Quantitation of antioxidant levels of soy-fern fermentation by DPPH assay

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Received: June 6, 2023 || Revised: March 1, 2024 || Accepted: 23 July 2024

ISSN: 1656-4707
E-ISSN: 2467-5903
Homepage: www.palawanscientist.org

How to cite:
Opena ELL, Cempron M, Remon J, Dela Cruz R and Guillena J. 2025. Quantitation of antioxidant levels of soy-fern fermentation by DPPH assay. The Palawan Scientist, 17(1): 26-34. https://doi.org/10.69721/TPS.J.2025.17.1.04

ABSTRACT

Fermentation biotechnology is one of the approaches to addressing the issues of food security worldwide, where the demand for healthier and safer foods is becoming mainstream. Even though fermentation has been practiced since ancient times, there are still an infinite number of topics that can serve as subjects for fermentative investigation. Among the popular nutraceutical research is the antioxidant properties of plants. In this study, the radical scavenging activities of soy-fern fermentation by mixed probiotics (13 species) have been quantified using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Four treatments were set up: soy-fern-probiotics, soy-fern, soy-probiotics, and soy. These treatments were fermented (submerged) for up to 72 h. Samples were taken at 0 h, 24 h, 48 h, and 72 h and were analyzed via a 96-well plate microplate photometer. Results showed that the production of antioxidants peaked at 24 h in soy-fern-probiotics and soy-fern (82.82% and 82.77%, respectively), suggesting that the presence of fern molecules could have affected the production of antioxidant molecules. It is also observed that the probiotics have less impact on the antioxidant levels. The analysis of variance (ANOVA) showed that there is a significant difference in this timeframe when compared to other timeframes. On average, the succession of antioxidant levels is as follows (highest to lowest): 24 h, 48 h, 0 h, 72 h. Overall, the level of antioxidants depends on the substrates, fermenting microorganisms, type of fermentation, and fermentation time. More studies on this matter are highly recommended.

Keywords: antioxidants, fiddlehead fern, soybean powder

INTRODUCTION

In recent years, there has been a shift in food research, where the focus has moved from being a source of energy to its role in the total well-being of an individual. This new focus is secondary to the growing interest of consumers in consumables that have something to do with the prolongation of life by preventing the development of chronic diseases such as cancer, obesity, and diabetes to name a few. Functional foods, or those foods that contain a nutritional value for maximal health impact when consumed regularly, are favored over their synthetic counterparts since the latter foods are traced to have certain degrees of negative health impacts (Cencic and Chingwaru 2010; Granato et al. 2010; Lobo et al. 2010; Gul et al. 2016). With this steady interest in functional foods, the agricultural sector has also seen

an increase in its contribution to the economy by supplying and processing raw materials (Bigliardi and Galati 2013), as well as in healthcare, as it is expected to reduce the number of hospitalizations in the coming years (El Sohaimy 2012).

Amid the desire for more efficient production of functional foods, fermentation biotechnology is arguably at the forefront. According to Zhu and Tramper (2013), the innovations in food biotechnology that can be seen today, particularly fermentation, have their roots in traditional food processing techniques; this means that the majority of the foods under investigation was already known since ancient times, only that there have been modifications to how they are prepared to include the use of new additives. The process of fermentation involves the utilization of microorganisms, specifically bacteria, and yeasts, that can improve the quality and safety of the food product, particularly its nutritional values, flavor, aroma, shelf life, and texture, and at the same time decrease the levels of anti-nutrition compounds (Hugenholtz 2013; Mukherjee et al. 2016; Leonard et al. 2021). Due to the realization that fermented products hold potential molecules that can address nutritional concerns, they are now considered more than just a side dish (Shin and Jeong 2015).

One of the foods that have been traditionally fermented is *Glycine max*, commonly known as soy and is known to contain one of the highest level of proteins that benefit both humans and livestock. The crop is known to have originated in East Asia and has been fermented in different ways, thus producing different products. Soymilk, soy sauce, natto, and tempeh are just a few examples of fermented soy products that are now produced worldwide (Cao et al. 2019). According to Jayachandran and Xu (2019), fermented soy unlocks more nutrition than its non-fermented counterparts. This means that microorganisms were able to maximize the transformation of soy components into more molecules that can have a positive nutritional impact, particularly its bioactive peptides, through the hydrolysis of proteins from soybeans (Sanjukta and Rai 2016).

