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Effect of Initial pH on the Microbial Growth, Final pH Value, Crude Protein and Ash Level of *Agaricus bisporus* Cap and Stem in Submerged Fermentation

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ABSTRACT

The aim of the study was to investigate the effect of submerged fermentation with *Lactobacillus* spp. on the nutritional composition of *Agaricus bisporus* cap and stem. Fresh *A. bisporus* was provided, and the cap and stem parts were separated and cut into small pieces. Afterward, distilled water (400 ml) and urea (8.4 g) were added to the mushroom parts (100 g) and placed in different fermentation flasks. The fermentation flasks containing mushroom caps or stems were divided into two groups, and the pH levels of the fermentation medium were adjusted to 6 and 7. Fermentation flasks were autoclaved at 121°C for 15 minutes and *Lactobacillus* spp. was inoculated to each flask at 1 ml (10^8 CFU/ml). A positive control group was formed by allocating one uninoculated flask for each replicate of each pH value. Fermentation flasks were incubated for 48 hours at 30°C. After fermentation, fermented and inoculated mushroom cap and stem were analyzed to determine the crude protein, ash content, *Lactobacillus* spp. count and pH value. *Lactobacillus* spp. count was higher (P=0.028) in the pH 6 group of mushroom cap and tended to be higher (P=0.078) in the pH 6 group of mushroom stems compared with the pH 7 group. Submerged fermentation decreased (P<0.001) the ash content of the mushroom cap and stem in both pH values except the cap with pH 7 compared with the uninoculated mushroom. Similarly, the fermented mushroom cap and stem had lower (P<0.01) final pH values in both initial pH values. *Lactobacillus* spp. increased (P<0.001) the crude protein content of the mushroom cap with pH 6 but did not alter the crude protein content with pH 7. Besides, submerged fermentation decreased (P<0.001) the crude protein content of mushroom stem with both pH values. The results indicate that submerged fermentation using *Lactobacillus* spp. can be used to improve the nutritional composition of mushroom caps with pH 6.

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Introduction

Mushrooms have been used medically since ancient times as an important source of bioactive compounds (Atila et al., 2021). *Agaricus bisporus* (white button mushroom) is an edible Basidiomycete fungus and the world's most widely produced and consumed mushroom. It has important bioactive phenolic components such as benzoic acid derivatives (p-hydroxybenzoic acid, protocatechuic acid, and gallic acid) and cinnamic acid derivatives (cinnamic acid, p-coumaric acid, ferulic acid, and chlorogenic acid) (Ramos et al., 2019). Mushrooms also have strong antimicrobial, antioxidant, anti-inflammatory, hypocholesterolemic and immunostimulatory effects on broiler chickens (Bederska-Łojewska et al., 2017).

Fermentation is a bioprocess used to improve the nutritional quality of agricultural products (Gungor et al., 2021). The fermentation method can be divided into solid-state and submerged fermentations. Solid-state fermentation is defined by microbial growth on moistened solid substrates without free water (Gungor and Erener, 2020). Submerged fermentation is the microbial growth in a medium containing large amounts of free water (Behera and Ray, 2019).

Mushroom caps that are not suitable for eating and mushroom stems that are contaminated with compost are discarded in large quantities during the packaging process in mushroom production facilities. The discarded by-products account for 20% of total mushroom production (Altop et al., 2021). This high amount of mushroom by-products causes environmental pollution if they cannot be utilized in any field (Yang et al., 2021). Developing the possibilities of using mushroom by-products in animal nutrition can contribute to preventing environmental pollution.

Fermentation can improve the nutritional composition of *A. bisporus*. Altop (2019) reported that solid-state fermentation using *Aspergillus niger* increased the crude protein and ash content of *A. bisporus* cap and stem. *Lactobacillus* spp. is used as a probiotic microorganism in broiler chickens. *Lactobacillus* spp. also improved the nutritional composition of agricultural products with submerged fermentation (Kumoro and Hidayat, 2018). This study aimed to investigate the effect of the submerged fermentation by *Lactobacillus* spp. on the final pH, *Lactobacillus* spp. count, crude protein and ash content of *A. bisporus* cap and stem at different pH levels. The hypothesis of this study is that submerged fermentation using *Lactobacillus* spp. will significantly affect the final pH, *Lactobacillus* spp. count, crude protein, and ash content of *A. bisporus* cap and stem, with these effects varying depending on the different pH levels applied during fermentation.

Materials and Methods

Preparation of Substrate

Fresh *A. bisporus* was supplied from a local market at Samsun, Türkiye. The cap and stem of the mushroom were separated from each other. The mushroom by-products were cut into small pieces before fermentation.

