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# Enrichment of casing soil with Fe and soy-flour under *Pseudomonas* inoculation on yield and quality of button mushroom

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## ABSTRACT

Effects of casing soil enrichment with soybean flour (SF) and iron (Fe) were explored on yield and quality of edible mushrooms inoculated with plant growth-promoting bacteria in a factorial experiment with four replications. Fe from Fe chelate source was applied at two levels of 0 ($Fe_0$) and 500 mg $L^{-1}$ ($Fe_{500}$), SF at three levels of 0% ($SF_0$), 1.5% ($SF_{1.5}$), and 3% ($SF_3$) of compost dry weight, and bacteria inoculation at two levels (non-inoculation and inoculation of mycelia with *P. putida*). The maximum fresh yield (20.3 kg $m^{-2}$), mushroom number (1041), biological efficiency (95.0%), vitamin C (3.74 mg 100 $g^{-1}$ FW), and yield of protein (6.48 kg $m^{-2}$) were obtained from $SF_{1.5}$ + *P. putida*. But, the maximum tryptophan (1.37 mg $g^{-1}$ DW), methionine (2.29 mg $g^{-1}$ DW), and antioxidant capacity (4.25 mg $mL^{-1}$) were related to $SF_3$ inoculated with *P. putida*. Furthermore, the maximum carbohydrate (5.64%) was related to $Fe_{500}$ + $SF_3$. Based on the results, casing soil enrichment with Fe did not have a significant influence on quantitative and qualitative traits of mushrooms, but SF application at the rate of 1.5%, especially when accompanied by *P. putida*, played a more essential role. Thus, it is recommended to use 1.5% SF along with *P. putida* to enhance the yield and qualitative traits of edible mushrooms.

**Keywords:** Antioxidant capacity, Casing soil, Fresh yield, Protein, Soy-flour..

## Enriquecimento do solo de cobertura com Fe e farinha de soja sob inoculação de *Pseudomonas* no rendimento e qualidade do cogumelo

## RESUMO

Os efeitos do enriquecimento do solo de cobertura com farinha de soja (SF) e ferro (Fe) foram explorados no rendimento e na qualidade de cogumelos comestíveis inoculados com bactérias promotoras de crescimento de plantas em um experimento fatorial com quatro repetições. Fe da fonte de quelato de Fe foi aplicado em dois níveis de 0 (Fe0) e 500 mg $L^{-1}$ (Fe500), SF em três níveis de 0% (SF0), 1.5% (SF1.5) e 3% (SF3) de massa seca do composto e inoculação de bactérias em dois níveis (não inoculação e inoculação de micélios com *P. putida*). O rendimento fresco máximo (20.3 kg $m^{-2}$), número de cogumelos (1041), eficiência biológica (95.0%), vitamina C (3.74 mg 100 $g^{-1}$ FW) e rendimento de proteína (6.48 kg $m^{-2}$) foram obtidos a partir de SF1.5 + P. *putida*. Porém, os valores máximos de triptofano (1.37 mg $g^{-1}$ DW), metionina (2.29 mg $g^{-1}$ DW) e capacidade antioxidante (4.25 mg $mL^{-1}$) foram relacionados ao SF3 inoculado com *P. putida*. Além disso, o carboidrato máximo (5.64%) foi relacionado ao Fe500 + SF3. Com base nos resultados, o enriquecimento do solo de cobertura com Fe não teve influência significativa nas características quantitativas e qualitativas dos cogumelos, mas a aplicação de SF na proporção de 1.5%, principalmente quando acompanhada por P. *putida*, teve um papel mais essencial. Assim, é recomendado o uso de 1.5% SF junto com *P. putida* para aumentar o rendimento e as características qualitativas dos cogumelos comestíveis.

