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RESEARCH ARTICLE

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Digital Analysis of Mycelium Growth and Mycelium Density *In Vitro* of *Pleurotus ostreatus* with Submerged Fermentation as Substrate Treatment

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ABSTRACT

Edible mushroom cultivation often involves sterilizing the substrate, or a similar heat process like pasteurization, to facilitate mycelial colonization. Chemical treatments are an alternative approach that is also employed in some regions. Mycelial growth and density were analyzed *in vitro* by capturing daily photographs using a digital camera, with sterilized substrate serving as the control treatment. Our findings revealed that both fermented and sterilized substrates had similar growth patterns, although fermented treatment required a longer incubation time for full substrate colonization. Mycelium in fermented substrate had a denser structure compared to sterilized treatment, reflecting the interactions with the naturally-present microorganisms within the substrate. Conversely, mycelium in sterilized treatment exhibited faster substrate colonization times but had a less dense mycelial structure. Yeast and bacterial colonies were present throughout the fermentation process and 7 days after *P. ostreatus* mycelium inoculation, indicating active microbial communities during colonization. An initial decrease in CFU on the 3rd day, followed by an increase by the 7th day, suggests a shift toward anaerobic and facultative microorganisms due to oxygen depletion during fermentation. Mold colonies disappeared by the end of fermentation. Despite the complex interactions between yeast, bacteria, and *P. ostreatus* mycelium, the naturally-present microorganisms in the substrate appear to have at least neutral effects, enabling mycelial growth.

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1. Introduction

Edible mushrooms can colonize and degrade a large variety of lignocellulosic substrates and other types of biomass that come from agricultural, forest, and food-processing industries [1]. Oyster mushroom belongs to the *Pleurotus* genus. This genus has high adaptability, aggressiveness, and productivity. They have the ability of growing in a wide range of residues, more than any other species in any other group of mushrooms [2]. The optimal substrate to use depends on various factors, such as a continuous and sufficient availability; physicochemical characteristics; an affordable acquisition price or method; its source location; and for the material to be easily transported and manageable [2].

Substrate is usually treated with some kind of heat process, such as sterilization, steaming, or pasteurization. Heat treatments require higher economic investment to handle high amounts of substrate for mushroom production. Chemical treatments are also employed but additional processes are needed to diminish their environmental impact. Farmers interested in edible mushroom production often have support from governmental research institutions or projects to obtain technical assistance and quality mushroom spawn. They also often have continuous access to agricultural wastes but the biggest limitation they face when trying to enter the mushroom growing sector is the inability to properly treat the biomass they intend to use as cultivation substrate [3]. Edible mushroom cultivation in developing countries can be done at a small scale but it needs simple technologies, simple conditions, and low costs [4].

One of the simplest methods for treating substrate used for edible mushroom growing mentioned in literature is the "Yeast Fermentation Method," "Anaerobic Fermentation," or "Cold Fermentation." In this method, the substrate is submerged underwater at room temperature from 2 to 5 days, with or without beer yeast inoculation or sucrose addition as fermentation aid. After this time, it is drained, exposed to the air, and inoculated with standard procedures. This method is based on some biochemical changes of the substrate made by the microorganisms that are naturally present. First, the anaerobic microorganisms

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metabolize available carbohydrates, making it harder for contaminants to grow (*Trichoderma spp., Penicillium spp.,* etc.). Secondly, pH value decreases significantly from the start to the end of the fermentation process. This changes the optimal pH range of contaminants, while staying within the range where *Pleurotus spp.* can grow. Third, the change in microorganism communities. While fermenting, aerobic microbes die, while facultative and anaerobic microbes are multiplying and active. After fermentation, the substate is exposed to air, killing the anaerobic microbes. Additionally, secondary metabolites formed during fermentation might also limit the growth of contaminants [4,5].

In the literature review it was found that there has been limited to no research conducted on the submerged fermentation method related to substrate treatment utilizing keywords such as "cold + fermentation," "anaerobic + fermentation," "submerged + fermentation," "mushroom + cultivation," in major article databases such as Web of Science and Google Scholar. Literature related to submerged fermentation and fungi has mostly been done with the objective of secondary metabolite production.

Composting is also applied as a lower-cost, simpler alternative to heat treatments for substrate preparation in mushroom cultivation. Several variations of the composting process have been tested, but they all employ calcium hydroxide ($Ca(OH)_2$) as part of the substrate composition [6,7], which could act as a chemical treatment to the substrate.

2. Materials and methods

This research aims to compare submerged fermentation and sterilization methods for substrate treatment by evaluating mycelial growth, its *in vitro* growth patterns, and digitally measuring mycelial density during the colonization period. Specifically, we investigate the feasibility of mycelium growth on naturally-fermented substrates submerged underwater for 7 days, without additional heat or chemical treatments, as an accessible alternative to traditional sterilization methods for *P. ostreatus* cultivation.