Ferns, on the other hand, are also the subject of fermentation studies. Products from fermented ferns show nutraceutical potential besides having insecticidal and fungicidal properties that help improve livestock and human gut health (Tamang et al. 2016; Mala et al. 2019).

The fern *Diplazium esculentum* Retz. is a common fern found in regions across Asia that have long been one of the food sources of different societies and cultures. Besides its role as an energy source, it is also known to have both pharmaceutical and nutraceutical potentials. It is considered to contain molecules that are antidiabetic, antioxidant, and proteins, can prolong shelf life, and can improve the sensory acceptability of certain food products (Saha and Deka 2017; Junejo et al. 2018; Samad et al. 2022). Thus, the addition of fern to already known food and its respective processes can enhance the overall quality of food in terms of its nutritive value.

As mentioned earlier, fermentation is primarily made possible through the complex metabolic interactions of microorganisms. Yeasts or bacteria act on biomolecules and transform them into other molecules that can enhance the components of a fermented product. Probiotics, a specific group of bacteria that improve human gut health, have already been utilized for fermentative production. For instance, *Lactobacillus plantarum*, a known probiotic, was employed to ferment soy in a study by Xiao et al. (2015). The study concluded that *L. plantarum* was able to elevate the antioxidant potential of soybean.

As the interests in newer non-synthetic functional foods continue to elevate, novel approaches must be utilized both in research and biotechnological designs. Devanthi and Gkatzionis (2019) emphasized that there should be continuous investigations into the fermentative processes that can maximize the nutritive potential of plants, including the search for new approaches to identifying starter cultures (pure and mixed) and the addition of other raw materials. Currently, the fermentation of soy and fern powders by mixed probiotics, particularly in powdered form, is not well reported. This paper therefore seeks to quantify the product of the fermentation of soy and fern powders through known probiotics in terms of their antioxidant properties via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay approach, which is currently one of the most reliable strategies in antioxidant property analysis.

METHODS

Fern Powder Preparation

The protocol used in fern powder preparation follows the work of Ang et al. (2022). Approximately 3 kg of the fern *D. esculentum* were collected from the fernery of Central Mindanao University, Maramag, Bukidnon. The ferns were obtained using pruning scissors and placed inside a plastic container. These samples were then brought to the laboratory of Tuklas Lunas Development Center of the university.

The samples were immediately washed with running tap water twice, and then rinsed with distilled water twice. Water from the samples was then drained for 10 minutes, and then weighed. After weighing, the samples were uniformly distributed inside the laboratory's air dryer and air-dried for 96 h. The samples were then placed in the oven and subjected to a temperature of 50 ºC for 4 h. Before the samples were removed from the oven for milling, they were tested for moisture content and found to have a reading

of less than 10% moisture content. The finely milled fern powder was stored in a clean plastic container.

Soy Powder Preparation

Freshly milled soybean powder (*Glycine max* L.) was purchased from a local grain store in a nearby farmer's market. The soybean powder was then stored in a plastic container and brought to the laboratory.

Probiotics Dilution

Probiotics packed from Atomy Probiotics 10+ served as the fermenting agent for this study. Each pack has 12 known probiotics which include *Bifidobacterium* spp. (*B. breve, B. bifidum, B. longum, B. lactis)*, *Lactobacillus* spp. (*L. rhamnosus, L. casei, L. plantarum, L. helveticus, L. acidophilus, L. paracasei, L. fermentum*), and *Streptococcus thermophiles.* One pack, which contains approximately 3 billion bacterial cells, was dissolved in 999 mL of ultra-pure water, making a dilution of approximately 3 million cells per ml.

