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Biopreservation Potential of Shimeji (*Hypsizygus* sp.) Mushroom Fermented with *Bifidobacterium* sp. InaCC B723

Hafidz Azhar Maskuri[1], Yaovapa Aramsirirujiwet[2], Ingorn Kimkong[2], Sari Darmasiwi[1*]

[1]Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sleman, Special Region of Yogyakarta 55281, Indonesia
[2]Department of Microbiology, Faculty of Science, Kasetsart University, 50 Ngam Wong Wan Rd., Lat Yao, Chatuchak, Bangkok, 10900 Thailand

*Corresponding Author: saridarma@ugm.ac.id

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Abstract. Nowadays, mushrooms are widely consumed as a source of functional food and health, including the shimeji (*Hypsizygus* sp) mushroom. Fresh shimeji is highly perishable, mainly due to its high-water content, high respiration rate, and the presence of microflora. The objective of this research was to examine the potential of *Bifidobacterium* sp. as a starter for fermentation and bio preservation of *Hypsizygus* sp. In this study, *Hypsizygus* sp. was fermented with addition of *Bifidobacterium* sp. (10^7 CFU/ml), continued with incubation in room temperature 25±2℃ for 18 days. Fermented samples were then analyzed for the number of microbial populations including lactic acid bacteria (LAB), yeast, and Enterobacteriaceae using Total Plate Count; chemical quality of pH, lactic acid using titration, nitrite using spectrophotometry, and volatile compounds using GC-MS. During fermentation, the LAB population increased rapidly until it reached its peak population on the 3rd day. The rapid growth of LAB was followed by an increase in lactic acid content and a decrease in pH. Organic acids can control the growth of other microorganisms such as yeasts, molds, and Enterobacteriaceae while preventing the damage and decay of mushrooms. GC-MS analysis of fermented mushroom extract exhibited major bioactive compounds of butanoic acid (14,25%), Hydroperoxide, 1-methylpentyl (10,01%), and n-hexadecanoid acid (9,756%). This research was the first report on the use of *Bifidobacterium* sp. for *Hypsizygus* sp. fermentation, with potential to be applied as a bio-preservation method of edible mushrooms with enhanced characteristics which can be applied to the wider community.

Keywords: *Bifidobacterium* sp.; fermentation; lactic acid; *Hypsizygus* sp.

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INTRODUCTION

Hypsizygus sp., commonly known as Shimeji mushroom, is an important mushroom cultivated in Japan, East Asia, and northern Europe (Chauhan et al., 2017). Shimeji has low protein, fiber, and fat content, but high in potassium, magnesium, and phosphorus (Gopal et al., 2022). Shimeji also has a nutrient-dense, flavorful, and high-water content which makes it easily damaged, yet susceptible to fungal disease, and nutritional loss (Yang et al., 2024). Therefore, the preservation process is needed to extend the shelf life of shimeji mushrooms. The most commonly used methods to preserve the mushroom are canning and salting. However, these methods can alter the physical and chemical properties of the mushroom, resulting in a darker color, less intense flavor, and reduced nutritional value (Liu et al., 2014). To preserve the nutritional and sensory value of mushrooms, another technique, such as fermentation, is required.

Fermentation is a method that can preserve food without changing its quality. Fermentation has been known to humans for a long time with the existence of conventional fermentation methods such as kimchi and sauerkraut. Conventional fermentation is conducted spontaneously without using bacterial starters, which requires a longer time (Zheng et al., 2017). Nowadays, single culture is more widely used as a starter in producing fermented foods because it has the advantage of food safety over spontaneous fermentation. In the fermentation process, microbes such as lactic acid bacteria (LAB) generally suppress the growth of pathogenic bacteria that can damage and spoil the mushrooms (Prayitno et al., 2020).

Lactic acid bacteria consume sugar and produce metabolites containing organic acid and bacteriocins as a result of metabolism, which can reduce the pH of the solution, inhibit the growth of pathogenic bacteria, and reduce nitrite levels in the solution(Abbasiliasi et al., 2017). Increased levels of organic acids in the solution can maintain the quality of the mushroom from damage and decay to extend the shelf life of the mushroom (Liu et al., 2016). Research by (Sun et al., 2022) reported the fermentation of ear mushrooms (*Auricularia auricula*) by *Lactiplantibacillus* and *Leuconostoc,* and found that apart from improving the nutritional quality and organoleptic properties, the fermentation improved storage stability even after 28 days of storage.