2.1. Strain

P. ostreatus sawdust spawn strain H67 was purchased from Kinox Co. in Sendai, Japan. To isolate the mycelium, 1 g of spawn was inoculated in potato dextrose agar under sterile conditions. After 7 days, 1 cm^2 of agar containing actively growing mycelium was transferred to potato dextrose liquid medium, made from boiling 200 g of potatoes in 1 liter of distilled water, cooling it down and adding 20 g of dextrose (Wako Pure Chemical Corporation). Mycelium suspended in liquid media was used as stock culture for reproduction of the strain and for spawn production.

2.2. Spawn

Using commercial barley grains, their average moisture content was adjusted by adding water gravimetrically and letting them absorb the water overnight until approximately 50% of moisture content was reached. This moisture content was selected for spawn production following the results of pre-experiments, because it gave the fastest colonization time while maintaining grain consistency and healthy mycelial morphology, compared to a lower or higher grain moisture content. After adjusting moisture content, 50 ml conical tubes were filled with barley grains and covered with aluminum foil to be sterilized at 121 °C for 20 min. After cooling down, 1 ml of liquid mycelium culture was inoculated in each tube and incubated at 25 °C until complete colonization was reached.

2.3. Substrate preparation and inoculation

Larch sawdust was obtained from the Forest Products Research Institute in Asahikawa, Hokkaido. Rice straw was obtained from the experimental farms of Hokkaido University. A mix of larch sawdust and rice straw at a 90:10 ratio was used as substrate, with the addition of sucrose at 7% of dry substrate weight for the fermentation treatment. Rice straw was chopped manually into pieces of 2 – 5 cm of length [8,9]. Fermentation was done by submerging 130 g of substrate under water in a sealable plastic bag for one week at 25 °C, carefully shaking the bag every day to ensure the submersion of all the substrate. After fermentation, the substrate was drained, spread evenly on disinfected metal trays, and dried for 3 h at 50 °C. For control treatment, the same formulation of substrate was used without the addition of sucrose. Substrate was hydrated for one hour and then sterilized twice at 121 °C for 20 min [10].

After substrate preparation, water content was adjusted by the squeeze test [11]. Eight Petri dishes per treatment were filled with 12 g of substrate (dry weight) each. Following aseptic technique under a laminar flowhood, each Petri dish was inoculated with 1 g of barley spawn. Then, it was covered and sealed with parafilm to avoid external contamination and drying of the substrate. Incubation was done at 25 °C until complete mycelial colonization was reached.

Daily measurements of pH during fermentation were taken with a double junction pH spear (OAKTON, Singapore) for 7 days at 24 h intervals from the start of the fermentation process.

2.4. Monitoring of mycelial growth and density of P. ostreatus

Mycelium monitoring was done following the procedure by [12] and [13] with some modifications. Photographs of the Petri dishes were taken every day at the same time, allowing a 24-hour cycle between every photograph until the mycelium had reached the end of the Petri dish or stopped growing. The Petri dishes were accommodated against a dark, flat surface and photographs were taken with a Fujifilm digital camera (model FinePix SL1000) with a resolution of 4624 × 3470 pixels, minimal zoom, macro automatic focus, ISO-200, F-stop at f 2.9, and an exposure time of 1/25 s. Montini et al. [13] used the Agfa ePhoto1280 digital camera to measure mycelial area (hereinafter, "mycelial growth") and mean pixel gray value (hereinafter, "mycelial density"), which takes photographs in JPEG format [14]. JPEG applies a lossy compression to images and modifies the value of pixels [15], which directly influences mycelial density measurements. To preserve all the original information and avoid image post-processing, all photographs were taken as RAW images in the RAF format [16]. Under the same conditions, photographs of a known distance were used for spatial calibration (pixel:mm ratio) in ImageJ (version 1.54 g) [17] software for the digital analysis.

2.5. Digital analysis of mycelial growth and density of P. ostreatus

In ImageJ software, the DCRaw Reader plugin [18] was employed to import the RAW images. The settings of the plugin were set as follows. White balance was set to None, interpolation quality was set to DHT, the options "Do not automatically brighten the image" and "Do not rotate or scale pixels (preserve orientation and aspect ratio)" were selected, output colorspace was set to raw, and the image was imported as an 8-bit grayscale image to ensure a dynamic range of pixel values between 0 (black) and 255 (white) [19], facilitating the measurement of mycelium density. A denser mycelium, characterized by more hyphae, would correspond to higher pixel values, and appear whiter in the image.

Using the polygon selection tool, the outer border of the mycelium was selected, and measurements of mycelial growth (mm^2) and mycelial density (mean gray value) were taken.

