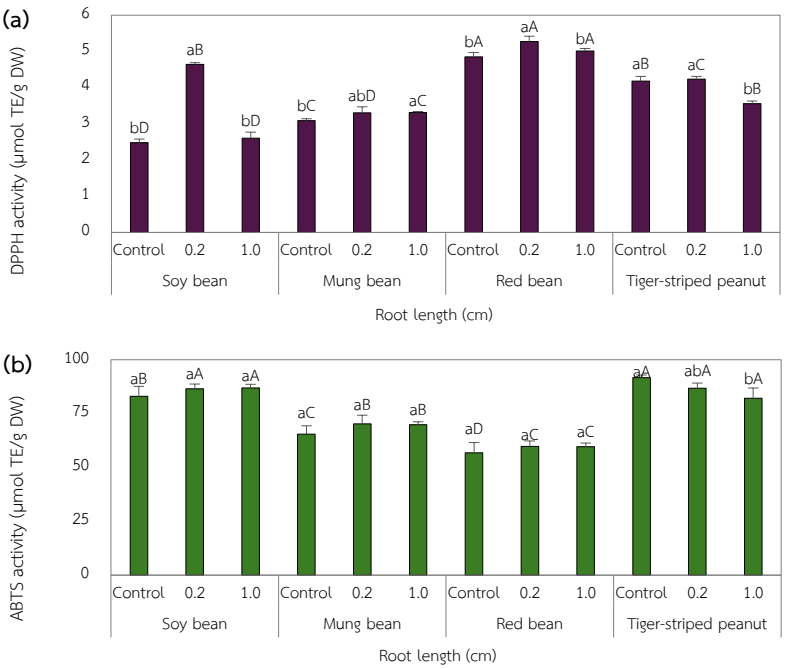

Figure 3 Antioxidant activity determined by DPPH (a) and ABTS (b) assays of the extracts. Data are presented as means ± standard deviations (n=3). Different lowercase letters indicate significant differences among root lengths within the same bean, and the different capital letters indicate significant differences among beans within the same root length (p-value < 0.05). Control represents the initial root length of the sample (0 cm) at 0-hour germination time.

The study showed that all four sprouted legume varieties with a root length of 1.0 ± 0.1 cm had higher levels of TPC and TFC compared to those with a root length of 0.2 ± 0.1 cm. As a result, seeds with a 1.0 ± 0.1 cm root length were used in the germinated legume tempeh study.

The correlations among the bioactive compound contents and antioxidant activities are presented in Table 2. The TPC exhibited a high positive correlation with the TFC, TPRC, TPEC, and ABTS results (r = 0.837–0.875, p-value < 0.01). The TPRC exhibited a high positive correlation with the TPEC and ABTS results (r = 0.807–0.911, p-value < 0.01). Moreover, TPEC exhibited a high positive correlation with the ABTS results (r = 0.909, p-value < 0.01). Meanwhile, a moderate positive correlation was found among TFC, TPRC, TPEC, and ABTS results (r = 0.587–0.729, p-value < 0.01). Moreover, a low positive correlation was observed among root length, TPC, and TFC (r = 0.238–0.409). These findings indicated that the TPC levels were representative of the flavonoid, peptide, and antioxidant activities determined by the ABTS assays for the four germinated legumes, and the peptide contents were effectively indicated by the ABTS activity in the samples. Bioactive peptides from fermented foods possess antioxidant properties and exhibit antihypertensive and antibacterial effects [30]. This finding agrees with the results

Table 2 Pearson's correlation coefficients (*r*) for correlations among bioactive compound contents, antioxidant activities, and root lengths of four legumes during germination

Variables	TPC	TFC	TPRC	TPEC	DPPH	ABTS	RL
TPC	1.000						
TFC	0.844**	1.000					
TPRC	0.757**	0.58**7**	1.000				
TPEC	0.875**	0.729**	0.911**	1.000			
DPPH	–0.332*	–0.212	–0.149	–0.199	1.000		
ABTS	0.837**	0.696**	0.807**	0.909**	–0.398*	1.000	
RL	0.238	0.409*	–0.117	–0.070	–0.134	–0.006	1.000

Note: * and ** indicate that the correlation is significant at *p*-value < 0.05 and *p*-value < 0.01, respectively.
TPC, total phenolic content; TFC, total flavonoid content; TPRC, total protein content; TPEC, total peptide content; DPPH, DPPH free-radical scavenging assay; ABTS, ABTS free-radical scavenging assay; RL, root length.

reported by Starzyńska-Janiszewska *et. al.* [13], who found that the TPC of spelt wheat tempeh fermented with *R. oligosporus* strongly correlated with antioxidant activity analyzed by ABTS (r = 0.975).