One species of lactic acid bacteria that can be used as a starter for fermentation is *Bifidobacterium* sp. It belongs to a species of probiotic microorganisms which are commonly found in the human gut, that provide health benefits to humans when administered appropriately (Chen et al., 2021; Turroni et al., 2019). Bifidobacterium strains were reported to have special characteristics such as tolerance to acidity, resistance to lysozyme, antimicrobial activity against pathogenic microorganisms (*Escherichia coli* and *Candida albicans*), and capability in producing exopolysaccharide(Kusharyati et al., 2020).

The application of *Bifidobacterium* sp. as a starter have been reported to be used in fruit and vegetable fermentation, including *B. longum* for the fermentation of orange, tomato, and carrot juices which produce lactic acid and acetic acid that inhibit the growth of *Campylobacter jejuni* (Havas et al., 2014). *B. breve* and *B. longum* were reported to be added in the fermentation of exopolysaccharide of *Cordyceps sinensis* mushroom with inhibition effect against *Escherichia coli* (Li et al., 2024). In addition, *B. longum* was also used as a fermentation starter for *Flammulina velutipes* mushroom extract which shows antioxidant activity and ACE inhibition (Peasura et al., 2025).

Research on the fermentation of *Hypsizygus* sp. mushrooms by *Bifidobacterium* sp. has never been done. Most research of lacto-fermented mushrooms used *Lactobacillus* sp. as the fermentation starter, such as the use of *Lactobacillus fermentum* in the fermentation of edible mushrooms *Termitomyces robustus* and *Pleurotus ostreatus* (Ogidi & Agbaje, 2021). *Lactobacillus acidophilus* was also used as a starter for fermentation of *Lactarius deliciosus* mushrooms (Venugopal et al., 2024). Lactobacillus strains are known to have good viability compared to Bifidobacterium in yogurt fermentation from buffalo milk (Bilal et al., 2021). Likewise, in the lactic acid fermentation of artichoke, pineapple, pumpkin, spinach, and cucumber showed better viability of *L. rhamnosus* compared to *Bifidobacterium animalis* subsp. *lactis* BB-12 (Güney & Güngörmüşler, 2021). However, (Cizeikiene & Jagelaviciute, 2021) stated that both *B. animalis* DSM 20105 and *B. pseudolongum* DSM 20099 showed excellent probiotic potential compared to *Lactobacillus* spp. in functional food/feed products as supplement.

The research aimed to examine the potential of *Bifidobacterium* sp. used as a starter in *Hypsizygus* sp. fermentation for biopreservation by examining microbial growth, chemical analysis, and the sensory quality of the fermented mushroom. It is envisioned that the study of lacto-fermented mushrooms using *Bifidobacterium* sp. will provide information to society about safe alternative biopreservation methods for edible mushrooms that keep their desirable characteristics. Additionally, it will advance scientific understanding of the development and advantages of lacto-fermented mushrooms as functional foods, including the biological activities and bioactive compounds they contain.

METHODS

Preparation of Bacterial Starter

Bifidobacterium sp. INACC B723 strain was procured from the Indonesian Culture Collection. The strain was subcultured on MRS (De Man, Rogosa, and Sharpe) broth medium, then incubated at 37°C for 24 hours. The cells were harvested using centrifugation at 4500 rpm for 20 minutes and then washed twice with 0.9% NaCl solution. The washed cells were measured in absorbance to match 0.5 McFarland (1.5×10^8 CFU/mL (Liu et al., 2016).

Mushrooms Fermentation

The preparation of fermentation is carried out under aseptic conditions. Approximately 300 g of *Hypsizygus* sp. was thoroughly cleaned, boiled, and placed into a 500 mL glass jar. The jar was then supplemented with 3% red chili and 2% garlic. These jars, containing the precisely measured ingredients, were sterilized in boiling water for 10 minutes. A solution of 2% salt and 1% sugar was prepared in 300 mL water, which was then added to a chilled jar and inoculated with

1 mL of LAB starter (10^7 CFU/mL). The fermentation was carried out at a consistent room temperature of 25±2˚C for a duration of 18 days (Zheng et al., 2017).