2.6. CFU quantification of substrate

Colony forming unit (CFU) quantification was done at the start of fermentation, after 3 days, and after 7 days, which is when the fermentation process was stopped. A fourth measurement was also done 7 days after inoculating the mycelium in the substrate. For the sample preparation, the procedure by [10] was applied with some modifications. A pooled sample was taken from 7 individual bags where the substrate was fermenting. In a NaCl 0.9% solution, 1 g of the pooled sample was crushed in a sterile mortar and pestle to break up the substrate particles. After this, the solution was transferred to sterile test tubes and shaked for 5 min at 120 r/min (BioShaker BR-41FL, Japan). Also, to recover tightly adhered bacteria, the tubes were vortexed for 1 min and sonicated for 30 s (AS ONE AS33GTU 50 Hz, output frequency 35 kHz, power 160 W, Japan). After the sample processing a serial dilution in NaCl 0.9% was done. Dilutions were plated on Potato Dextrose Agar (PDA) (Wako Pure Chemical Corporation) for yeast and mold enumeration [20]. Tryptic Soy Agar (TSA) (Wako Pure Chemical Corporation) [21] and Luria-Bertani Agar (LB Agar) (Wako Pure Chemical Corporation) [22] were used for non-fastidious bacterial enumeration. Each CFU analysis was done with 3 independent repetitions.

2.7. Statistical analysis

Statistical analysis for all tests and graphs was done with R (v4.2.2) [23] in RStudio (v2023.12.1.402) [24]. Additional R packages were also used in the analysis [25–28]. The distribution of mycelial growth and mycelial density was assessed with histograms and quantile-quantile plots. The nonparametric Friedman test for repeated measures was employed to assess the overall differences between treatments in both mycelial growth and mycelial density across all days. Treatments served as the grouping factor, while days were considered as the blocking factor. For daily pairwise comparison between treatments, Dunn's test was applied [29,30].

3. Results

3.1. pH of fermented substrate

Fermented substrate for one week with the addition of sucrose had an initial average pH value of 6.35 (SD = 0.05) and it decreased as fermentation went on until it reached a final pH of around 4.0 (SD = 0.09) (Figure 1).

3.2. Mycelial growth

The Friedman test revealed a statistically significant difference in growth area between fermented and

sterilized treatments across all days $\chi^2(1)$ =9, p = 0.002, indicating substantial variation in growth between the two treatments (Figure 2).

Dunn's test for daily pairwise comparison revealed consistent significant differences across all days (Table 1). Differences are more prominent between days 2 through 6, and on day 8. Sterilized treatment grew to higher area measurements faster compared to fermented treatment, reaching full colonization by the 8th day of incubation. Variation between treatments also differed. Fermented treatment had a notably higher variation in growth in each day compared to sterilized treatment, which was increasing as days passed. Sterilized treatment maintained a stable variation throughout the incubation period.

3.3. Mycelial density

The Friedman test suggests that there are no significant differences in mycelial density between treatments among all days $\chi^2(1)$ = 3.6, p = 0.057. Figure 3 shows changes in mycelial density throughout the incubation period. Dunn's test for daily pairwise comparison revealed significant differences from days 2 to 7 (Table 2). Differences are more prominent between days 2 through 5. Fermented treatment showed higher mycelial density compared to sterilized treatment in most days, except at the start and the end of the incubation period. Variation between treatments seemed constant. Fermented treatment had a higher variation in mycelial density compared to sterilized treatment, but the behavior of both treatments was similar.

3.4. CFU quantification of fermented substrate

Changes in the number of CFU during substrate fermentation and 7 days after mycelium inoculation are shown in Figure 5. Data was transformed to logarithmic scale given the difference in magnitude of the collected data. After 3 days of substrate fermentation, CFU counts of all types of microorganisms decreased, followed by an increase in counts for non-fastidious bacteria and yeast colonies. Mold colonies did not appear on samples taken at day 7 of fermentation, nor 7 days after mycelium inoculation. After inoculation, bacterial colony counts remained stable, while yeast colonies decreased, reaching a similar count as the start of fermentation.

4. Discussion

4.1. pH of fermented substrate

Zi et al. [31] found that adding water-soluble carbohydrates during silage fermentation of stylo (*Stylosanthes guianensis*) improved microbial diversity, increased the presence of acid-producing bacteria, and increased the yield of lactic acid while lowering silage pH values. A similar pH trend was