3.2 Effect of *R. oligosporus* Fermentation on the Bioactive Compounds and Antioxidant Activities of Germinated Legumes

Tempeh production varies based on the type of legume. The nutritional content of each legume, including protein, starch, fiber, fat, and minerals content, influences the tempeh fermentation time because they are crucial in promoting microbial growth during fermentation [31]. Additionally, a physical barrier, with or without a legume seed coat, could hinder the growth of *R. oligosporus* by affecting nutrient assimilation. In this study, the full mycelium growth stage, typically the consuming stage, was used for each type of legume. This approach resulted in varying inoculation conditions of *R. oligosporus* and fermentation times across the four legumes, which were based on our preliminary study.

Changes in the TPC of germinated legume tempeh by *R. oligosporus* are illustrated in Figure 4(a). The TPC of germinated tempeh samples was higher than that of the control samples, except for the case of germinated red bean tempeh (*p*-value < 0.05). The germinated tempeh from tiger-striped peanuts had the highest level of TPC, followed by soybean, mung bean, and red bean, respectively (*p*-value < 0.05). The percentage TPC increases in germinated legume tempeh compared to the control sample (the germinated beans at 0-hour fermentation time) of tiger-striped peanuts, soybeans, mung beans, and red beans were 115%, 71%, 50%, and 0%, respectively. In contrast, tempeh fermentation caused a dramatic decrease in the TFC for all samples (Figure 4(b), *p*-value < 0.05). The TPC results of this study were similar to those reported by Lim *et. al.* [32], who reported that TPC increased in wild turmeric fermented with *R. oligosporus*. The increase in TPC during *R. oligosporus* fermentation could have been due to α/β-amylase, β-glucuronidase, and β-glucosidase, which break down the cell

wall matrices and release polyphenols from carbohydrate-conjugated phenolic compounds. Similarly, fermentation with the lactic acid bacteria (*Latilactobacillus delbrueckii* subsp. *bulgaricus*) increased the TPC and TFC in quinoa sprouts due to the activity of glycosidases and polyphenol oxidases during fermentation [33]. Meanwhile, the decrease of TFC during fermentation may have resulted from microbial enzyme activity, which can lead to either an increase or decrease in flavonoids [12]. Moreover, the decrease in TFC in germinated red bean tempeh may have been associated with the degradation of anthocyanins during the boiling process involved in preparing the legumes for tempeh fermentation, as well as with the leaching associated with their water-soluble properties.

The TPRC of germinated legume tempeh decreased for all samples when compared to the controls (Figure 4(c), p-value < 0.05). On the other hand, the TPEC of the germinated legume tempeh increased for all samples when compared to the controls (Figure 4(d), p-value < 0.05). The germinated legume tempeh from soybeans had the highest level of TPEC, followed by tiger-striped peanuts, mung beans, and red beans, respectively (p-value < 0.05). The percentage TPEC increases in the germinated soybean, tiger-striped peanut, mung bean, and red bean tempeh samples compared to the control (the germinated beans at 0-hour fermentation time) were calculated as 36%, 23%, 215%, and 43%, respectively. Similarly, Zhang *et. al.* [34] found that the protease produced by *R. oligosporus* played a crucial role in converting proteins into small peptides and amino acids during the fermentation of soybeans into tempeh, resulting in an increase in peptides within the tempeh. Song *et. al.* [35] noted that the Folin reagent method for total peptide measurement may interfere with the detection of phenols, citric acid, and sulfhydryl compounds present in the samples. To enhance accuracy by removing polyphenols, polyvinylpolypyrrolidone (PVPP) can be used for sample cleanup. Additionally, the ninhydrin, trinitrobenzene sulfonate (TNBS), and o-phthaldialdehyde (OPA) methods can also be employed.

The change of antioxidant activity measured by the DPPH assay of germinated legume tempeh corresponded to the ABTS assay results (Figure 4(e) and 4(f)). The results showed that the DPPH and ABTS activities of the germinated tempeh significantly increased compared to the control samples (p-value < 0.05), except in the case of red bean tempeh. The percentage increase in DPPH activity for germinated soybean, mung bean, and tiger-striped peanut tempeh samples compared to the control was calculated at 80%, 103%, and 52%, respectively. Additionally, the percentage increases in ABTS activity for these germinated varieties were 33%, 26%, and 41%, respectively, compared to the control. Meanwhile, the decrease in DPPH and ABTS activities in germinated red bean tempeh could have been due to the loss of anthocyanins. The results indicated that antioxidant activity of germinated legume tempeh was related to the bioactive compounds present. Liu *et. al.* [17] reported that soybean tempeh fermentation increased isoflavone aglycone content by more than 20 times that of unfermented seed, and correlation between isoflavone aglycone content and FRAP and DPPH assayed antioxidant activities were found (p-value < 0.05).