Microbial Enumeration

At specific intervals of day 0, 3, 6, 9, 12, 15, 18, about 1 mL of fermentation solution was carefully extracted and gradually diluted to a concentration of 10^{-5}. These dilutions were then poured into specific media: MRS agar medium for LAB, PDA for yeast, and Violet Red Bile Glucose Agar for Enterobacter. The media were incubated at precise temperatures: 37˚C for 48 hours for LAB and Enterobacter, and yeast at 30˚C for 48-72 hours (Liu et al., 2016).

Measurement of pH and Acid Content

About 5 mL of sample solution at intervals of day 0, 3, 6, 9, 12, 15, and 18 were measured using a pH meter. For measurement of acid content, fermentation sample (10 mL) was diluted in a volumetric flask to 100 mL, then 10 mL of the dilution was taken and added with phenolphthalein indicator 2% (w/v) as much as 1 mL. The solution was titrated with 0.1 M NaOH for 2 minutes until the right pink color was formed (Jablonska-Rys et al., 2022).

Nitrite Content Analysis

A 5 ml sample is taken and diluted into a 50 mL volumetric flask with distilled water. The solution is filtered with filter paper. After filtering, 10 mL of solution was taken into a test tube, and 0.25 mL of sulfanilamide solution was added, then vortexed and rested for 8 minutes. After 8 minutes, 0.25 mL of NED solution was added, vortexed, and rested for 10 minutes. After resting, the solution was measured for absorbance using a spectrophotometer with a wavelength of 538nm. Nitrite levels were determined using a nitrite standard curve with concentrations of 0 mg/L, 0.01 mg/L, 0.02 mg/L, 0.05 mg/L, 0.1 mg/L, 0.15 mg/L, 0.2 mg/L (Liu et al., 2016)

GC-MS Analysis

Filtered sample (100 mg/mL) was added to methanol with KOH (2 N; 0.1 mL) and heated in a water bath at 45°C for 1 hour. After the esterification reaction, the sample was cooled, and hexane (1 mL) was added and stirred thoroughly to extract the fatty acid esters. The upper hexane layer was dried with sodium sulfate, dissolved in 0.1 mL hexane and analyzed by GC-MS using an HP5MS-09012024 instrument (Agilent, USA) with an HP-5MS UI column (30 m× 250 µm × 0.25 µm), under the following operating conditions: 250°C injection temperature and 280°C detector temperature. The temperature was programmed from 50° to 280°C. The temperature increase was 5°C/minute and held at 280°C for 5 minutes. The carrier gas was used with a flow rate of 1 mL/min. The injection volume was one µL. Components separated from the GC column enter the MS, where they are ionized and fragmented. The MS then analyzes the fragments based on their mass-to-charge ratio, creating mass spectra for each component with a GCMS library database (Balakrishnan & Agrawal, 2014)

Sensory Analysis

The results of 14 days of fermentation were given to 10 respondents consisting of 5 women and 5 men aged between 20 and 25 years, non-smokers. Samples were given to respondents and assessed based on appearance, color, taste, smell, and texture. Assessment with levels 1-5 (5 = very like, 4 = like 3 = neutral, 2 = dislike, 1 = very dislike) (Jablonska-Rys et al., 2022).

Data Analysis

The data obtained were processed into tables and graphs in the Microsoft Excell application and then statistically analyzed using the IBM SPSS statistic 25 application. Statistical analysis was carried out using the One-way ANOVA method and further testing was carried out using the Duncan test with a significance level of 0.05% and 95% confidence level.

RESULTS AND DISCUSSION

Microbial Growth

The population of lactic acid bacteria was seen to increase significantly during the first 3 days of fermentation, then there was constant growth and began to show a decline on the 15th to 18th day as shown in Figure 1. The rapid increase in LAB population at the beginning of fermentation up to the third day was due to environmental parameters supporting LAB growth, such as an anaerobic environment, and carbon sources, such as glucose, that are still widely available. It was reported previously by Liu et al. (2016) and Jabłonska-Rys et al. (2022), that the LAB population increased rapidly up to day 3, then decreased as the fermentation time increased. The decrease in the LAB population at the end of fermentation time occurs because there are fewer carbon sources.