**Palavras-chave**: Capacidade antioxidante, Solo de cobertura, Rendimento fresco, Proteína, Farinha de soja..

## 1. Introduction

Edible mushrooms are among the biggest and most valuable natural resources for protein-rich foodstuff production from low-value food in a short time so that they have a special place in the world (Chang and Wasser, 2017). Mushrooms grow on composts including straw, chicken manure, chalk, and other additives, which are costly inputs for mushroom production (Roise et al., 2016). So, mushroom producers look for methods to reduce their production costs by increasing bio-efficiency and more production from composts (Kim et al., 2008). To gain higher yields, the substrate should be enriched by adding such nutrients as nitrogen (N), phosphorus (P), potassium (K), zinc (Zn), and iron (Fe). Although chicken manure that is incorporated into the compost contains these nutrients, it sometimes fails to supply all mushroom demands, so Desrumaux et al., (2000) emphasize the addition of Fe to the casing soil since Fe is the main component of catalase and cytochrome in *A. bisporus* required at a rate of 0.1-0.3 mg $kg^{-1}$ (Pardo-Giménez et al., 2018). Research shows that individual micronutrients, e.g. copper (Cu), boron (B), and Fe, added to the substrate at spawning do not influence the yield significantly (Weil et al., 2006). Nonetheless, it seems that micronutrient supplements provide mushroom producers with a potential opportunity to improve the efficiency and quality of freshly harvested mushrooms (Kumar et al., 2020).

Compost enrichment has drawn interest as a good way to increase crop yields. Enrichment is performed by using various supplements, perhaps with a plant origin or derived from compounds whose components are released gradually to feed the mycelia (Schisler, 1967; Delphina and Royse, 2016). Supplements are mostly applied to compost or spawn to allow complexes to take up them (Nurudeen et al., 2014; Sing and Jain, 2016; Carrasco et al., 2018). Research has identified cottonseed meal, peanut meal, wheat bran, and especially soy-flour to be the best supplements with plant origins (Sing and Jain, 2016). Soy-flour, which is derived from grounding dry soybeans, is an ideal protein-based supplement containing a high concentration of carbon (Zied et al., 2011).

The use of soy-flour as the main source of organic N in the substrate of Agaricus has been reportedly successful (Mascarin et al., 2018). According to Carrasco et al., (2018), soy-flour partly replaces chicken manure and is a good alternative to reduce the C/N ratio and boost microbial activity in the *A. bisporus* substrate so that it not only increases the yield but is also good for enhancing mushroom protein content. Pardo-Giménez et al., (2018) report that soy-flour has a high protein content varying in the range of 40-50% and performs as an excellent source of nitrogen and carbon, permitting better growth and fastest pinhead formation of fruit bodies.

By studying N concentrations of different soybean cultivars, Mascarin et al., (2018) found that the total N content of soybeans was in the range of 7.1-13.4%, which is much higher than that of cottonseed and corn flour. Nurudeen et al., (2014) compared some nutrient supplements and revealed that soy-flour significantly outperformed corn flour, wheat flour, and cow bean flour in biological efficiency, fresh yield, and mushroom population.

To bolster edible mushroom yields, biofertilizers can greatly influence the profitability along with breeding techniques and supplement applications (Pratiksha et al., 2017). The most important microorganisms, applied in biofertilizer production, plant growth-promoting rhizobacteria (PGPRs) including *Pseudomonas* (Prathap and Ranjitha Kumari, 2015), which exists in the casing soil used in *A. bisporus* mushroom production and interacts with the mycelia of this species (Siyoum et al., 2015; Pratiksha et al., 2017).

The bacteria of the genus *Pseudomonas* are from the family Pseudomonadaceae, which are non-sporing, rod-shaped or slightly curved, polar-flagellated, and Gram-negative. Among the species of *Pseudomonas*, *P. putida* is more effective than other species in the growth of white button mushroom mycelia (Mohammad and Sabaa, 2013; Chen et al., 2013). This bacterium plays a key role in the next growth stages of the mushroom including fruit-bearing (Colauto et al., 2016) and improves the yield, dry weight, and protein content of this mushroom (Zarenejad et al., 2012). *P. putida* has a positive role in its host as it produces indole-3-acetic acid (IAA), improves the solubility of mineral phosphate, reduces growth-inhibiting compounds e.g. ethylene, and secretes siderophore to cope with pathogens (Roca et al., 2013). These mechanisms of *P. putida* with the mycelia of edible mushrooms can be studied as they can enhance mushroom yields.

Research shows that IAA-producing bacteria increase fresh weight, dry weight, protein content, population per unit area, and cap diameter of *A. bisporus* (Mohammad and Sabaa, 2013). Chen et al., (2013) enumerate ethylene as a candidate inhibitor of the *A. bisporus* growth. They revealed that the *P. putida* strains contributed to the growth of the *A. bisporus* hyphae by producing the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, reducing ACC, and thereby reducing ethylene level, but the mutant bacteria that produced only slight amounts of ACC deaminase inhibited the growth of hyphae. Therefore, the *A. bisporus* and *P. putida* growth-inhibiting ethylene increase the growth of fungal mycelia by absorbing and consuming ethylene precursors. *P. putida* contributes to maintaining crop quality and increasing host yield by producing siderophores that control pathogenic microorganisms and facilitate Fe uptake (Gülser and Pekşen, 2003; Colauto et

al., 2016). The application of Fe to the substrate of *A. bisporus* induces the formation of pinhead structures (Mohammad and Sabaa, 2013).