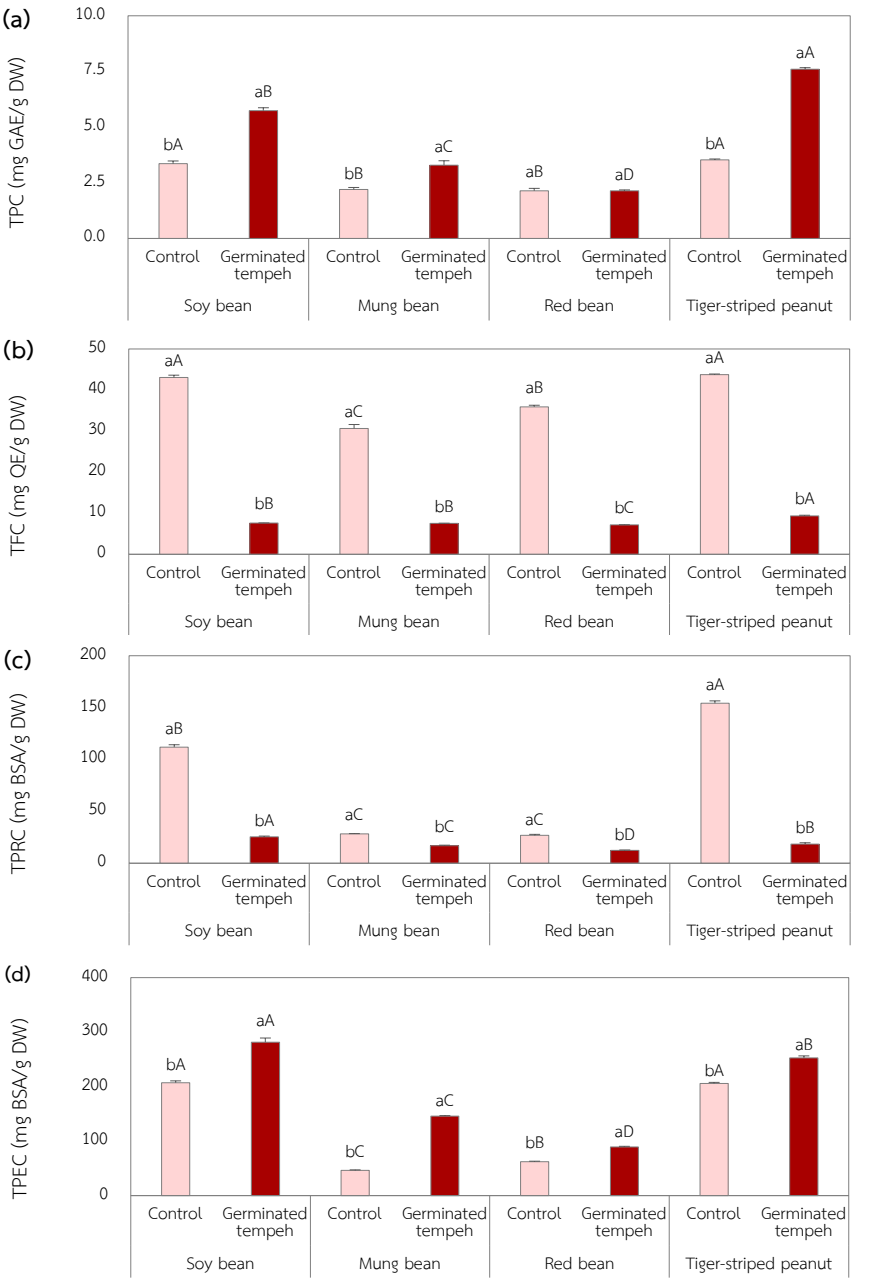

Figure 4 Total Phenolic Content (TPC), (a) Total Flavonoid Content (TFC), (b) Total Protein Content (TPRC), (c) Total Peptide Content (TPEC), (d) DPPH activity, (e) and ABTS activity, (f) of the germinated tempeh extracts. Data are presented as means ± standard deviations (n=3). Different lowercase letters indicate significant differences between the same bean, and the different capital letters indicate significant differences among beans within the same condition (p-value < 0.05). Control represents the germinated beans with a root length of 1.0 ± 0.2 cm at 0-hour fermentation time.