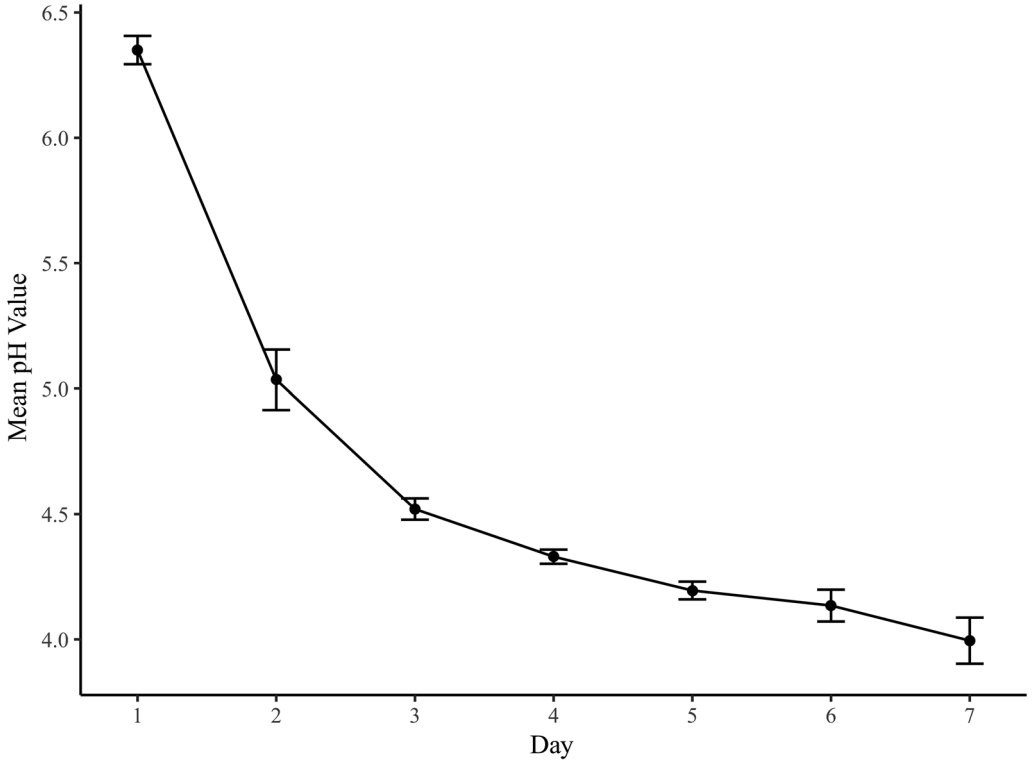

Figure 1. Scatterplot of pH value over time (days) of substrate fermentation process. Mean with standard deviation (black bars) from 2 replications.

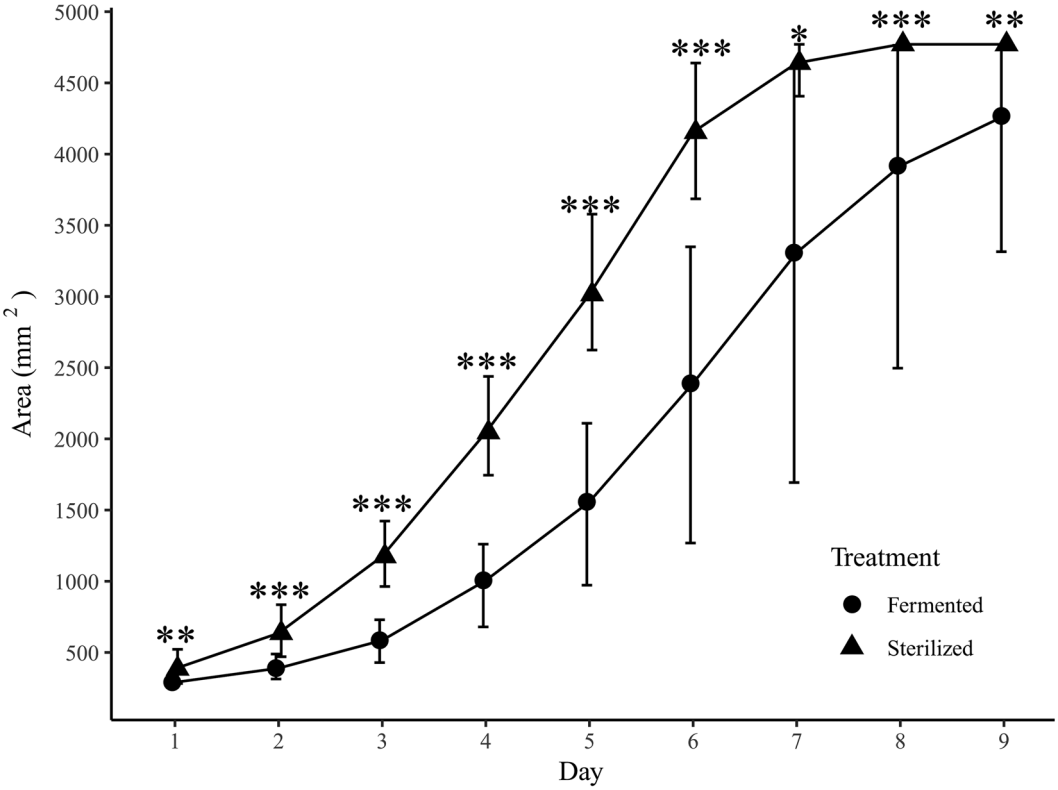

Figure 2. Scatterplot of mycelial area (mm^2) over time (days) of fermented and sterilized substrate treatment. Mean with 95% confidence intervals (black bars) from 8 replications. Significance labels related to level of statistical significance between treatments each day, are also included on the plot.

Table 1. Pairwise comparisons of fermented vs. sterilized treatments for growth area.

