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Sustainable improvement of nutrition quality and biological activity from cassava residue and okara through solid-state fermentation by *Pleurotus citrinopileatus* mycelium

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ABSTRACT

Vietnam's agri-food sector produces 1.64 million tons of byproducts yearly and contributes almost 26% of the country's GDP in 2023. A very small portion of this waste was turned into compost, with the majority being disposed of as waste in the environment. However, there has not been much done in the way of research or technical applications to utilize this residue up to this point. Hence, this study investigated the effect of solid-state fermentation with *Pleurotus citrinopileatus* mycelia on polysaccharide (PS) and protein contents, antioxidant properties, probiotic growth stimulation, pathogenic inhibition, and bio-physicochemical properties of extracts from culture medium before and after fermentation was investigated. The findings indicated that when cassava (CASS) residue and okara are mixed in a 1:1 ratio, the mycelium develops swiftly uniform, even white, very thick, and high density. The total protein, ash, and PS contents from this fermented mixture were raised by 65.12%, 70%, and 57.24%, respectively. The PS extract inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* with sterile ring diameters of 3.47 ± 0.38 and 3.06 ± 0.27 cm, respectively; stimulated the growth of *Lactiplantibacillus plantarum* with a colony density of 9.34 logCFU/ml after 24 hours of culture; and increased antioxidant capacity with IC_{50} = 3,287.62 g/ml. Heavy metals content, bacteria, yeasts, and mold levels were all lower than the allowable thresholds as recommended for animal feed purposes. The results show that *P. citrinopileatus* mycelium can ferment CASS residue and okara to produce a safe and nutritious source of animal feed supplements. This offers a viable approach for enhancing the added value of agricultural residue. Thus, more research is required to assess the financial viability of using fermented substrates produced by oyster mycelium as an additional feed source for animals. Researchers can also concentrate on conducting additional studies on the safety, use, and biological activity of the isolated PS fractions in the functional food sector.

1. INTRODUCTION

Vietnam's agricultural industry produces 1.64 million tons of by-products yearly and accounts for almost 26% of the country's GDP in 2023. A very tiny quantity of this residue was employed as compost or cattle roughage, and even less was used to grow edible mushroom fruit bodies [1]. The majority of this residue was thrown into the environment as waste. In the meantime, there has not been much done in the way of academic research or technological applications to make use of this by-product. Additionally, because of the traits of a growing nation, urbanization and industrialization are occurring at an incredibly fast rate, which is rapidly reducing the amount of land available for agricultural production. Due to this, the livestock business is compelled to grow focally with a very high animal density per unit area. Convenience, vaccinations, antibiotics, disinfection chemicals, and weight-gaining diets were therefore frequently overused in the livestock sector. This has been having detrimental effects on human health and the environment, including a rise in antibiotic resistance, a weakened immune system, antibiotic residues in cattle, and growth boosters like salbutamol and clenbuterol [2,3].

Edible mushrooms have physiological effects on both humans and animals [4]. These effects include changes to the immune system [5,6], cardiovascular vessel functioning [7,8], digestive system [9,10], anticancer [11,12], antioxidation [13,14], and antihyperglycemic effects [15,16]. Polysaccharides (PS) isolated from the fruiting bodies of those mushrooms exhibit antitumor and immune-boosting properties [17]. Some *Pleurotus* species are edible and have bio-physiological effects that make them versatile in study because they include vital

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nutritional elements [18], enzymes [19], and PS [20,21]. Studies have demonstrated antitumor [22,23], antifungal [24,25], anticancer [26], and antioxidant effects [27] and have shown capacity in the prevention of cardiovascular disease [28], improvement of immune function [29], and the lowering of blood sugar [30]. *Pleurotus citrinopileatus*, a species in the Basidiomycetes subphylum, has been shown to contain PS [31], which when extracted shows anticancer action [32–34]. *Pleurotus citrinopileatus* also has a high concentration of bioactive compounds such as proteins, amino acids, minerals, dietary fiber, and trace elements. PS derived from *P. citrinopileatus* fruiting bodies have a variety of activities, including antioxidant [35], hepatoprotective [36,37], anti-inflammatory [38], and anti-obesity [39]. The production of fruiting bodies, however, is not ideal for mass production because of the extended cultivation period and difficult-to-control growing conditions [40]. Solid-state fermentation (SSF) is an efficient process that has a shorter cycle time and is easier to manage. As a result, the preparation of *P. citrinopileatus* mycelia via SSF and PS analysis has become more popular as the best way to culture mushrooms under controlled conditions and for industrial applications [41]. *Pleurotus citrinopileatus* has recently seen growing market demand and production as a result of its possible involvement in medicine, food for the human diet, and feed supplements for animals [42].

Because of its rich and diverse nutritional makeup, okara has a short shelf life and degrades quickly [43]. Furthermore, insoluble fiber is the primary source of carbohydrates in okara. This reduces its nutritional value, makes it difficult to digest, and results in an unpleasant texture for food manufacture. As a result, okara use in food technology production applications is limited [44]. Okara is an insoluble residue from protein extraction produced during the production of soy protein isolate, soymilk, and soybean curd (also known as tofu) [45]. About 1.1 kg of okara is produced for each kg of dry soybeans used to make soymilk or tofu [46]. To be more specific, 53% of the initial soybean dry mass is recovered in tofu, 34% in okara, and 16% in whey on average. Tofu recovers approximately 72% of the protein, okara recovers 23%, and whey recovers 8%; soybean oil recoveries are 82% in tofu, 16% in okara, and less than 1% in whey, respectively [47]. Okara contains more than 50%–60% dietary fiber, 9%–25% protein, 8% ash, 10% lipid, and predominant sugar components such as galactose (46%), arabinose (22%), and galacturonic acid (18%), as well as other sugars such as rhamnose (5%), fucose (3.2%), xylose (3.7%), and glucose (1.2%) [45,48]. It is high in dietary fiber and has been linked to health benefits, chronic illness prevention, and bowel conditioning [49]. As a result, okara can be used in a variety of foods, including salads, soups, sauces, baked products, sweets, sausages, and okara burgers [50]. Furthermore, it can be employed not only as a raw material source for fiber-fortified foods [51], but also for pharmaceutical and industrial uses [52] and animal feed production [48,53].