Preparation of microorganism

Lactobacillus spp. were isolated from the cecum of a healthy 42-day-old broiler chicken (Ross 308). The cecum samples were serially diluted and diluations were inoculated to the *Lactobacillus* spp. selective medium (MRS agar). The grown *Lactobacillus* spp. colonies were selected by the morphology and biochemical tests (catalase, oxidase, indole). MRS broth was used for the cultivation of *Lactobacillus* spp. Cultivation was made in a shaking incubator at 120 rpm and 30 °C for 48 hours (Kumoro and Hidayat, 2018). Fresh bacterial suspension containing 10^8 CFU *Lactobacillus* spp. per milliliter (10^8 CFU/ml) was prepared before fermentation.

Submerged Fermentation

The cap and stem of the mushrooms were each weighed to be 100 grams separately and placed in seperate fermentation bottles. A mushroom cap or stem (100 grams) was mixed with distilled water (400 ml) in the fermentation flasks. Urea (8.4 g, 46% N) was added to the mixture as a nitrogen source. Two pH groups (6 and 7) were formed and pH levels of the fermentation medium were adjusted to 6 or 7 with 1 N NaOH and 1 N HCl. The mushroom parts were autoclaved for 15 min at 121 °C for sterilization.

One ml of *Lactobacillus* spp. suspension (10^8 CFU/ml) was inoculated into the sterilized mushrooms. A positive control was also formed by the separation of one uninoculated flask for each inoculated flask. The uninoculated and inoculated cap and stem were incubated for 48 hours at 30 °C.

Determination of Lactobacillus spp. Count, pH and Nutritional Composition

Crude protein, ash content, *Lactobacillus* spp. count and pH value of raw, fermented, and uninoculated mushroom caps and stems were determined at the end of the fermentation.

The counts of *Lactobacillus* spp. were determined from the fermentation liquid.. One milliliter of fermentation liquid was transferred to a sterile test tube and diluted with 9 ml sterile Ringer solution. Fermentation liquid and Ringer solution were mixed well, and the mixed solution was serially diluted (1:10). Each dilution was plated onto MRS (de Man, Rogosa and Sharpe) Agar (Merck 110660). Plates were incubated in anaerobic conditions at 30 °C for 72 hours. After incubation, *Lactobacillus* spp. colonies on plates were counted.

Mushroom pH was determined from different pieces of mushroom in triplicate. The pH value of the fermentation liquid was also determined from three different points after mixing the liquid. The pH values were recorded using a digital pH meter.

The crude protein and ash content of the raw, uninoculated, and inoculated mushrooms were determined according to the AOAC (2000) methods.

Statistical Analysis

A one-way ANOVA was performed to evaluate the statistical differences using SPSS 21.0. Differences between the means of the treatment groups were determined by Duncan's multiple-range test. Differences were considered statistically significant when $P \leq 0.05$.

Results

The Lactobacillus spp. counts for the fermented mushroom caps and stems are shown in Figure 1. *Lactobacillus* spp. count was higher (P=0.028) in the pH6 group than in the pH7 group for mushroom cap. Similarly, the pH 6 group tended (P=0.078) to have higher *Lactobacillus* spp. count compared with the pH7 group for mushroom stems.

The pH levels of the uninoculated and inoculated mushrooms are presented in Figure 2, while the pH levels of the fermentation liquid are given in Figure 3.Fermentation significantly decreased (P<0.01) the pH levels of both the mushrooms and the fermentation medium, compared to the uninoculated mushrooms in both pH groups.

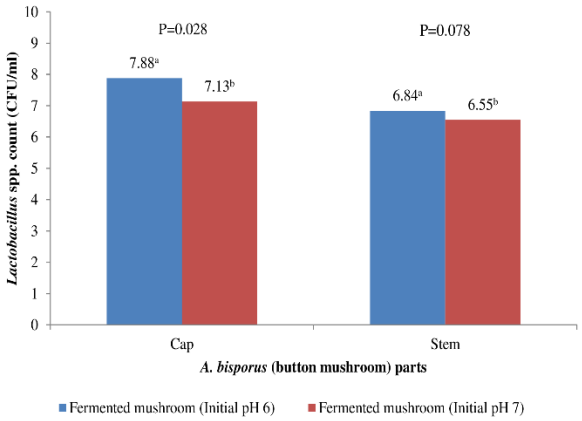

Figure 1. Final *Lactobacillus* spp. count of fermented *A. bisporus* cap and stem parts using *Lactobacillus* spp. with different initial pH

[a,b]Means with different superscripts within each mushroom part are significantly different (P<0.05)