Day	Z-value	Adjusted p-value	Significance
1	−2.41	0.007	**
2	−3.25	<0.001	***
3	−3.36	<0.001	***
4	−3.36	<0.001	***
5	−3.36	<0.001	***
6	−3.36	<0.001	***
7	−2.83	0.02	*
8	−3.24	<0.001	***
9	−2.2	0.01	**

Note: If the adjusted p-value is the same as the original p-value, only the adjusted p-value is reported.

observed when sucrose was added during substrate fermentation.

Lactic acid-producing microorganisms involve a variety of bacteria and fungi, including *Bacillus* strains and yeast [32]. Gbolagade [33] analyzed the bacterial community of composted hardwood sawdust and wheat bran used as substrate for *Pleurotus* cultivation. Four out of nine isolates found were *Bacillus* species, which are able to ferment most sugars, and can aid in the fermentation process. Sándor et al. [34] established a lower pH limit of 4.2 for *P. ostreatus* growth, while [35] determined that a low pH (3.8 – 4.0) inhibited bacterial and fungal contamination in *Pleurotus sajor caju* submerged cultivation while supporting normal mycelium growth. The pH values observed after substrate fermentation align with the ranges described by [34] and [35].

The addition of sucrose might have changed the naturally present microbial community, aiding to limit the growth of contaminants by modifying the pH of the substrate where contaminants cannot grow optimally.

4.2. Mycelium growth

After reviewing the existing literature related to substrate treatments for edible mushroom production, research regarding *P. ostreatus* mycelial growth in substrate that has not been through some kind of heat or chemical treatment to reduce or eliminate microbial presence is lacking. Hernández et al. [7] employed wooden crates for composting the substrate used for *P. ostreatus* cultivation. Their procedure is simple and effective for mushroom cultivation, but they also added 2% of $Ca(OH)_2$ to their substrate composition, which can be categorized as a chemical treatment. Calcium hydroxide has been commonly used to treat substrate, usually by submerging the substrate in a 2% $Ca(OH)_2$ solution for several hours [6,36,37], to increase pH until it reaches alkaline levels, effectively changing the optimal pH range where contaminants and competitor organisms can grow. Only one article found in the literature review applied a water immersion, anaerobic fermentation process to treat the substrate, similar to our experiments [38]. The

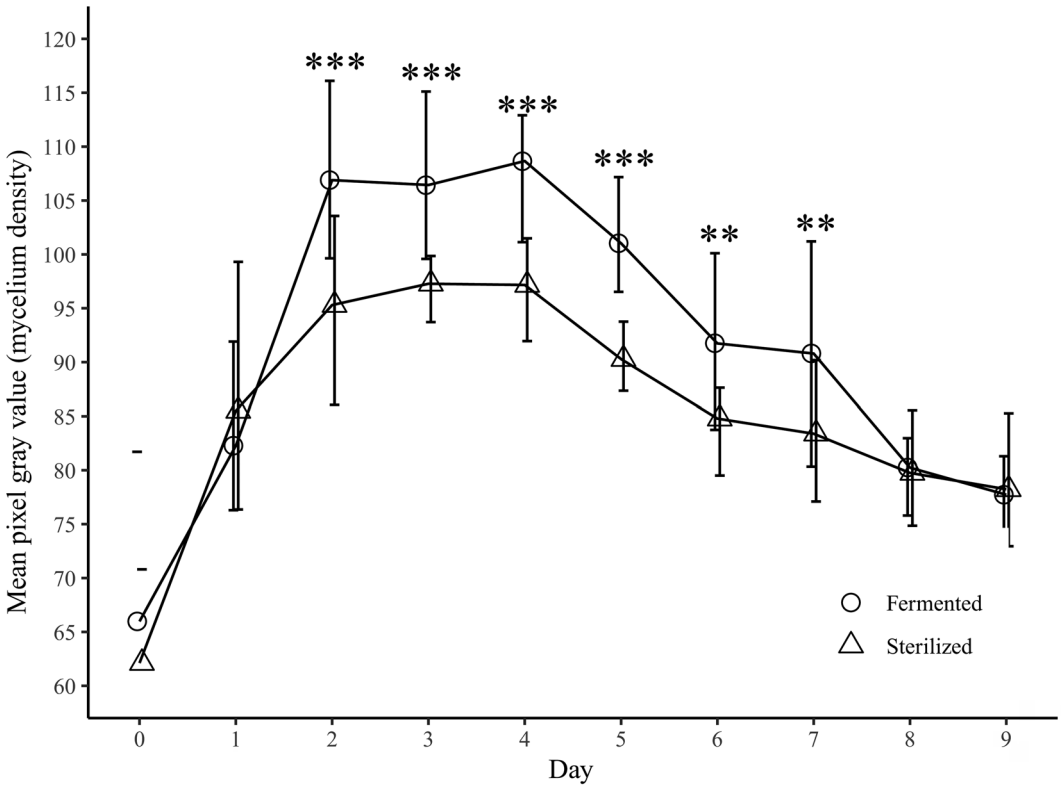

Figure 3. Scatterplot of mean pixel gray value (mycelium density) over time (days) of fermented and sterilized substrate treatment. Mean with 95% confidence intervals (black bars) from 8 replications. Significance labels related to level of statistical significance between treatments each day, are also included on the plot.