Similar to numerous other crop by-products, cassava (CASS) residue frequently possesses certain drawbacks, including a low protein content, a relatively high carbohydrate content consisting of non-starch PS (cellulose, hemicellulose, pectin, and lignin) that are indigestible, and additional compounds like tannin, phytate, and cyanide [54]. CASS processing and usage generates a large amount of residue. Unused CASS residue typically rots, pollutes the environment, and endangers both human and animal health [55]. CASS residue has a nitrogen-free extract percentage of 74.4%, a crude energy content of 3,519 Kcal/kg [56], and is high in amino acids and minerals such as copper, potassium, manganese, and iron [57]. Furthermore, because CASS residue is strong in fiber and starch, it could be a viable option for dairy feedstuff for poultry and livestock [58]. However, because of a lack of suitable and current technology to convert these potentially rich resources to other value products in Vietnam, the bulk of okara and CASS residue is being used ineffectively and is regarded as agricultural waste that causes environmental issues.

In the field of utilizing agricultural residues via a fermentation process, Sabater *et al.* [59] introduced new ways of valorization of vegetable food waste and by-products through fermentation processes to improve nutritional value, or to produce biologically active compounds from those waste and by-products. Oktaviani *et al.* [60] applied bioconversion of CASS peel residue into yeasts to produce cell wall mannoprotein as an antioxidant. Suriyapha *et al.* [61] delved into how to make bioconversion of agro-industrial residues as a protein source supplementation for cows. Verardi *et al.* [62] reviewed research on agricultural residue recovery through fermentation technology and analyzed the key steps in the agro-residue bioconversion process. Bala *et al.* [63] mentioned pathways to convert agro-residues, into valuable bioproducts and bioactive compounds, as well as their applications. Blasi *et al.* [64] addressed the valorization methods for the biotransformation of lignocellulosic agricultural waste into economically and environmentally valuable products. Cruz *et al.* [55] and Adnane *et al.* [65] provided a thorough examination or evaluation of anaerobic co-digestion technology as a biochemical recovery pathway of CASS residue and other agricultural residues for the production biogas that fulfill the global target of renewable energy. Therefore, in this study, through the SSF process, we utilized okara and CASS residue as substrates for *P. citrinopileatus* mycelium growth and development to convert this organic waste into profitable, food-based feedstocks, reduce agricultural waste, and facilitate the release of important PS in order to increase the nutritional content of these residues for animal feed supplementary purposes in the future. In addition, this research helps achieve the sustainable development goals that the United Nations and its member states have set forth, including promoting good health and well-being, and responsible consumption and production.

2. MATERIALS AND METHODS

2.1. Materials, Strains, and Culture Medium Preparation

Soybean and CASS residues were used as substrates for *P. citrinopileatus* mycelium via the SSF process. The soybean residue (okara) was purchased from local markets in Danang city, Vietnam. The CASS residue was provided by the Quang Nam flour factory. Both residues were dried at 50°C until constant weight, then ground to a fine powder and passed through a sieve 0.2 mm.

Pleurotus citrinopileatus was supplied by the Mycology Laboratory, Faculty of Biology and Environmental Science, Da Nang University of Science and Education, The University of Danang, Vietnam. The commercial probiotic strain, *Lactiplantibacillus plantarum* WCFS1, and pathogenic strains, including *Staphylococcus aureus* ATCC 25023 and *Escherichia coli* ATCC 85922 were provided by the Laboratory of Microbiology, Faculty of Chemical and Food Technology, Danang University of Polytechnic, The University of Danang, Vietnam. The commercial prebiotics (the powder contains inulin, fructose-oligosaccharide, and galactose-oligosaccharide with a ratio of 1:1:1) were distributed by Southeast Asia Pharmaceutical and Trading Joint Stock Company. The other chemicals were purchased from Sigma (St. Louis, MO).

Pleurotus citrinopileatus was employed in the treatment of okara and CASS residue. The prebiotic properties of PS extracted from *P. citrinopileatus* mycelium cultured on those residues were evaluated by using *L. plantarum*. *Staphylococcus aureus* and *E. coli* were utilized to assess the resistance potential of fermentation extract against Gram-positive and Gram-negative bacteria.

Table 1. The ratio of substrates used in SSF tests.

Formulas	Number of samples and repetitions	Compositions
CASS	5 × 3 = 15	100% CASS residue
OKAR	5 × 3 = 15	100% okara
C_5O_5	5 × 3 = 15	50% CASS residue + 50% okara
C_7O_3	5 × 3 = 15	70% CASS residue + 30% okara
C_3O_7	5 × 3 = 15	30% CASS residue + 70% okara

Solid breeding medium [Potato Dextrose Agar (PDA)] was prepared by commercial PDA powder (20 g dextrose, 15 g agar, and 4 g potato starch) and was mixed in 1,000 ml deionized water and autoclaved at 121°C for 15 minutes before distributing the hot liquid to sterile glass test tubes (50 ml, 25 × 180 mm), up to a depth of 90 mm, resulting in approximately 25 ml of PDA in each tube. Liquid breeding medium (PDB+) was prepared by commercial PDB powder (20 g glucose, 4 g potato extract) mixed with 2 g yeast extract and 2 g peptone in 1,000 ml deionized water. Pour (150 ml) liquid medium into 500 ml-flasks before autoclaving (WAC-60 Steam Sterilizer, Witeg Labortechnik GmbH - Wertheim, Germany) at 121°C for 30 minutes.

The solid-state medium was prepared by various ratios of substrates (Table 1) to select the optimal ratio of CASS residue and okara for the highest yield, performance, and appearance of *P. citrinopileatus* mycelium biomass production. 150 g of each formula was put into polypropylene disposable culture boxes and sterilized for 20 minutes at 121°C.