Fermentation

Submerged, anaerobic fermentation was employed in this study. Here, four treatments with three replicates per treatment were set up with the following compositions:

Treatment A: Soybean with fern

A_1 (with probiotics): 1 g soybean powder + 1 g fern powder + 1 ml probiotics solution + 37 ml sterile distilled water

A_2 (without probiotics): 1 g soybean powder + 1 g fern powder + 38 ml sterile distilled water

Treatment B: Soy without fern

B_1 (with probiotics): 1 g soybean powder + 1 ml probiotics solution + 38 ml sterile distilled water

B_2 (without probiotics): 1 g soybean powder + 39 ml sterile distilled water

Each treatment was placed in 50 ml conical tube and sealed with a tube cap for submerged fermentation. The samples were centrifuged for 80 s at 1860 rpm, and then incubated at 37 ºC. One milliliter of each sample was taken and stored in a 2.0 ml microfuge tube for DPPH assay at the following time frames: 0 hours, 24 hours, 48 hours, and 72 hours. Seventy-two hours of fermentation is considered by Oyewole et al. (2001) to be one of the peaks of certain fermentation processes.

DPPH Assay

To quantify the antioxidant content of the fermented materials, the DPPH assay was employed (Porquis et al. 2018). The DPPH radical scavenging percentage was obtained following the formula by Shah and Modi (2015), where A_0 is the scavenging value of the ascorbic acid as a positive control (PC), and A_S is the value of the extracts:

$$\%DPPH\ radical\ scavenging\ activity = [\frac{A0 - AS}{A0})]\ x\ 100$$

A_S was generated using the following formula:

$$AS = (APC - ASB)$$

Where: $APC = PC + extract$
and $ASB = solvent + extract$

The solvent used was 95% ethanol, and the values for radical scavenging were generated in a 96-plate Thermo Scientific Multiskan GO version 100.40.

The schematic diagram of the experimental set-up is shown in Figure 1.

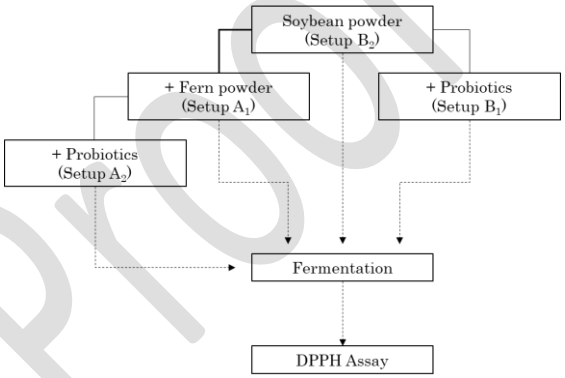

Figure 1. Flow of the experimental approach for setting up the fermentation process of the different treatments (soy-fern-prebiotics (A2), soy-fern (A1), soy-probiotics (B1), soy (B2)).

Statistical Analysis

The analysis of variance (ANOVA) was employed to determine the degree of variability of the radical scavenging properties among different treatments as well as across different timeframes. The degree of variability was also determined in each of the treatments to timeframes. A pairwise comparison was further employed for treatments that showed statistical significance using either $P < 0.05$ or $P < 0.001$. This comparison was used to further compare different timeframes. For data visualization, the program Python was used.

RESULTS

Antioxidant Levels Across Different Treatments on Different Timeframes

Figure 2 shows the pattern of the radical scavenging activities of different treatments across different timeframes based on the DPPH assay. Here, it shows that soy-fern-probiotics and soy-fern treatments have similar patterns where, at 24 h, the

antioxidant properties are at their highest, ranging from 82.82% to 82.77% (soy-fern-probiotics and soy-fern, respectively). From these peaks, both treatments tend to have reduced their antioxidant properties (72.37–37.43%for soy-fern probiotics, and 73.16–48.99%for soy-fern). Furthermore, for these two treatments, it is noteworthy that 0 h has higher antioxidant levels compared to the levels after 72 h of fermentation.

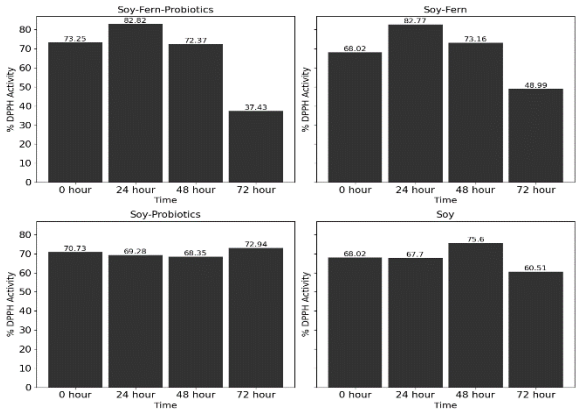

Figure 2. Comparative radical scavenging activities of different treatments across 4 fermentation timelines between the four treatments (soy-fern-prebiotics, soy-fern, soy-probiotics, soy) (in percentage).