Table 2. Pairwise comparisons of fermented vs. sterilized treatments for mycelial density.

Day	Z-value	Adjusted p-value	Significance
0	0.63	0.26	–
1	−0.89	0.18	–
2	3.04	0.001	***
3	3.04	0.001	***
4	3.15	<0.001	***
5	3.36	<0.001	***
6	2.62	0.004	**
7	2.2	0.01	**
8	0.63	0.26	–
9	−0.42	0.33	–

Note: If the adjusted p-value is the same as the original p-value, only the adjusted p-value is reported.

researchers applied 2% calcium carbonate ($CaCo_3$) to the substrate formulation. Whether calcium carbonate has a similar effect as calcium hydroxide on substrate used for mushroom cultivation is not well-documented. The addition of calcium carbonate to the substrate may have been the reason it got similar results as processes that employed calcium hydroxide. Besides this, our search could not find other research related to the submerged fermentation method without any chemical or heat-related processes, to obtain mycelial colonization of substrates. Nevertheless, we recognize that the limited availability of research on this topic within our search parameters does not rule out the existence of other potentially relevant studies.

The presence of mycelial growth in a substrate fermented by its natural microbial community and devoid of any heat process or chemical additives is promising. Despite drying the substrate at 50 °C for 3 h to reduce moisture content, bacterial and yeast CFU counts remained unaffected, as shown in Figure 5. As expected, sterilized treatment had consistent, rapid mycelial growth, and it reached full colonization by the 8th day of incubation. Fermented treatment had a slower growth rate, with just half of the Petri dishes reaching full colonization by the 9th day of incubation. The growth of the rest of the Petri dishes seemed to have slowed down considerably or just stopped completely (Figure 4). This variability in growth rates within the fermented treatment may be attributed to differences in the distribution and activity of microbial species across individual Petri dishes, even though all were exposed to the same fermentation conditions. Variations in the relative abundance and metabolic activity of these microbial species likely influenced local substrate conditions, such as nutrient availability or competitive interactions, resulting in the observed growth patterns. Furthermore, pH measurements for the sterilized substrate, taken after the substrate had cooled down after sterilization, showed a mean value of 6.05 (± 0.15), which is conducive to fungal growth. Unlike the fermented substrate, the absence of