2.2. Inoculation and Culture Condition

A quantity of 3 g *P. citrinopileatus* was inoculated on PDA test tubes under sterile circumstances and cultured in an incubator at 27°C for 6 days. The mycelium (3 g) was then picked with a sterile tweezer from activated *P. citrinopileatus* test tubes and inoculated into 500 ml sterile flasks containing the liquid fermentation medium (PDB+) by shaking culture (ES-20 Orbital Shaker-Incubator, Thermo Fisher Scientific Inc, Germany) at 150 rpm at 27°C for 10 days.

5 ml of the mycelium from the liquid fermentation medium was taken and then poured into the center of the disposable sterile plastic culture boxes containing solid substrate (treated okara and/or CASS residue). The SSF of *P. citrinopileatus* was operated at 28°C for 10–15 days.

2.3. Measurement of Mycelium Surface Area

Every 3 days during the SSF process, samples were taken to measure the surface area of the mycelium on the substrate. For each formula, five parallel trials were performed and repeated in triplicates to get the average value. *Pleurotus citrinopileatus* mycelium on the surface of each experimental plastic box was captured (Canon Kiss X7, Canon Inc., Japan) with a normal scale attached beside the box [66]. Then the surface area of mycelium was measured by ImageJ open-source software.

2.4. Extraction and Preparation of Crude PS

When the *P. citrinopileatus* mycelium completely covered the substrate surface, the entire fermented substrate was collected and subjected to further processing. First, it was dried at 50°C until reaching a constant weight by the oven (ESCO OFA-32-8 Isotherm, Singapore), then milled and sieved through a mesh with a pore diameter of 0.2 mm. After that, PS was extracted by using deionized water, followed by alcohol precipitation.

To begin the extraction process, the powder sample was mixed thoroughly with hot deionized water (80°C) in a ratio of 1:20. The mixture was shaken at 150 rpm for 4 hours at 28°C and then centrifuged at 8,000 rpm for 10 minutes to obtain supernatant. The liquid extract was filtrated and concentrated using rotary evaporation at 60°C under reduced pressure. Next, a quadruple volume of anhydrous ethanol was added to remove the tiny particles and to reduce sugar. This solution was precipitated for 24 hours at 4°C, then centrifuged (Hermle Z446, Germany) at 8,000 rpm for 10 minutes, the supernatant was discarded, and the filter residue was dried (60°C) to get the fermented crude PS extract [67].

The negative control was performed by extracting crude PS from the original substrate before conducting the SSF process.

2.5. Determination of PS Content

The phenol–sulfuric acid method was used to determine the crude PS content, which is based on the principle of PS hydrolysis reaction into monosaccharides, which will color with phenol, and the resulting solution has a maximum absorbance at OD 490 nm wavelength. The crude PS content is calculated from the D-glucose standard curve equation computed according to the following equation.

$$\text{PS content}(\%) = \frac{\text{OD} + 0.0366}{0.0091} \times V \times \frac{100}{m(1-w)} \times \frac{162}{180} \quad (1)$$

where OD is the optical density of target sample; V is the volume of sample solution after dissolved; m is the initial weight of crude PS extracted; and w is the moisture content of initial crude PS.

2.6. Assessment of PS Antioxidant Activity

The antioxidant activity of PS was measured using the ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate) test. ABTS+ solution was prepared by blending ABTS aqueous solution (7 mM) with $K_2S_2O_8$ (4.9 mM) and incubating the mixture in the dark at room temperature for 16 hours. After that, the ABTS+ solution was diluted with (phosphate-buffered saline, 0.01 mM, pH 7.4) to obtain an absorbance of 0.70 ± 0.02 at 734 nm [42].

The crude PS obtained above was dissolved in deionized water to make sample solutions with final concentrations of 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, and 4.0 mg/ml. 200 µl of the test sample at different concentrations was mixed with 3 ml of ABTS+ solution, the absorbance was measured at 734 nm after 1 hour of incubation in the dark at room temperature. Ascorbic acid (vitamin C) was used as a positive control. ABTS inhibitory activity was expressed as the percentage inhibition (%) of ABTS, calculated by formula 2, where A_0: the absorbance of positive control sample at 734 nm, and A_i: the absorbance of test sample at 734 nm. Furthermore, an IC_{50} (inhibitory concentration, 50%) value was also calculated for each sample.

$$\text{The scavenging activity of ABTS}^{+}(\%) = \left(1 - \frac{A_i}{A_0}\right) \times 100 \quad (2)$$

2.7. Evaluate the Probiotic Growth Stimulation

Lactiplantibacillus plantarum strain was cultured on five different formulations of modified-De Man Rogosa Sharpe (MRS) medium

with a pH of 7 at 37°C for 24 hours under anaerobic conditions. The MRS medium supplemented with 10 mg/ml glucose (F1); 10 mg/ml commercial prebiotics (F2); 10 mg/ml of crude PS extracted from substrate after SSF (F3); 10 mg/ml of crude PS extracted from substrate before SSF (F4); and MRS eliminated glucose (F5). The optical density at 620 nm (OD_{620}) of *L. plantarum* cells was evaluated using a UV-VIS spectrophotometer [68]. The plate culture was then used to calculate cell density via *L. plantarum* colonies using the following equation.

$$N = \frac{\sum C}{V \times \left(n_1 + 0.1n_2\right) \times d} \quad (3)$$

where C is the number of *L. plantarum* colonies on two consecutive plates; n_1 and n_2 denote the number of plates on two consecutive plates; V denotes the volume of cell suspension added to each plate (ml); and d denotes the dilution ratio corresponding to n_1.

2.8. Evaluate the Pathogenic Growth Inhibition

The crude PS (1 g) obtained from substrate extractions before and after SSF were diluted in deionized water (5 ml) and then utilized for inhibitory tests. Pathogenic bacteria (*E. coli* and *S. aureus*) were grown in Luria-Bertani medium (3 g/l yeast extract, 5 g/l peptone, 5 g/l sodium chloride) for 48 hours at 37°C. Culture a 50 µl solution of *E. coli* and *S. aureus* onto plates with NA medium (same NB medium added 15 g/l agar). Each PS solution was poured onto plates harboring pathogenic bacteria. After 24 hours of incubation at 37°C, inhibitory efficiency was determined by comparing the diameter of the clear zone from the PS extract-containing plates to that of the control zone with deionized water [69].