Unlike the previous two treatments, soy-probiotics and soy seem to have established patterns in terms of the levels of antioxidant production across the timeframes. For soy-probiotics, the lowest is at 48 h at 68.35% and the highest in 72 h at 72.94%. Meanwhile, in soy, the lowest antioxidant level is during 72 h with 60.51%, and the highest is during 48 h with 75.6%. It can be noted that soy-probiotics and soy display higher antioxidant levels at 72 h compared to the previous two treatments.

Two points can be of interest in this result. First, the presence of ferns in the first two treatments can potentially change in the patterns of the levels of antioxidants in the timeframes, as well as their higher radical scavenging activities compared to treatments without ferns. Secondly, the levels of antioxidants in soy-probiotics, and soy seem to be steady within the timeframe, especially in the first three timeframes. This could mean that if more timeframes are extended (>72 h), more distinct patterns of antioxidant levels could be established. But even with the absence of the aforementioned patterns, the antioxidant levels of soy-probiotics and soy are high, especially going up to 60%.

The heatmap of the average antioxidant levels per replicate is shown in Figure 3. Here, it shows that the highest radical scavenging activities lie inside the 24-h timeframe and are seen to be highest within the replicates of soy-fern-probiotics (SFP) and soy-fern (SF). In contrast, the lowest radical scavenging activities can also be found in these treatments after 72 h of the fermentative process.

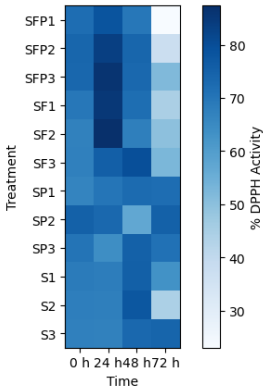

Figure 3. Heatmap which shows the %DPPH radical scavenging activity per replicate in each treatment over the experimental timeframe. Here, 24 h fermentation of soy-fern-probiotics and soy-fern shows the highest antioxidant properties.

To further visualize the data, a contour plot was generated, this time using the average antioxidant levels of each treatment, as shown in Figure 4. This contour plot validates the previous data, as it shows that the highest antioxidant levels are concentrated between the treatments soy-fern probiotics and soy-fern. Furthermore, the figure also reveals that it is in the treatment soy-fern that has the highest radical scavenging activity within the 24-h range.

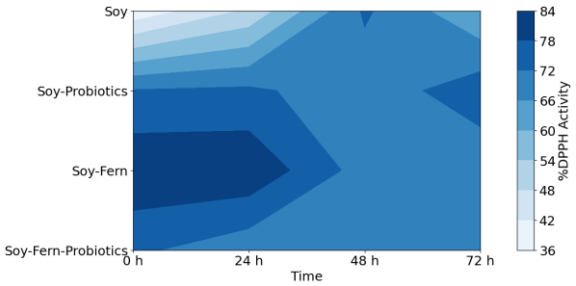

Figure 4. Contour plot showing that soy-fern and soy-fern-probiotics having the highest peaks of radical scavenging activities.

Figure 5 shows the average radical scavenging levels using the DPPH assay of all treatments across different timelines. Here, it shows that on average, the antioxidant level is highest after 24 h of fermentation, followed by 48 h, 0 h, and 72 h. As seen in the graph, the initial antioxidant level (0 h) is higher compared to the level after 72 h of fermentation. It can be inferred that after 24 h, the microorganisms' capacity and efficiency to ferment and release radical-scavenging molecules have diminished over time. Furthermore, it can also imply that for soy-fern fermentation, if there should be any attempts, the isolation of antioxidants should be conducted between 24-48 h.

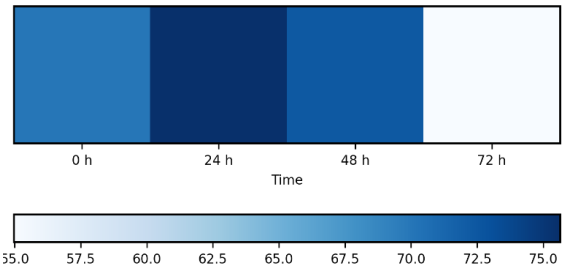

Figure 5. Heatmap showing the average antioxidant levels (in %) of all treatments (soy-fern-prebiotics, soy-fern, soy-probiotics, soy) in different timeframes. Here, 24 h fermentation shows the highest antioxidants produced.