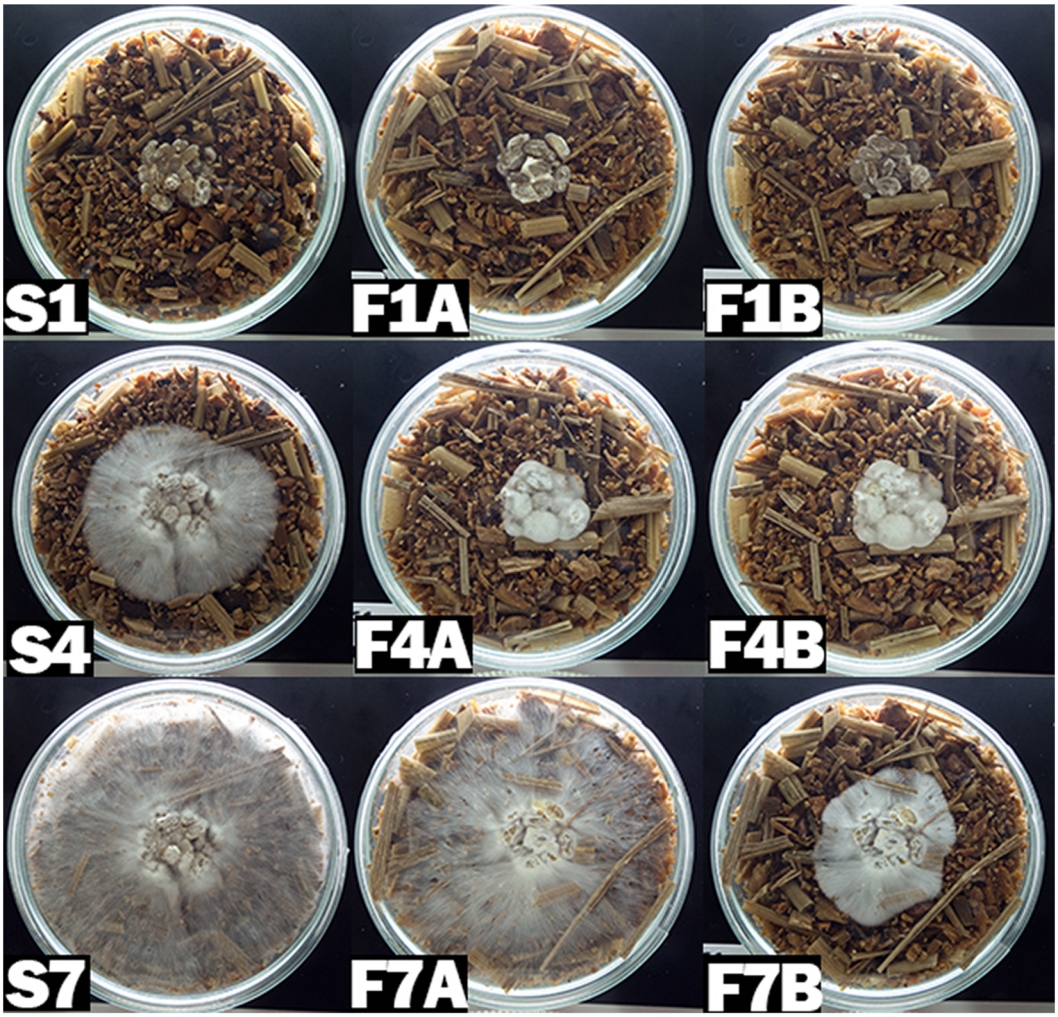

Figure 4. Comparison of mycelium growth and density in sterilized versus fermented substrates. Sterilized substrate: Day 1 (S1), day 4 (S4), day 7 (S7). Fermented substrate: Day 1 (F1A, F1B), day 4 (F4A, F4B), day 7 (F7A, F7B). Two different growth patterns emerged on fermented substrate.

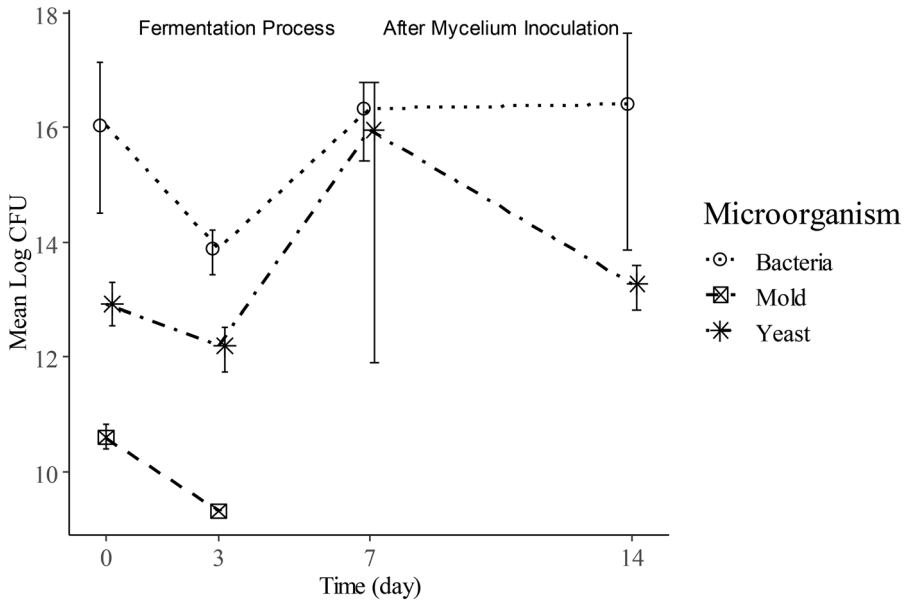

Figure 5. Mold, yeast, and non-fastidious bacterial CFU log counts over one week of fermentation, and 7 days after mycelial inoculation on fermented substrate. Means and standard deviation (black bars) taken from at least 2 replications. There were no CFU counts in substrate after sterilization.

microbial activity in the sterilized treatment likely stabilized the pH throughout the incubation period, reducing the impact of environmental fluctuations on fungal growth. Tariang et al. [39] evaluated the growth rate of *P. ostreatus* under different conditions, one of them being levels of pH ranging from 4.0 to 7.5. A pH of 4.0 had a significantly lower growth rate compared to values of 6.5 to 7.5. These lower and higher pH values correspond with our experimental treatments, and the results seem consistent.

4.3. Mycelial density

Mycelial density is often assessed visually and categorized as "abundant," "regular," or "scarce," or to equivalent categories in a subjective manner [39–43]. Utilizing digital images to analyze mycelial density provides less subjective measurements, offering insights that might have been overlooked through visual assessments alone.

Both treatments showed the same general trend in mycelial density. From the day of inoculation, we saw an increment up until day 2, followed by stable density levels until day 4, where it started decreasing until the end of the incubation period. We consistently observed higher mycelial density in the fermented substrate compared to the control, except at the beginning and the end of the incubation period. Similar mycelial density values at the start of the incubation can be attributed to the same spawn being used to inoculate both treatments, which had the same color and amount of mycelium on it. The decrease in mycelial density by the end of the incubation period might be due to the depletion of available resources or the initiation of recycling mechanisms within the mycelial network, effectively changing the fungal structure [44].