2.9. Assessment of Substrate Quality

Moisture, crude protein, lipid, ash, and PS contents were analyzed in the unfermented okara and CASS residue before and after SSF.

Heavy metals (Pb, Cd, Hg, and As), and aflatoxin contents; microbial levels (aerobic mesophilic bacteria, coliforms, yeast, and mold counts) were measured in the product containing entire spent substrate after SSF by *P. citrinopileatus* mycelium.

2.10. Data Analysis

All the tests were carried out in triplicates, and the results were expressed as mean standard deviation (SD). Analysis of Variance was used to compare variances across the means of different combinations of substrate, and growth stimulation of probiotics. Then, Tukey's Honest Significant Difference (HSD) test was used to assess the significance of differences between pairs of those formulas means. The Welch's *t*-test was used to determine if there is a statistically significant difference between the means of PS, protein, starch, and ash contents, and anti-oxidation capacity of PS extracted in substrate before and after fermentation. R for Windows (Ver 4.3.1) and MS Excel (MS Office 365) were employed to plot the data and execute the comparison analysis.

3. RESULTS AND DISCUSSION

3.1. Effects of Different Substrates on Growth of *P. citrinopileatus* Mycelium

The properties of the substrate utilized have a significant impact on the growth and development of the mushroom mycelium. Agricultural byproducts with high fiber and nutrient content that remain after processing are viable raw materials for harvesting edible mushroom mycelium. The variations in the mycelium area of *P. citrinopileatus* throughout SSF in formulas are shown in Table 2 and Figure 1.

The mycelium in CASS had extended to practically the whole substrate surface by the 10th day of SSF. After 13 days of culture, the mycelium covered the whole substrate surface in all treatments. The time it took the mycelium to cover the substrate surface was faster in treatments with a high proportion of CASS residue (CASS and C_7O_3) than in other treatments. This could be because CASS residue has more fiber content than okara, and the structure is more porous, allowing the mycelium to easily occupy pores in the substrate, resulting in faster mycelium spreading [42]. Although okara is a nutrient-rich substrate suited for mycelium growth, as in the OKAR formula, when 100% of the substrate is okara, the mycelium is thicker and has greater biomass, its propagation speed is slower.

Furthermore, there was a substantial difference in the formulations when assessing the thickness of the mycelium using sensory perception. The C_5O_5 mycelium is the thickest, with spongy mycelium and a uniform white tone. The nutritional status of the substrate, such as C/N ratio, vitamins, plant hormones, micro- and multi-minerals, determines the yield and quality of mycelial biomass [70]. As a result, it is likely that mycelium will grow better when grown on okara coupled with CASS residue, where there might be a balance of this ratio, rich in nutrients, and suitable porosity. The issues of mycelium quality and speed of spread were concurrently handled in the C_5O_5 formula with a mixing ratio of 50% CASS residue and 50% okara, hence, C_5O_5 was selected as the optimal media for further experiment and analysis in the following sections. Some microorganisms such as *Rhodococcus* strain UCC 0010 in the study of Maniyam *et al.* [71] have the ability to produce a high titer of concentrated intracellular laccase activity at highly acidic conditions to detoxify Congo red when cultured on coconut waste. Okara and CASS residue do not directly produce effective

Table 2. Changes in area (cm^2) and external morphology during SSF of *P. citrinopileatus* mycelium on different formulas.

Formulas	Duration of SSF (days)				Mycelium external morphology
	Day 1	Day 4	Day 7	Day 10	
CASS	19.42 ± 0.89 ^{c}	36.96 ± 1.49 ^{a}	60.06 ± 2.16 ^{a}	96.18 ± 4.32 ^{a}	Thin, low density
OKAR	19.44 ± 1.39 ^{c}	29.25 ± 1.30 ^{c}	42.53 ± 3.46 ^{c}	72.11 ± 5.05 ^{c}	Thick, porous, even white, high density
C_5O_5	23.09 ± 1.13 ^{ab}	34.96 ± 1.11 ^{b}	52.00 ± 2.08 ^{b}	87.27 ± 4.55 ^{b}	Very thick and high density, even white
C_7O_3	23.39 ± 0.61 ^{a}	33.98 ± 1.06 ^{b}	55.00 ± 1.76 ^{b}	89.99 ± 1.95 ^{b}	Moderate thick and density, even white
C_3O_7	21.81 ± 2.07 ^{b}	34.87 ± 2.29 ^{b}	48.93 ± 2.32 ^{b}	81.16 ± 1.34 ^{b}	Thick, even white, high density

CASS: 100% CASS residue; OKAR: 100% okara; C_5O_5: 50% CASS residue + 50% okara; C_7O_3: 70% CASS residue + 30% okara; and C_3O_7: 30% CASS residue + 70% okara. The values with different letters in the same column indicate significant differences ($p < 0.05$). Data is expressed as mean ± SD of 15 samples in triplicates.

enzymes like *Rhodococcus* strain UCC 0010 produced rhodococcal laccase. However, they are preferable substrates for the growth and development of oyster mycelium. Then, this edible mushroom produced enzymes to decompose carbohydrates such as cellulose, hemicellulose, pectin, and lignin into digestible PS [72,73].