Different Timeframes and Treatments Show Statistical Differences

Statistical analysis for the difference in radical-scavenging activities between different treatments across different timeframes is shown in Table 1. In the 0 h and 48 h timeframes, there is no significant difference in antioxidant rates between the different treatments. This is in contrast to the 24 h and 72 h timeframes, where a significant difference has been observed with *P*-value=0.004 and 0.19 respectively (for $P<0.01$ and $P<0.05$, respectively). These results validate the values reflected in Figure 2.

The Duncan test for pairwise comparison in the 24 h timeframe reveals that soy-fern probiotics and soy-fern treatments are statistically different from soy-probiotics and soy treatments. This means that the antioxidant levels of soy-fern-probiotics and soy-fern are significantly higher compared to soy-probiotics and soy. This statistical difference suggests that the presence of fern may contribute to this difference through its distinct molecules that are absent in soy alone.

For the 72-h timeframe, the Duncan test for pairwise comparison shows varied statistical significance. For instance, soy-fern-probiotics are significant against soy-probiotics and soy. Soy-fern is significant against soy-probiotics and soy. On the other hand, soy-probiotics are significant against soy-fern-probiotics and soy-fern. Lastly, soy is significantly different from soy-fern-probiotics. The varying metabolites that are formed in different treatments at this timeframe could be the potential reason for this varying significance.

Furthermore, since there is no statistical difference between treatments with and without probiotics across different timeframes, it can be concluded that the contribution of probiotics to the radical-scavenging activities is minimal. Therefore, it can be hypothesized that the resident microbial species in fern and soy powders have a significant contribution to the antioxidant properties of the study.

The within-subjects test on the treatment soy-fern probiotics shows a statistically significant *P*-value of 0.000, indicating a difference in antioxidant rates at different time points (Table 2). Due to this significance, the data was subjected to a pairwise comparison which showed in Table 3 that only the 0 h timeframe is not statistically significant when compared to 48 h (*P*-value = 0.435). This lack of significance suggests that both timeframes have similar antioxidant rates.

Table 1. Descriptive and ANOVA statistics result for radical scavenging activities between different treatments across different timeframes (Note: $^{**}P<0.01$, $^{*}P<0.05$, ^{ab}Duncan test for pairwise comparison).

Time (h)	Treatment	Mean (antioxidant%)	SD	n	F-value	*P*-value
0	Soy-Fern-Probiotics	73.25	0.85	3	3.233	0.082
	Soy-Fern	68.02	1.44	3		
	Soy-Probiotics	70.73	4.50	3		
	Soy	68.02	0.59	3		
	Total	70.01	3.07	12		
24	Soy-Fern-Probiotics	82.82^{b}	3.98	3	10.668**	0.004
	Soy-Fern	82.77^{b}	6.22	3		
	Soy-Probiotics	69.28^{a}	4.74	3		
	Soy	67.70^{a}	0.45	3		
	Total	75.64	8.37	12		
48	Soy-Fern-Probiotics	72.37	2.37	3	0.756	0.549
	Soy-Fern	73.16	6.11	3		
	Soy-Probiotics	68.35	9.80	3		
	Soy	75.60	2.16	3		
	Total	72.37	5.79	12		
72	Soy-Fern-Probiotics	37.43^{a}	14.40	3	6.075*	0.019
	Soy-Fern	48.99^{ab}	4.19	3		
	Soy-Probiotics	72.94^{c}	2.11	3		
	Soy	60.51^{bc}	15.15	3		
	Total	54.97	16.54	12		

As mentioned earlier, the 24-h timeframe appears to be when antioxidant rates peak due to the release of metabolites as antioxidant molecules. After this time, a decrease in these antioxidant activities is observed until the 72-h timeframe.