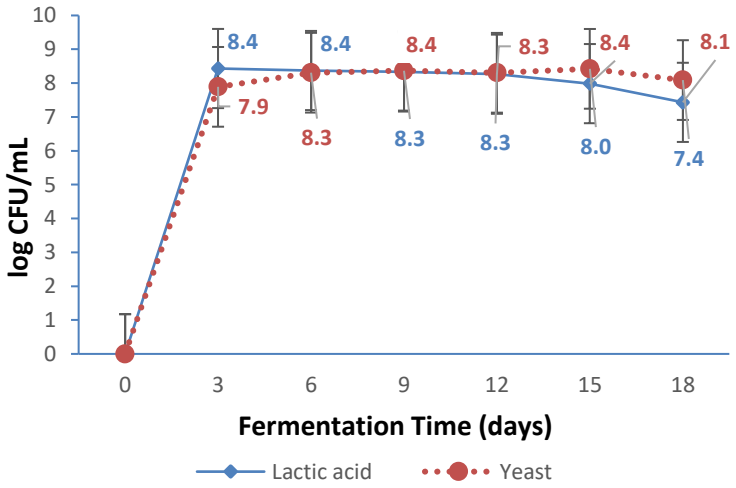


Figure 1. Lactic acid bacteria and yeast growth of lacto-fermented *Hypsizygus* sp. using *Bifidobacterium* sp.

Figure 1 also shows the growth of yeast in lacto-fermented shimeji, with a trend that is almost the same as the growth of lactic acid bacteria. Yeast growth also experienced a significant increase until the day when it showed an exponential phase, then the population showed static growth until day 15 and decreased slightly on day 18 to 8.1 log CFU/mL. Lactic acid bacteria and yeasts often coexisted throughout all stages of fermentation, and during the first six days, the growth of the yeast population was comparable to that of LAB. A controlled yeast population can help avoid spoilage and maintain the quality of fermented mushrooms (Liu et al., 2016). When the LAB population is higher than the yeast, the fermentation that occurs tends to produce products with more sour taste and lower pH. However, conversely, when the yeast population is higher than the LAB population, it will produce more ethanol so that it is less acidic and preservation is insufficient.

In this study, *Bifidobacterium* sp. was used to control the yeast population through competition for nutrient assimilation during fermentation. The results obtained are from research conducted by Liu (2016), which found that yeast growth was lower than LAB until 6th day, and then yeast decreased at the end of fermentation time. This higher growth of LAB caused a decrease in the pH of the solution due to an increase in acid levels produced by LAB.

Meanwhile, there was no Enterobacteriaceae detected on violet bile glucose agar medium from day 0 until the end of fermentation or 18th days. This could possibly happen because the fermentation condition was unsuitable for the Enterobacteriaceae to grow, where the appropriate temperature for Enterobacter was around 25-41 C, and pH above 4. The rapid growth of lactic acid bacteria and yeast created acidic conditions where both populations of microorganisms are able to ferment carbon sources into organic acids that might damage the cell wall structure of pathogenic bacteria, such as enterobacter, that are susceptible to acidic environments (Alebiosu et al., 2017; Wang et al., 2015). This result was in accordance with the results of Liu (2016) that showed the use of LAB starter could efficiently inhibit Enterobacter growth during fungal fermentation.

Changes in pH, Lactic Acid, and Nitrite Content

Based on Figure 2, it was found that the pH of lacto-fermented shimeji decreased from 4.94 at 0th day then continued to decrease until 3.51 at 6th day, while remain stable in 9th to 15th day, and significantly decrease to 3.52 at 18th days. This result follows research conducted by Liu et al. (2016) that the pH of the solution dropped drastically on 3rd days, and then the decrease in pH from 6th days to the end of fermentation tended to be stable. Research by Zheng et al. (2017) reported similar results in acidification and sauerkraut experiments on King oyster Mushroom. The study found that fermented shimeji have pH values between 3.52 and 4.92, varying based on fermentation temperature, carbohydrates, and additions. A pH below 4.0 ensures preservation of fermented vegetables and anaerobic conditions as reported previously (Jablonska-Rys et al., 2022; Meshram et al., 2018)