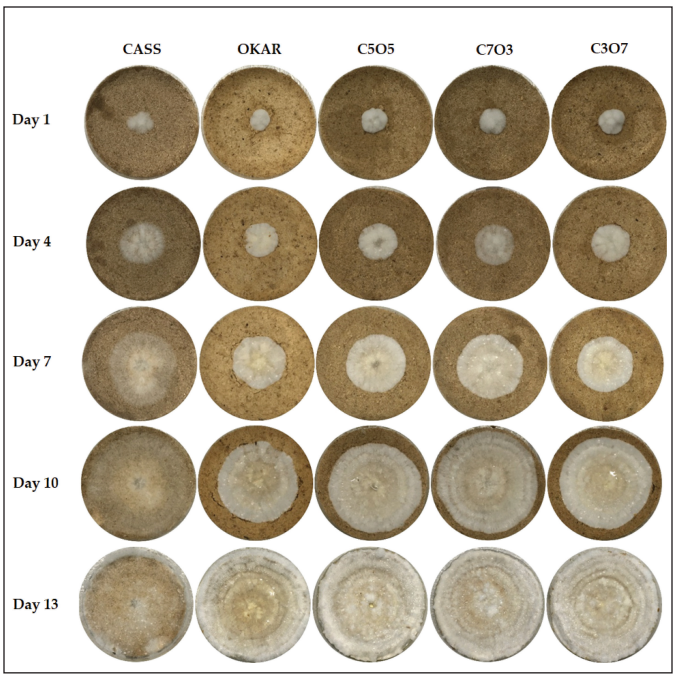

Figure 1. Growth of *P. citrinopileatus* mycelium during SSF in five formulations. CASS: 100% CASS residue; OKAR: 100% okara; C_5O_5: 50% CASS residue + 50% okara; C_7O_3: 70% CASS residue + 30% okara; and C_3O_7: 30% CASS residue + 70% okara.

3.2. Chemical Compositions of Substrates Before and After SSF

The protein, lipid, carbohydrate, and ash content of animal feed are all essential considerations. Furthermore, numerous research demonstrates that feeding with β-glucans helps animals gain weight and boost immune system. Some nutrients are relatively low in agricultural byproducts prior to mycelial fermentation. However, after SSF, the composition and concentration of these nutrients, particularly the protein and carbohydrate content, are greatly increased. Figure 2 depicts the results of nutritional composition analysis in C_5O_5's substrate (50% CASS residue and 50% okara) before and after SSF by *P. citrinopileatus* mycelium.

The protein content increased by almost 65.12% after solid fermentation, from 10.48 to 19.57 g/100 g. The rapid rise in single-cell protein biomass of the mycelium during fermentation, according to Bakratsas *et al.* [74], can explain the increase in protein content. *Pleurotus* species are highly adaptable to biotransformation using carbon sources. *Pleurotus* species' acidophilic nature, as well as their ability to reduce pH through the production of organic acids, has been found to prevent nitrogen losses due to ammonia volatilization [42]. The lipid concentration tends to drop (71.27%) after solid fermentation, from 1.77 to 0.71 g/100 g, probably due to mycelium breakdown and bioconversion of lipids into energy for cell survival. In addition, the decrease in lipid content may be the result of lipid use by fungus, possibly in the synthesis of phospholipids constituents of the cell membrane of fungal tissue [75]. Abui *et al.* [76] reported the same trend for fermentation of sweet potato with *P. ostreatus*, observing a decrease in total lipid content from 1.93% to 0.54%. The total ash content increased 70% from 2.04 to 4.77 g/100 g, which could be attributed to the presence of minerals in the liquid spawn supply prior to the inclusion of the substrate for SSF. The rise in PS content might be attributed to the mycelium's usage of components such as starch, cellulose, and lignocellulose to grow and synthesize intracellular PS or crude

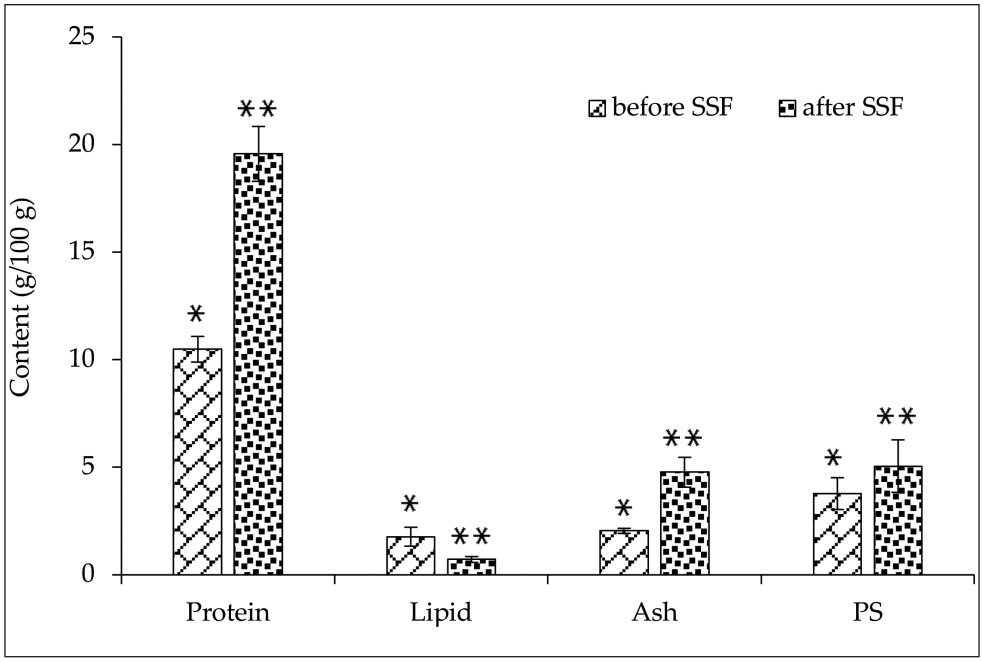

Figure 2. Contents of some compounds before and after SSF in the C_5O_5 substrate. Values are expressed as means ± SD (n = 10). Compound (protein, lipid, ash, PS) with the same symbol above the bar is not significant difference (Welch's t-test, conf.level = 0.95). Error lines represent ± SD of the mean.