A within-subject analysis on soy-fern also showed that radical-scavenging activities are significantly different across different timeframes with a *P*-value of 0.002 (significant at $P < 0.01$) (Table 4). A further analysis employing pairwise comparisons showed that 0 h against 72 h, 24 h against 72 h, and 48 h against 72 h are all significant with *P*-values of 0.027, 0.025, and 0.017, respectively, based on $P < 0.05$ confidence (Table 5). This means that the radical-scavenging rate at the 72-h timeframe has a broader difference compared to other timeframes. Since antioxidant rates are showing a downward trend, this could mean that the antioxidant activity at this timeframe is now significantly lower compared to the other timeframes. Based on the means, the highest radical scavenging activity falls at the 24 h timeframe (82.77%), and the lowest is at 72 h (48.99%).

For the treatments soy-probiotics and soy, there has been no significant difference when antioxidant activities are analyzed in these respective treatments (Tables 6 and 7). And since no statistical difference is detected in these treatments, a pairwise comparison is no longer needed. Within these treatments, levels of antioxidant activities are similar across timeframes; but even then, based on the means of these treatments, radical scavenging activities are considerable, with the least being at least 60.51% (soy, 72 h) and the highest being 75.60% (soy, 48 h).

Table 2. Tests of within-subjects effects of soy-fern-probiotics across time (Note: ****P*<0.001).

Treatment	Time	Mean (antioxidant %)	SD	n	F-value	P-value
Soy-Fern-Probiotics	0 h	73.25	0.85	3	30.137***	0.000
	24 h	82.82	3.98	3		
	48 h	72.37	2.37	3		
	72 h	37.43	14.40	3		

Table 3. Pairwise comparison of DPPH activity across time (Soy-Fern-Probiotics) (Note: **P*<0.05, [a]Adjustment for multiple comparison: Least Significant Difference).

Comparison	Mean Difference	P-value[a]
0 h vs. 24 h	-9.570*	0.037
0 h vs. 48 h	0.877	0.425
0 h vs. 72 h	35.817*	0.046
24 h vs. 48 h	10.447*	0.013
24 h vs. 72 h	45.387*	0.017
48 h vs. 72 h	34.940*	0.040

Table 4. Tests of within-subjects effects of Soy-Fern across time. (Note: ***P*<0.01).

Treatment	Time	Mean (antioxidant %)	SD	n	F-value	P-value
Soy-Fern	0 h	68.02	1.44	3	19.257**	0.002
	24 h	82.77	6.22	3		
	48 h	73.16	6.11	3		
	72 h	48.99	4.19	3		

Table 5. Pairwise comparison of DPPH activity across time (Soy-Fern) (Note: **P*<0.05, [a]Adjustment for multiple comparison: Least Significant Difference).

Comparison	Mean Difference	P-value[a]
0 h vs. 24 h	-14.747	0.052
0 h vs. 48 h	-5.143	0.296
0 h vs. 72 h	19.025*	0.027
24 h vs. 48 h	9.603	0.308
24 h vs. 72 h	33.772*	0.025
48 h vs. 72 h	24.169*	0.017

Table 6. Tests of within-subjects effects of Soy-Probiotics across time (Note: *P*>0.05-not significant).

Treatment	Time	Mean (antioxidant %)	SD	n	F-value	P-value
Soy-Fern	0 h	70.73	4.50	3		
	24 h	69.28	4.74	3		
	48 h	68.35	9.80	3	0.251	0.858
	72 h	72.94	2.11	3		

Table 7. Tests of within-subjects effects of Soy across time (Note: *P*>0.05-not significant).

Treatment	Time	Mean (antioxidant %)	SD	n	F-value	P-value
Soy	0 h	68.02	0.59	3		
	24 h	67.70	0.45	3		
	48 h	75.60	2.16	3	1.759	0.254
	72 h	60.51	15.15	3		