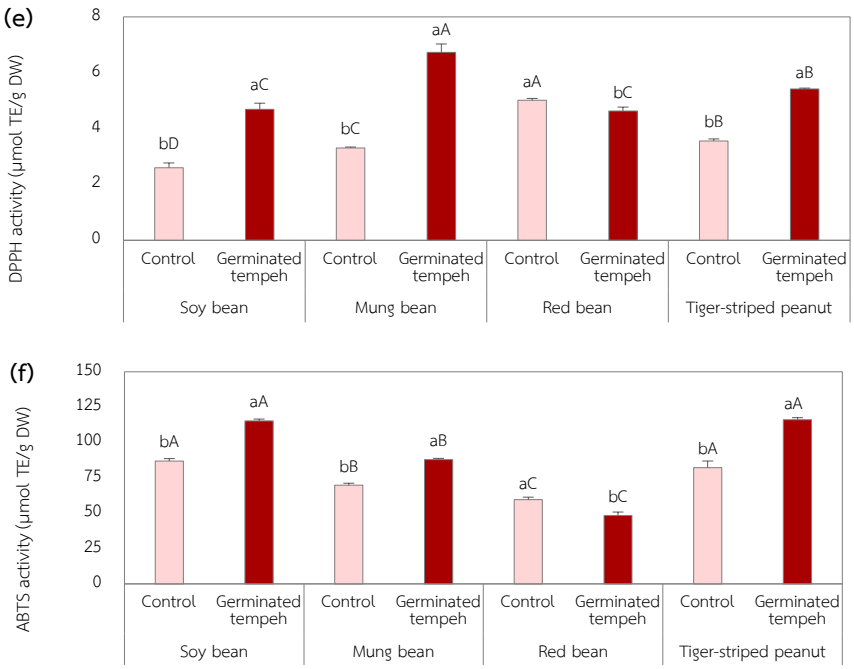

Figure 4 (*Continued*) Total Phenolic Content (TPC), (a) Total Flavonoid Content (TFC), (b) Total Protein Content (TPRC), (c) Total Peptide Content (TPEC), (d) DPPH activity, (e) and ABTS activity, (f) of the germinated tempeh extracts. Data are presented as means ± standard deviations (n=3). Different lowercase letters indicate significant differences between the same bean, and the different capital letters indicate significant differences among beans within the same condition (p-value < 0.05). Control represents the germinated beans with a root length of 1.0 ± 0.2 cm at 0-hour fermentation time.

Our results indicated that combining germination of sprouts to a root length of 1.0 cm and fermentation by *R. oligosporus* in the soybean, mung bean, and tiger-striped peanut sprouts effectively enhanced the levels of phenolic compounds, peptides, and antioxidant activities as determined by DPPH and ABTS assays. The increase in phenolic compounds during the fermentation of sprouts primarily resulted from the breakdown of cell walls, and subsequent activities of the enzyme, β-glucosidase produced by *R. oligosporus*, which converted the bound phenolics into free forms, enhancing the availability of phenolics and antioxidant activity [36]. According to Lim *et. al.* [37], solid-state fermentation with *R. oligosporus* for 3 days produced efficient enzymes that degraded cell wall matrices and thus increased the TPC of wild-simulated ginseng leaf. Meanwhile, the enhancement of peptides could have been attributed to the partial hydrolysis of proteins during the germination of 1.0 cm root length sprouts, which activated the protease enzymes produced by *R. oligosporus* during tempeh

fermentation. This process led to a higher concentration of peptides. Similarly, Hsieh *et. al.* [38] found an increase in free peptide content during the fermentation of *Chenopodium formosanum* (Djulis) sprouts by *R. oligosporus*, using both traditional plate and bioreactor fermentation methods.

4. Conclusions

We can conclude that germination significantly enhanced the TPC, TFC, and antioxidant activity of the legumes. A strong positive correlation was observed among TPC, TFC, TPRC, TPEC, and ABTS results in germinated legumes (p-value < 0.01). Additionally, fermentation by *R. oligosporus* effectively enhanced the TPC, TPEC, and antioxidant activities of the germinated legumes as measured by DPPH and ABTS assay. This study suggests that germination is an inexpensive method for producing phenolic compounds, while fermentation of legume sprouts effectively enhances both phenolics and peptides, especially in tiger-striped peanut and soybean sprouts. The increase of peptides in fermented sprouts may result from the partial degradation of protein structures during germination, which activates protease activity in fermentation. Therefore, these methods, individually or especially in combination, have the potential to produce legumes and legume-based foods of improved nutritional value, making them worthy of further investigation into the functional properties and sensory qualities of the samples and extracts for food applications.

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