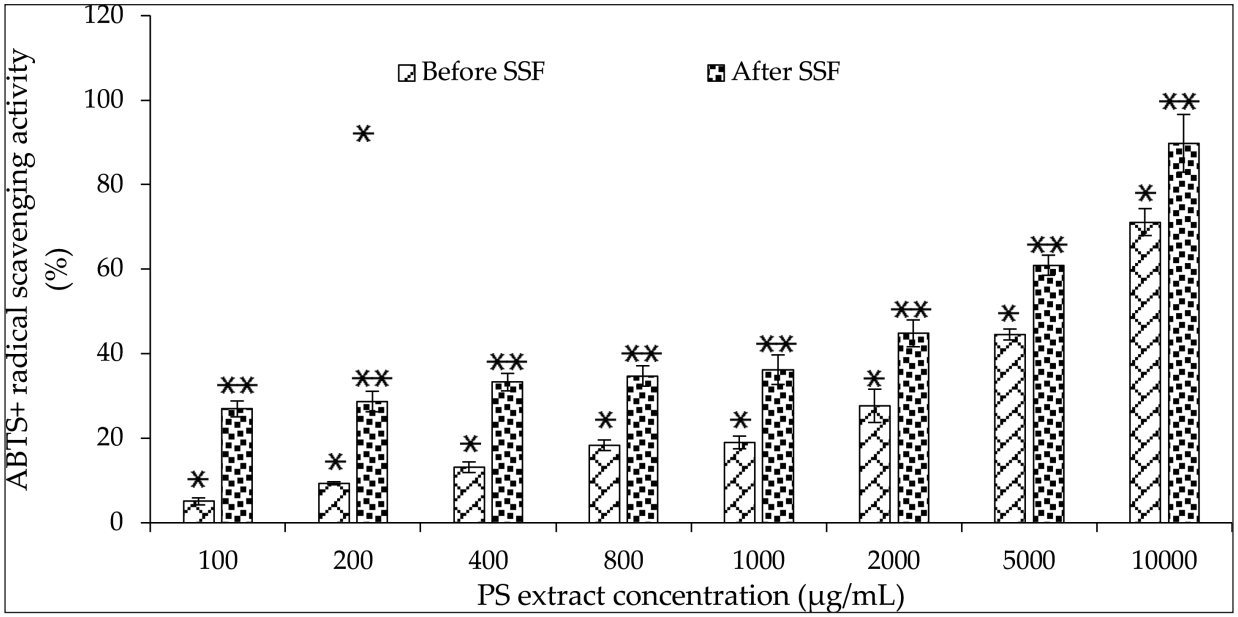

Figure 3. The ABTS+ radical scavenging activity (%) of PS extracts from before and after fermentation C_5O_5's substrate. Values are expressed as means ± SD (n = 10). PS concentration (from 100 to 10,000 µg/ml) with the same symbol above the bar is not significant difference (Welch's t-test, conf.level = 0.95). Error lines represent ± SD of the mean.

PS that was not utilized after lignocellulosic decomposition. The comparatively high PS content (3.78 g/100 g) measured in the substrate extract prior to SSF could be attributed to the fact that CASS residues frequently contain substantial quantities of water-soluble starch after extraction.

The phenol-sulfuric acid method is one of the most common methods applied to the analysis of total sugar content during PS study. In this method, the concentrated sulfuric acid breaks down PS to monosaccharides. These compounds then react with phenol to produce a yellow-gold color. The color depth is proportional to the sugar content, which can be determined at OD 490 nm wavelength. However, according to Yue *et al.* [77], it was found that the total sugar content obtained from the phenol-sulfuric acid method was generally lower than the real total sugar content, particularly in cases where the samples included acidic monosaccharides. In this study, only the formula C_5O_5 (50% okara and 50% CASS residue) was selected for further research. Maybe it is because the unfermented CASS residue still contains starch, an insoluble PS at room temperature. Then under the effect of high temperature (autoclaving process), starch is partially hydrolyzed into monosaccharides. This might result in not much significant difference in the PS content determined in the substrate before and after fermentation by the phenol-sulfuric acid method.

Agricultural residues are the primary substrate utilized in SSF because they still contain a significant amount of nutrients. After being inoculated, microorganisms that produce enzymes proliferate on the surface and start hydrolyzing primary polymeric substrates like proteins and indigestible PS [78]. Additionally, SSF offers an environment with physicochemical characteristics akin to those of microorganisms' native habitat. This benefit is particularly pertinent to fungi, as they have evolved to function better on solid substrates, where mycelium may more easily develop and spread. Because the structure of mushroom cells includes components such as chitin, glucan, soluble PS, and secondary compounds, the SSF process that follows will have positive consequences like the release of nutrients that are tightly bound in complexes or the creation of some biologically active molecules. These help SSF increase nutrient content compared to other methods [79].

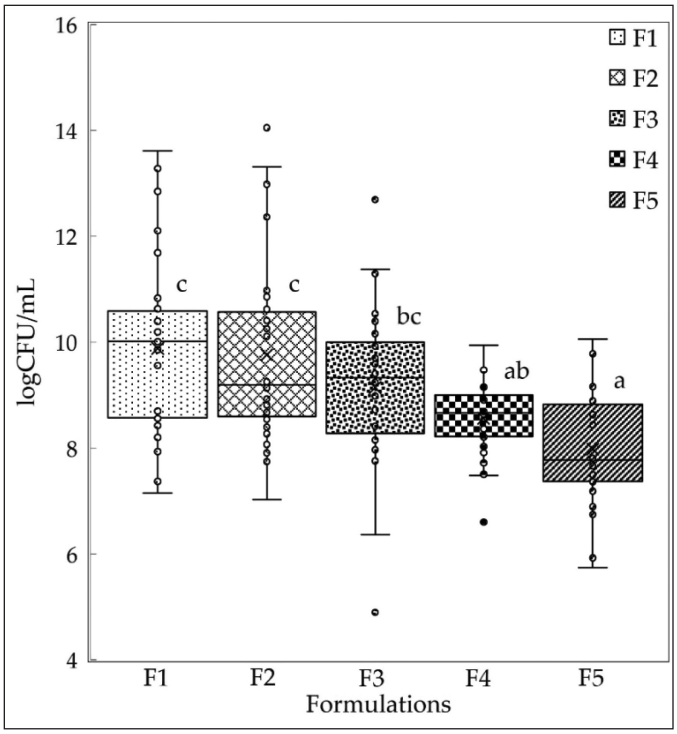

Figure 4. Colony density of *L. plantarum* in different culture media. Means that do not share a letter are significantly different at $p \leq 0.05$ applying Tukey's HSD test. The F1: MRS medium supplemented with 10 mg/ml glucose; F2: 10 mg/ml commercial prebiotics; F3: 10 mg/ml of crude PS extracted from fermented substrate; F4: 10 mg/ml of crude PS extracted from unfermented substrate; and F5: MRS eliminated glucose. Values are expressed as means ± SD of triplicates (n = 30).