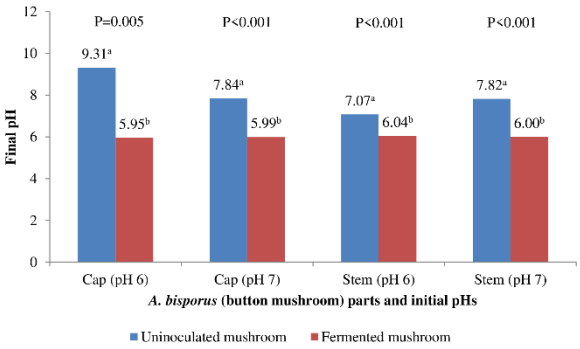

Figure 2. Final pH of fermented *A. bisporus* cap and stem parts using *Lactobacillus* spp. with different initial pH

[a,b]Means with different superscripts are significantly different within each initial pH level of each mushroom part (P<0.05)

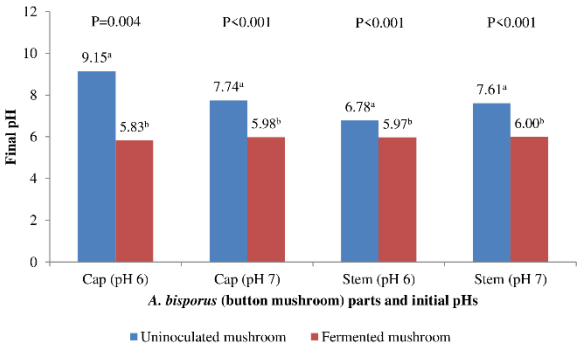

Figure 3. Final pH of fermentation medium of fermented *A. bisporus* cap and stem parts using *Lactobacillus* spp. with different initial pH

[a,b]Means with different letters are significantly different within each initial pH level of each mushroom part (P<0.05)

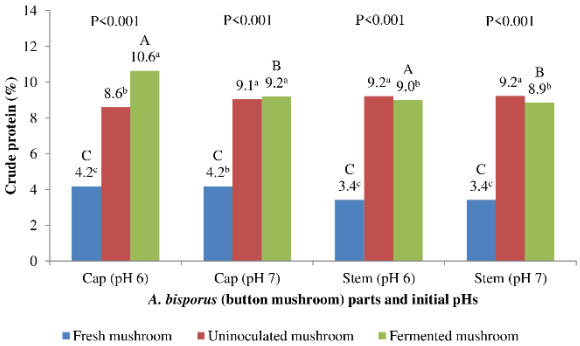

Figure 4. Crude protein content of fermented *A. bisporus* cap and stem parts using *Lactobacillus* spp. with different initial pH

[a,b,c]Means with different superscripts are significantly different within each initial pH level of each mushroom part (P<0.05)

[A,B,C]Means with different letters within each mushroom part are significantly different (P<0.05)

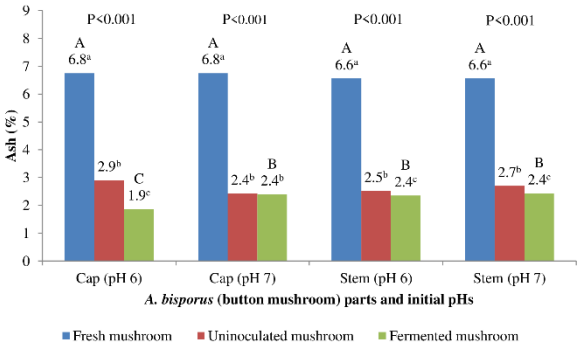

Figure 5. Ash content of fermented *A. bisporus* cap and stem parts using *Lactobacillus* spp. with different initial pH

[a,b,c]Means with different superscripts are significantly different within each initial pH level of each mushroom part (P<0.05)

[A,B,C]Means with different letters within each mushroom part are significantly different (P<0.05)

The effect of submerged fermentation on the crude protein content of mushroom cap and stem with different pH values is shown in Figure 4. Submerged fermentation increased (P<0.001) the crude protein content of the mushroom cap with pH 6. However, no effect was observed on the crude protein content of the mushroom cap by submerged fermentation with pH 7. In addition, *Lactobacillus* spp. decreased (P<0.001) the crude protein content of mushroom stem with both pH values. The highest crude protein content was observed by submerged fermentation with pH 6 among the fresh or fermented mushrooms in both mushroom part.

The effect of submerged fermentation on the ash content of mushroom cap and stem with different pH values is given in Figure 5. Submerged fermentation decreased (P<0.001) the ash content of the mushroom cap and stem in both pH values except cap part with pH 7 compared with the uninoculated mushroom.

Discussion

Submerged fermentation using *Lactobacillus* spp. caused a higher final *Lactobacillus* spp. count with a pH 6 level compared with a pH 7 level in *A. bisporus* cap and stem, which indicates better fermentation performance at a pH 6 level. Similar to the results of the present study, Aasen et al. (2000) reported that *Lactobacillus* spp. count was higher at pH 6 level than at pH 6.5 and 6.75. Tang et al. (2016) also noted that pH 6 showed a better performance on proliferation of *Lactobacillus* spp. compared with the pH 8 level. The higher final *Lactobacillus* spp. count at pH 6 indicates that pH 6 is the desired pH level for *Lactobacillus* spp. in the submerged fermentation conditions performed in the present study.