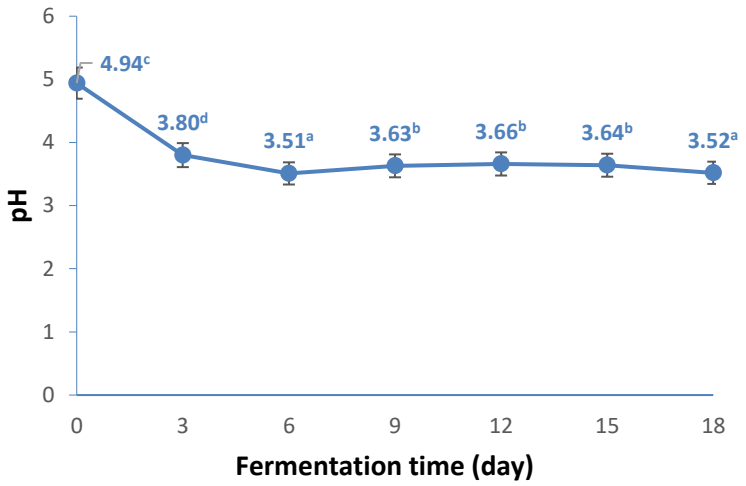


Figure 2. pH of *Hypsizygus* sp. fermented with *Bifidobacterium* sp. Means in the graphic with different superscript letters (a, b, c) are significantly different ($p < 0.05$).

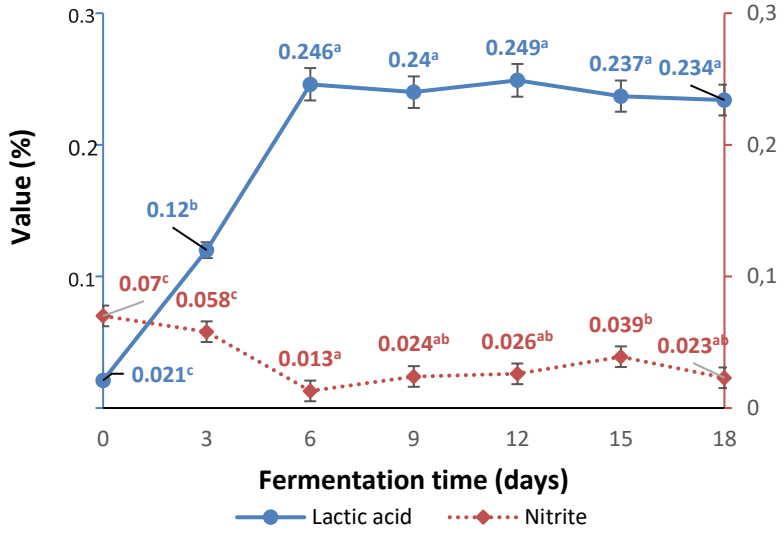


Figure 3. Lactic acid and nitrite content of *Hypsizygus* sp. fermented with *Bifidobacterium* sp. Means in the graphic with different superscript letters (a, b, c) are significantly different ($p < 0.05$).

A reduction in pH during fermentation is linked to the production of organic acids, including lactic acid, during fermentation which were the primary metabolite of LAB as shown in Figure 3. The increase in lactic acid levels indicates conformity with the growth of lactic acid bacteria and yeast which showed an exponential phase in the 3rd days of incubation until the 6th days (Figure 1) which was also almost the same as the trend shown by the levels of lactic acid produced. This was also supported by pH measurement (Figure 2) which shows a decrease starting from 3rd days until 6th days. This indicates that LAB could use carbon sources available of fermented mushroom as also reported by (Bartkiene, E. et al., 2023). A slight increase of pH at 9th days to 15th days might related with reduction of LAB and yeast growth, while slight increasing at 18th days was due to organic acids accumulation in the end of fermentation. It was reported that different LAB strains produce variable amounts of organic acid depending on their distinctive characteristics. *Bifidobacterium* sp. is a heterofermentative lactic acid bacteria that can reduce pH levels during the production of lactic acid and other organic acids, such as acetic acid, with a simultaneous reduction in oxygen levels through the production of carbon dioxide (Skrzypczak et al., 2020).