3.3. Antioxidant Capacity of PS Extract Before and After SSF

Free radicals such as O-, OH, and reactive oxygen species are powerful oxidizing agents that can react with any macromolecule in the cell, generating mutations and cancer. A compound's antioxidant activity in cells is determined not only by its concentration, but also by other parameters such as lipid and protein content, temperature, oxygen level, and the presence of other antioxidants. To test the antioxidant capacity of the PS extract before and after SSF, ABTS+ was utilized as a free radical to receive electrons or hydrogen in this investigation, and ascorbic acid as the standard control substance.

As shown in Figure 3, the ABTS+ radical scavenging of PS extracted from substrates before and after SSF was directly proportional to concentration; as concentration increased from 100 to 10,000 g/ml, the free radical scavenging efficiency increased gradually, from 5.05% to 71.13% for the PS extract from unfermented substrate, and from 26.94% to 89.78% for the PS extract from fermented one. The extracts' IC_{50} values before and after SSF were 6,078.88 and 3,287.62 g/ml, respectively. This demonstrates that the antioxidant capacity of PS obtained from the extract after SSF is greater than that of the unfermented substrate. Many B-vitamins, including vitamin B3, vitamin B5, and vitamin B2, are found in *P. citrinopileatus* that have the potential to remove free radicals [80], and phenolic compounds have antioxidant characteristics [81].

In addition, as shown in Figure 2, although PS content did not seem to be changed by the fermentation (from 3.78 to 5.05 g/100 g), the antioxidant capacity of PS extracted in the fermented substrate was significantly higher than unfermented one, as shown in Figure 3. Mateos-Aparicio *et al.* [82] and Vong and Liu [83] report that okara has a total content of between 40% and 65% dietary fiber, comprising both soluble and insoluble fiber, with soluble fiber making up around 10% of this total. This explains PS's (a soluble fiber type) ability to function as an antioxidant when isolated from the unfermented substrate [82,83].

3.4. Effects of PS Extract on Growth of *L. plantarum*

Probiotics are beneficial living bacteria and yeasts for human or animal health [84]. Probiotics, such as *L. plantarum*, may assist to maintain or restore a healthy balance of bacteria in the gut, potentially alleviating diarrhea, constipation, and other digestive issues [69,85]. *Lactiplantibacillus plantarum* is also used to treat or prevent illnesses such as eczema, seasonal allergies, irritable bowel syndrome, excessive cholesterol, and inflammatory bowel disease. *Lactiplantibacillus plantarum* is a Gram-positive lactic acid bacterium that is typically found in fermented foods and the gastrointestinal system. It is widely exploited in the food business as a possible starter probiotic, and as a result, the consumption of food combined with probiotics has skyrocketed [84]. The addition of crude PS extract to *L. plantarum* culture media to evaluate its growth stimulation after 24 hours of culture was presented in Figure 4.

The *L. plantarum* proliferated quickly in the formula added with 10 mg/ml glucose (F1), and the colony density (9.879 logCFU/ml) was greater than in the other treatments. This is because glucose is a monosaccharide that bacteria may take directly through the cell membrane without being broken down. Meanwhile, the culture media in the formulations F2, F3, and F4 with carbon source contain complex carbohydrates that must be decomposed before being absorbed, therefore bacterial growth is slower than in F1. Furthermore, colony density was greater in the medium formula added with commercial prebiotics (F2, 9.757 logCFU/ml) than in the formulations supplemented with crude PS derived from substrate extracts before and after fermentation (F3, 9.141 logCFU/ml and F4, 8.560 logCFU/ml). This might be because commercial prebiotics provide a direct food source for probiotics, which have a selective growth-stimulating effect on the bacteria in the intestines. The colony density was greater in the formulation supplemented with PS extracted from the substrate after SSF (F3, 9.141 logCFU/ml) than before SSF (F4, 8.560 logCFU/ml). This is explained by the fact that during solid fermentation, the mycelium synthesizes internal PS, secretes external PS, and a part of crude PS is degraded from the original substrate's accessible cellulose. These PS, like commercial prebiotics, serve as a food supply for probiotics [86]. The findings suggest that using mycelium to ferment agricultural residues boosted their prebiotic activity, fulfilling the potential application as feed supplements for the livestock and aquaculture industries. Figure 5 presents the outer morphology of *L. plantarum* colonies on Petri dishes containing different modified-MS medium.

3.5. The Growth Inhibition of PS Extract on *E. coli* and *S. aureus*

Only PS extracts taken from the substrate after SSF by *P. citrinopileatus* mycelium exhibit the aseptic ring (clear zone) on *E. coli* and *S. aureus* plates with the diameters of 3.47 ± 0.38 and 3.06 ± 0.27 cm (Fig. 6), respectively. This finding indicates that PS derived from this extract inhibits the growth of Gram-negative and Gram-positive bacteria. This is explained by mycelium development, which utilizes the nutrients in the substrate to produce enzymes like β-glycosidase and cellulase. These enzymes degrade intracellular macromolecules and liberate individual phenolic compounds from the matrix, raising the total polyphenol concentration substantially [87]. Furthermore, the enzyme β-glycosidase can hydrolyze extracellular polymers of microbial biofilms, inhibiting microbial cell development [88].