The final pH level of *A. bisporus* cap and stem were decreased after submerged fermentation using *Lactobacillus* spp. in the present study. Brinques et al. (2010) reported that *Lactobacillus* spp. can produce lactic acid in submerged fermentation. The decrease in the final pH of the mushroom can be due to the production of lactic acid by *Lactobacillus* spp, during submerged fermentation. Indeed, Indrastuti et al. (2019) showed that submerged fermentation reduced the pH value of cassava flour.

The pH of the mushrooms with an initinal pH of 6 or 7 were approximately 6 after fermentation in this study. This means that the pH level of the pH 6 groups remained the same, while the pH level of the pH 7 groups decreased to 6. This may be due to the ability of *Lactobacillus* spp. to lower the pH level of the fermentation medium to the desired pH level of 6. In addition, the final pH levels of the uninoculated groups were numerically higher than their initial pH levels. This may be due to an increase in the final

pH of the fermentation medium caused by the dissolution of the nutrient content of the mushroom in the fermentation medium during sterilization and incubation.

Submerged fermentation using *Lactobacillus plantarum* increased the crude protein content of durian seed (Kumoro and Hidayat, 2018). The crude protein content of the mushroom cap was also increased by submerged fermentation with pH 6 in the present study. Similarly, Batbayar et al. (2023) reported that *L. plantarum* increased the pea protein-enriched flour in submerged fermentation. The increase in the crude protein level of the mushroom cap in the present study can be due to the microbial protein produced by *Lactobacillus* spp. during submerged fermentation. Indeed, the highest *Lactobacillus* spp. count was observed in *A. bisporus* cap with pH 6.

The crude protein content of mushroom stem was reduced by submerged fermentation using *Lactobacillus* spp. in this study. Similarly, Kumoro et al. (2020) showed that *L. plantarum* decreased the protein content of gadung (*Dioscorea hispida* Dennst) tuber flour with submerged fermentation. *Lactobacillus* spp. can digest the protein of substrates into smaller molecules to produce the enzyme protease (García-Cano et al., 2019). The decrease in the crude protein content of the mushroom stem after fermentation can be because of the digestion of protein in the mushroom stem by *Lactobacillus* spp.

The ash content of pea protein-enriched flour was increased by submerged fermentation using *L. plantarum* (Batbayar et al., 2023). However, *Lactobacillus* spp. decreased the ash content of *A. bisporus* cap and stem with submerged fermentation in the present study. Similarly, Kumoro et al. (2020) reported a decreased ash content in gadung tuber flour after submerged fermentation using *L. plantarum*. The reduced ash content of *A. bisporus* by fermentation can be due to the dissolution of soluble minerals by the fermentation medium or consumption by *Lactobacillus* spp. to use in metabolic activities for growth and development (Kumoro et al., 2020).

Conclusion

Submerged fermentation can decrease the ash content and pH value of *A. bisporus* cap and stem. In addition, *Lactobacillus* spp. increased the crude protein content of the cap part but decreased the crude protein content of *A. bisporus* stem. The higher *Lactobacillus* spp. was observed in mushrooms fermented with pH 6 compared with the pH 7 group. The obtained result showed that submerged fermentation using *Lactobacillus* spp. can be used to improve the nutritional composition of *A. bisporus* cap, and the pH 6 level provided a better fermentation environment for *Lactobacillus* spp., as evidenced by a higher *Lactobacillus* spp. count and increased crude protein level in the mushroom cap. The results also showed that submerged fermentation can be used to produce feedstuff or feed additive for poultry from *A. bisporus* cap with higher nutritional value and also containing probiotic bacteria *Lactobacillus* spp. Further studies investigating the effect of submerged fermentation on the detailed nutritional composition of mushrooms are needed to recommend the use of submerged fermented mushrooms with *Lactobacillus* spp. in animal nutrition.

Acknowledgement

This study is a combined and expanded version of the paper titled “Effect of Liquid State Fermentation Using *Lactobacillus* spp. on the Nutritional Composition of the Stalk Parts of *Agaricus bisporus* in Different pH Levels” presented at the IV. International Agricultural, Biological & Lifescience Conference held in Edirne, Türkiye on 28-31 August 2022, and the paper titled “Liquid State Fermentation Using *Lactobacillus* spp. Affect the Nutritional Composition of the Cap Parts of *Agaricus bisporus*” presented at the IV. Balkan Agricultural Congress held in Edirne, Türkiye on 31August-3 September 2022, in line with the recommendations of the referees.

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