Nitrogen is necessary for the growth and development of plants. Nitrogen fixation adds nitrate, which is the nitrogen that plants absorb. Plant absorption rate and nitrate reductase determine the amount of nitrate in vegetables, including mushrooms (Ranasinghe & Marapana,

2018). During the fermentation process, nitrate can change into nitrite. In this matter, LAB strains could effectively degrade nitrite during the process of vegetable fermentation (Xia et al., 2017). Results of the nitrite level of fermented mushrooms showed that the nitrite level dropped to the lowest nitrite level on 6th days at 0.013 mg/l. Nitrite levels then increased at 15th days by 0.039 mg/l and decreased again until 18th days by 0.023 mg/l (Figure 3). This study is in accordance with (Liu et al., 2016) who conducted an analysis of nitrite reduction in *Pleurotus* spp. fermentation by *Lactobacillus pentosus* which resulted in a 38% decrease in nitrite, while in this study there was a 67% decrease in nitrite levels so that the use of *Bifidobacterium* sp. as a starter was quite effective in reducing nitrite levels. The decrease in nitrite levels is in line with the growth of lactic acid bacteria which can degrade nitrate into nitrite through the production of the nitrate reductase enzyme. However, the decrease became constant on 9th-18th days, possibly due to the decreasing growth rate of lactic acid bacteria towards the stationary phase.

The accumulation of nitrite levels in foods, particularly fermented foods, is a matter of grave concern. Consumption of high levels of nitrite can pose serious health risks, including the potential for cancer and several other diseases. High nitrite levels can facilitate the formation of nitrosamines, which are carcinogenic compounds, and met-hemoglobinema, which are detrimental to health (Karwowska & Kononiuk, 2020; Liu et al., 2016). Therefore, it is imperative to control nitrite levels in food. The use of *Bifidobacterium* sp. starter has been proven to be effective in reducing nitrite levels in fermentation solutions. LAB, through the production of organic acids and the synthesis of the enzyme nitrite reductase, can also significantly reduce nitrite concentration (Yan et al., 2008).

GC-MS Analysis

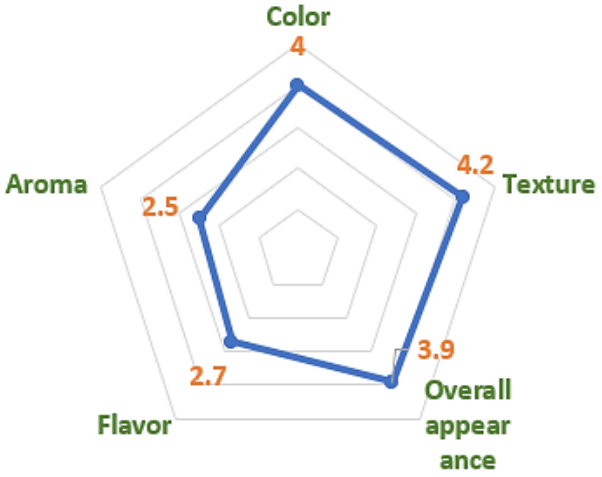
GC-MS analysis was conducted to determine the compounds from the 18th day of the fermentation sample. Twenty compounds were detected during the test, as shown in Table 1. The compounds that have been obtained are grouped into seven groups based on their functional groups: acids, peroxides, alcohols, ketones, esters, furans, and hydrocarbons.

Table 1. GC-MS analysis on *Hypsizygus* sp. fermented with *Bifidobacterium* sp.

Compound	**%Area**	**Compound Area**
Acids		
Butanoic acid	14. 25	155117.45
n-Hexadecanoic acid	9.76	106266.84
Octadecanoic acid	2.50	27196.61
Hydroperoxide		
Hydroperoxide, 1-methylpentyl	10.01	108999.36
Hydroperoxide, 1-ethylbutyl	8.96	97578.94
Ketone		
2-Hexanone	5.54	60310.72
Alcohol		
2-Hexanol	3.91	42602.53
3-Hexanol	2.48	27017.36
1-Nonanol	2.31	25091.17
Ester		
Hexanedioic acid, mono(2-ethylhexyl)ester	2.22	24110.57
Oxalic acid, allyl nonyl ester	1.23	13335.26
Sulfurous acid, 2-ethylhexyl isohexyl ester	1.18	12798.74
Furan		
3,7-Dimethyl-2,3,3a,4,5,6-hexahydro-1-benzofuran	2.18	23778.52
Hidrocarbon		
Octane, 2,4,6-trimethyl-	3.18	34563.26
Octane, 3,5-dimethyl-	2.50	27259.99
Hexane, 3,3-dimethyl-	1.86	20260.38
Hexane, 3,3-dimethyl-	1.55	16884.57
2-Decyne	1.32	14312.89
Nonane, 3,7-dimethyl-	0.97	10586.46