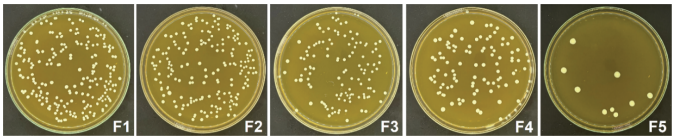

Figure 5. The number of *L. plantarum* colonies on formulations. The F1: MRS medium supplemented with 10 mg/ml glucose; F2: 10 mg/ml commercial prebiotics; F3: 10 mg/ml of crude PS extracted from fermented substrate; F4: 10 mg/ml of crude PS extracted from unfermented substrate; and F5: MRS eliminated glucose.

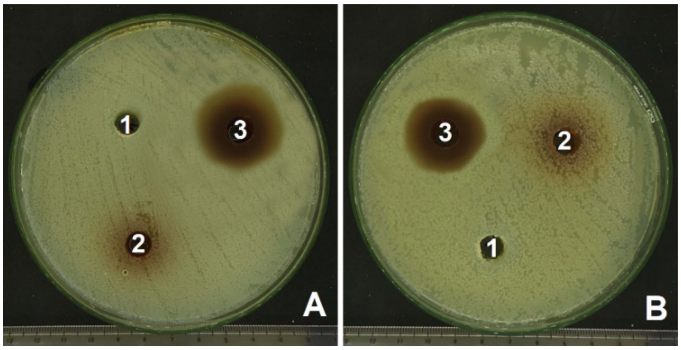

Figure 6. The aseptic ring (clear zone) of pathogenic inhibition, *E. coli* (A) and *S. aureus* (B) from: ❶deionized water; ❷PS extract from unfermented substrate, and ❸PS extract from fermented substrate by *P. citrinopileatus* mycelium.

Table 3. Biomass safety analyses after SSF.

Parameters	Unit	Applied analysis methods	Tested results	Allowable threshold[1]
Lead (Pb)	mg/kg	AOAC 999.11	0.138 ± 0.004	5
Cadmium (Cd)	mg/kg	AOAC 999.11	0.116 ± 0.002	3
Mercury (Hg)	mg/kg	AOAC 971.21	LDL	0.1
Inorganic arsenic (As)	mg/kg	AOAC 986.15	LDL	2
Total aflatoxin (B1, B2, G1, G2)	µg/kg	AOAC 991.31	ND	10
Total aerobic mesophilic bacteria	CFU/g	ISO 4833-1:2013	ND	10
Coliforms	CFU/g	ISO 4832:2007	ND	10
Total yeast and mold counts	CFU/g	ISO 21527-1:2008	ND	10
Salmonella	CFU/25 g	ISO 6579-1:2017	ND	ND

[1]Vietnam technical regulation for aquaculture feed (QCVN 02-31-2: 2019/BNNPTNT). Part 2: Feed supplements (Vietnam's Ministry of Agriculture and Rural Development). LDL = "lower detection limit". ND = "not detected". Values are expressed as means ± SD (n = 9).

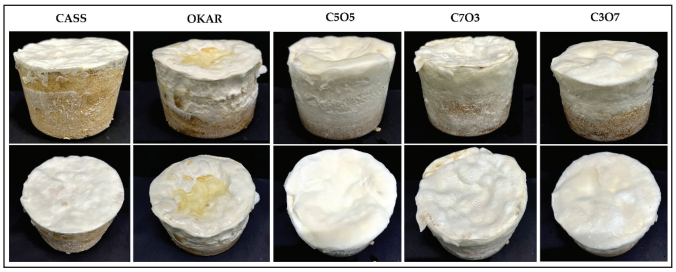

Figure 7. The external morphology of *P. citrinopileatus* mycelium on different combinations of CASS residue and okara formulations. CASS: 100% CASS residue; OKAR: 100% okara; C_5O_5: 50% CASS residue + 50% okara; C_7O_3: 70% CASS residue + 30% okara; and C_3O_7: 30% CASS residue + 70% okara.

3.6. The Biomass Quality After SSF

The use of crop residue in the final composition of animal feed is an economically appealing alternative. Many of these residues, however, have qualities that may make it difficult or impossible to use for this purpose, such as the presence of poisonous or antinutritional chemicals or insufficient amounts of necessary amino acids [89]. In this respect, the study's goal is to manufacture feed supplements for animal breeding by using SSF to turn these agricultural residues into a culture medium that can be used in animal feed supplements. As a result, the biomass after SSF was evaluated to ensure its quality for the purpose of animal feed. As shown in Table 3, the results of the analysis indicated that the level of the parameters was lower than the allowed limit under QCVN 02-31-2: 2019/BNNPTNT regulations on prebiotic products for aquaculture feed. This finding pointed out the huge potential of using CASS residue and okara to produce the animal feed industry. Figure 7 shows the external morphology of *P. citrinopileatus* mycelium on different formulations after the SSF process.

4. CONCLUSION

The study found that SSF by *P. citrinopileatus* mycelium had a good effect on the PS content, antioxidant characteristics, probiotic growth stimulation, pathogenic growth inhibition, and bio-physicochemical properties of CASS residue and okara. Fermentation of these residues had significantly improved protein, ash, and PS profiles; antioxidant capacity (as measured by the ABTS+ assay); stimulated the growth of beneficial bacteria; and inhibited pathogenic strains. *Pleurotus citrinopileatus* mycelium SSF offers a possible technique to improve the characteristics of CASS residue and okara through the bioconversion of agricultural residues into value-added products. These fermented substrates have the potential to replace commercial-industrial animal feed supplements, resulting in significant economic and human health benefits, and SSF is a promising bioprocess with excellent results in the detoxification and nutrient enrichment of agricultural residues. Hence, more research on the economic efficiency and safety of SSF products utilized in the livestock industry is necessary. There is further research focusing on the changes in the active compositions of other edible mushrooms and other types of substrates as a result of SSF. The safety, use, and biological activity of the isolated PS fractions in the functional food sector.

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6. CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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