Mycelial density levels in fermented substrates being higher than the control could be influenced by the presence of microorganisms in the substrate. While microorganism CFU measurements for sterilized substrates after inoculation yielded no growth or counts below the threshold for accurate results (10 – 30 colonies) [45], the fermented substrate retained an active microbial community throughout the experiment. In such substrates, microbial interactions likely affect resource availability and competition, forcing the mycelium to adapt metabolically. According to Fricker et al. [44], these interactions can contribute to the formation of dense hyphal networks as a response to environmental challenges. Conversely, sterilized substrates, which lack competing microorganisms, promote straightforward radial hyphal expansion, resulting in less dense but faster-growing mycelium. While microorganisms play a significant role in influencing density, additional factors, including changes in substrate microstructure or inherent metabolic responses of the mycelium to their environment, may also contribute to these differences. Shrestha et al. [42] evaluated *Cordyceps militaris* mycelial density and other growth characteristics under several culture media compositions, incubation durations, and light conditions. They found that, under the same culture media and light conditions, mycelial density varied with the duration of the incubation period. The mycelium in some media was moderate to abundant during the first weeks and then decreased to moderate or poor density by the fourth week of incubation. The opposite result was also observed in some culture media

Tariang et al. [39] also evaluated *P. ostreatus* mycelium morphology, including mycelial density, under different pH levels. They found that pH values of 4.0, 4.5, 6.0, 6.5, 7.0, and 7.5 had all similar mycelial growth morphology during all 9 days of incubation. These results are also consistent with our findings, in terms of mycelial density patterns over time.

4.4. CFU quantification of fermented substrate

Yeast and bacterial CFU were present during the whole fermentation process and were also present 7 days after inoculation of the mycelium, which was actively growing despite the presence of these microorganisms. The initial decrease of yeast and bacterial CFU on the 3rd day of fermentation and subsequent increase on the 7th day of fermentation suggests a shift in the microbial community. Most likely due to the depletion of oxygen, reducing the counts of aerobic microorganisms, and promoting the growth of facultative and anaerobic microorganisms.

Yeast CFU counts increased from the 1st to the 7th and final day of fermentation. These results are in accordance with the findings of [46], where they analyzed fungal CFU changes during oyster mushroom substrate composting and preparation. Yeast CFU were also present in substrate colonized by *P. ostreatus*. Whether yeasts have a slightly positive, neutral, or inhibitory effect on filamentous fungi is dependent on its source (phyllosphere-derived or soil-derived yeast), the species of yeast, and the species of filamentous fungi that it is interacting with [47]. This could mean that the naturally-present yeast in the substrate could have a neutral or positive effect on *P. ostreatus*, while having antagonistic

effects on other possible contaminant filamentous fungi.

Bacterial CFU counts increased slightly from the 1st to the 7th day of fermentation, and then remained somewhat stable after inoculation of *P. ostreatus* mycelium. It has been observed that *P. ostreatus* can coexist with certain bacterial communities during its colonization phase [10]. The presence of *P. ostreatus* mycelium influences the microbial community within the substrate, reducing or increasing specific bacterial populations. Some interactions observed between bacteria and *P. ostreatus* vegetative mycelium are the bacteriolytic breakdown of bacterial biomass by the fungal hyphae, the attachment of bacteria to the mycelium, and the promotion of hyphal growth through the secretion of extracellular enzymes and exudates by bacteria [48]. The presence of bacterial CFU counts even after one week of colonization by *P. ostreatus* mycelium highlights the complex interactions between them within the substrate.

The disappearance of mold colonies during fermentation may result from their preference for air-level oxygen environments, where they utilize oxygen as substrate for respiration [49]. Mold colonies were observed on the 1st day of fermentation, but by the 3rd day there was a decrease in CFU counts. This decline suggests that molds may have utilized the available oxygen near the surface of the substrate, particularly in areas not submerged underwater. By the end of fermentation, the absence of mold colonies is likely attributed to the depletion of oxygen within the fermentation bags, or the inability to perform other types of metabolic mechanisms under oxygen-limited conditions. Additionally, daily mixing of the substrate throughout the fermentation process ensured uniform submersion of the biomass, further inhibiting mold growth.

While bacterial CFU counts remained relatively stable throughout the fermentation and post-inoculation periods, these counts do not capture the diversity or activity of bacterial communities within the substrate. Differences in microbial community composition across Petri dishes could have influenced nutrient availability and competitive interactions, shaping local conditions that impacted fungal growth.

5. Conclusions

The presence of *P. ostreatus* mycelial growth in substrate fermented underwater without chemical or heat treatments is a promising, low-cost alternative to traditional substrate sterilization methods. Digital analysis showed that, despite slower colonization, mycelial density in the fermented substrate was generally higher compared to sterilized substrates, reflecting interactions with naturally-present microorganisms. While these findings suggest that submerged fermentation can make the substrate preparation process more accessible and reduce costs, further research is needed to validate its effectiveness and scalability in mushroom cultivation. Incorporating microbial community profiling techniques in future studies would also help clarify the role of microbial diversity and pH fluctuations in shaping mycelial growth dynamics, providing deeper insights into the potential of this method as an accessible alternative for substrate treatments.

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Disclosure statement

The authors declare no potential conflict of interest.

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