It was shown that the most widely detected compounds in GCMS are acids, especially butanoic acid or butyric acid. Butyric acid is synthesized by butyrate-producing bacteria, such as clostridial bacteria from clusters IV and XIVa, commonly found in the human colon (Rivière et al., 2016). The metabolism of *Bifidobacterium* bacteria and butyrate-producing bacteria are related to each other due to the cross-feeding interaction between Bifidobacteria and butyrate-producing bacteria (Moens et al., 2016; Rivière et al., 2016). Another compound found in the mushroom fermentation sample is hydrogen peroxide. The presence of this compound indicates the absorption of oxygen by *Bifidobacterium* sp., thus forming hydrogen peroxide (Shimamura et al., 1992). Oxygen absorption occurs due to the fermentation process, which is facultative anaerobic or not fully aerobic. Organic acids, hydrogen peroxide, and diacetyl are produced by LABs with acidifying ability, which lowers harmful microorganisms (de Souza & Dias, 2017).

During fermentation, the sample bottle is opened several times for test sampling, allowing air exchange, including oxygen, to enter the sample. Another compound that has a high concentration in the solution is hexadenoic acid (16:0), which is one of the most common saturated fatty acids found in the human body that can be found in food or synthesized endogenously from other fatty acids, carbohydrates, and amino acids (Carta et al., 2022)**.** The compounds produced in the fermentation process will affect the sensory of shimeji mushroom fermentation, thus affecting the level of panelist preference for the mushroom, as shown in Figure 4.



Characters	Score
Color	4±0.82
Texture	4.2±0.92
Overall appearance	3.9±0.74
Flavor	2.7±0.67
Aroma	2.5±1.68

Figure 4. Organoleptic test on *Hypsizygus* sp. fermented with *Bifidobacterium* sp.

(A) (B)

Figure 5: (A) unfermented *Hypsizygus* sp. (B) *Hypsizygus* sp. fermented with *Bifidobacterium* sp.

Sensory Analysis

The overall organoleptic test showed that the panelists liked the shimeji mushroom fermentation product with an average value of 3.9. In addition, the panelists gave an average value of 4 for color and 4.2 for texture (Figure 4), respectively, since the color and texture of the fermented mushroom did not differ much from fresh mushrooms (Figure 5). However, for the aroma and flavor parameters, the panelists gave a score of 2.5 and 2.7 for the fermented mushroom, indicating that the panelists did not like these two parameters. The results obtained are in line with research conducted by (Jablonska-Rys et al., 2022) on button mushrooms fermented by *Lactobacillus plantarum*, with the highest value obtained by the texture parameter followed by color and overall appearance and the lowest value obtained in the aroma and taste parameters. It may happen because organic acids resulting from bacterial metabolites can prevent food spoilage and the growth of other microorganisms that can damage the mushroom. Then, the panelists did not like the taste of the fermented mushroom because it tasted sour due to the organic acids present. The odor produced by the mushroom after fermentation was also not favored by the panelists; this is because, during fermentation, several types of compounds are formed that can produce unpleasant odors, such as esters. This was first research explores the potential of *Bifidobacterium* sp. as a starter for *Hypsizygus* sp. fermentation, highlighting their potential for biopreservation and advancing scientific and society understanding of lacto-fermented mushrooms as functional foods with desirable characteristics.

CONCLUSION

The use of *Bifidobacterium* sp., as a starter in lacto-fermented shimeji could enhance the growth of lactic acid bacteria, while increasing lactic acid production and lowering pH, causing inhibition of Enterobacteriaceae growth and reduction of nitrite content. The sensory quality of the mushroom was influenced by bacterial metabolites, with color, texture, and appearance had higher preference, but not for taste and aroma. Thus, the application of *Bifidobacterium* sp. has the potential for biopreservation of *Hypsizygus* sp., however it requires further research such as optimization of the number of starters added or addition of other spices that could improve organoleptic and mushroom quality.

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