DISCUSSION

The peak of antioxidant properties as shown in Figure 2 at the 24-h timeframe, can be attributed to the capacity of the microorganisms that act on the different substrates. Basically, at this stage, microbial growth enters the stationary phase, where most of the metabolites have already been produced. As fermentation progresses, theoretically, there will be an increase in the population of the microbial species that break down complex molecules via enzymatic reactions. This can result in the consumption of more substrates. As the quantity of substrates decreases, more cells are deprived of nutrients, which prevent further cellular division. This then follows a continuous downward spiral of population decline, coupled with a decline in substrate. Since various probiotics species were initially added at the start of the procedure, it is theoretically possible that, along the timeline, some of these species are already in their death phase. This could be due to either the possible formation and accumulation of toxins as metabolic by-products from the substrates (Doekes et al. 2019), the activation of the antagonistic behavior of different species included in the probiotics additive, or from the resident microbial species from the substrates (soy and fern). Wang et al. (2021) reported in their study that among eight species of lactic acid bacteria, only one strain persisted throughout the fermentation process. They also reported that a plateau of the microbial growth curve was recorded after 20 h. Similar studies on the fermentation peak, as well as the reduction of the number of bacterial species and strains over time, have also been reported (Magala et al. 2015; Pereira et al. 2016). Such results seem to be consistent with what was observed in this study, particularly in the timeframe where antioxidant levels are at their highest (24 h), and might suggest that other probiotics that were initially added were gradually terminated in the progress of the fermentation process.

The peaks at the 24-h timeframe for soy-fern-probiotics and soy-fern are suspected to be primarily due to the fern. It may be that the release of fern antioxidant compounds, or it could also be a result of the synergistic reactions between soy, fern, and the acting microorganisms. Zannah et al. (2017) identified that alkaloids, terpenoids, flavonoids, polyphenols, and saponins are found in the aqueous extracts of *D. esculentum*.

On the other hand, the treatments soy-probiotics and soy showed different patterns of antioxidant levels. As observed, only at 72 h was a significant movement of the antioxidant levels observed. It can be inferred, then, that the reaction time of soy alone (without regard to the probiotics added) is much different compared to the substrates that have ferns. In this case, there are two possible reasons for such behavior. First, Xue et al. (2016) identified that bioactive compounds could reach their maximum availability after 120 h (compared to this study at 72 h). This implies that there could potentially, be increase in the levels of radical scavenging activity if the timeframe is increased. Second, specific antioxidant molecules are already available when soy is dissolved in water, and through the fermentation process, such molecules may have served as the primary substrates of the fermenting microorganisms. This could then account for the decline of specific molecules, as reflected in Figure 2.

Barus et al. (2019) identified that the process of fermentation, the types of microorganisms, and the length of the fermentative process directly affect the antioxidant activities of substrates by employing the DPPH assay. Here they demonstrated that soybeans that underwent fermentation have higher antioxidant activities compared to unfermented soybeans.

Furthermore, the varying antioxidant levels were also recorded when using different microorganisms, specifically *Rhizopus*, *Bacillus*, and *Klebsiella* species.

In a similar study, Cui et al. (2020) also identified the crucial role of selecting microbial species that will ferment substrates, either in a single culture or mixed culture. They stipulated that such microbial strains can vary the outcome of fermentation, specifically in the formation of bioactive compounds like those that are categorized as antioxidants. Based on this study, it is evident that synthesis of antioxidants is the result of the interplay of different substrates (in this case, soy and fern), the length of fermentation, and the types of fermenting microorganisms. Such results agree with the principles of fermentation biotechnology.

In conclusion, fermentation biotechnology is indeed a work in progress, where several methods must be tested to optimize the production of molecules that are of pharmaceutical, nutraceutical, and agricultural significance.I In this case, the production of compounds that are capable of scavenging free radicals that can affect cellular health. Hence, fermentation biotechnology should involve various innovative approaches to keep up with the demand of a growing society for healthier, safer, and more affordable food products and derivatives.

FUNDING

The DOST-SEI is acknowledged for the funding contribution.

ETHICAL CONSIDERATIONS

This research ethically observed all pertinent methods in meeting the objectives of the study.

DECLARATION OF COMPETING INTEREST

The authors declare that there are no competing interests among the authors.

ACKNOWLEDGMENTS

The authors would like to acknowledge Central Mindanao University's Tuklas Lunas Development Center and Natural Science Research Center for allowing us to conduct this research. We would also like to acknowledge the Department of Science and Technology – Science Education Institute for the scholarship provided. Lastly, the editorial staff and reviewers for this article is greatly recognized.

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ROLE OF AUTHORS: *ELLO – led the conduct of the experiment as well as on the analysis and interpretation of data; MC- assisted in the collection of samples and experimental setup; JMR – assisted in the collection of samples and experimental setup; RDC – refined the methodology and over-all content of the paper, and allowed the conduct of the experiment in her laboratory; JG – statistical analysis and interpretation of data.*