Since no report has ever been published on the effects of casing soil enrichment by the interaction of a micronutrient (Fe) and an organic supplement (soybean flour) along with *P. putida* on the quantitative and qualitative traits of *A. bisporus*, the present research aims to shed light on the influence of casing soil enrichment with nutritional supplement and *P. putida* on improving the quantitative and qualitative traits of *A. bisporus*.

## 2. Material and Methods

The research was conducted at the Horticultural Research Department of Agricultural and Natural Resources Center of Ahvaz, Southern Iran (38°21' N., 48°50' E., 23 m. altitude from sea level) in 2019. It was a factorial experiment based on a completely randomized design replicated four times in which the treatments included casing soil enrichment with iron (as Fe-chelate at a rate of 0 or 500 mg $L^{-1}$), soybean flour as the complementary casing soil (at a rate of 0, 1.5 or 3% of the wet weight of compost), and phase II compost inoculated/not inoculated with *Pseudomonas putida* strain R53W.

The soy-flour (40% protein, 19% lipid, and 8% moisture) was supplied by Soyan Toos Company. The Fe chelate, which included 9% Fe, was procured from Khazra Company, Iran. Also, mushroom spawn, casing soil, and compost were supplied by Qarch-Jolgeh-Dez Company. Tables 1 and 2 present the results of the physical and chemical analysis of the casing soil and compost.

The experiment was conducted by the shelf method as it has a high production efficiency and low space requirement. The first shelf was placed 15 cm above the ground and the shelves, which were 140 cm wide, were spaced by 65. Also, to cultivate the mushroom, bag blocks were placed on the shelves. The blocks were polyethylene 140 × 160 cm rectangular containing 17 kg compost. Each block was considered a single experimental plot.

*Pseudomonas putida* strain R156 was supplied by the microbial collections of Water and Soil Research Institute, Karaj. It is one of the best *Pseudomonas* strains and can produce siderophores used to solubilize organic and mineral phosphates and generate auxin and ACC-deaminase (Table 3). To prepare the *P. putida* inoculant, adequate amounts of the nutrient culture media containing cycloheximide were first prepared.

Then, a loop of the target bacteria was inoculated to the Erlenmeyer containing the sterile culture medium in sterile conditions and was placed in a shaker-equipped incubator at 150 rpm at 28°C. The counting of bacteria by the plate method showed that the population of the bacteria reached $10^8$ cells $mL^{-1}$ in four days. Each 17-kg substrate was added with 85 mL of the propagated bacterial suspension (Kim et al., 2008).

**Table 1**. The properties of the casing soil

| Moisture (%) | EC dS $m^{-1}$ | pH | S.P | OM | N | P | K | C/N | Cu | Fe | Mn | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | (%) | | | | | (mg $kg^{-1}$) | | | |
| 25 | 2.1 | 5.25 | 10.0 | 20.1 | 1.35 | 0.19 | 0.13 | 8.5 | 10 | 3800 | 80 | 25 |

EC: Electrical conductivity, S.P: Saturation percentage, OM: Organic matter, N: Nitrogen, P: Phosphorus, K: Potassium, C/N: Carbon/Nitrogen, Cu: Copper, Fe: Iron; Mn: Manganese; Zn: Zinc.

**Table 2.** The properties of the phase II compost

| Moisture (%) | EC dS $m^{-1}$ | pH | OM | OC | N | P | K | C/N | Cu | Fe | Mn | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | (%) | | | | | (mg $kg^{-1}$) | | | |
| 73.6 | 7.7 | 7.63 | 44.1 | 26.3 | 1.95 | 2.65 | 3.35 | 13.4 | 48 | 190 | 330 | 195 |

EC: Electrical conductivity, S.P: Saturation percentage, OM: Organic matter, N: Nitrogen, P: Phosphorus, K: Potassium, C/N: Carbon/Nitrogen, Cu: Copper, Fe: Iron; Mn: Manganese; Zn: Zinc.

**Table 3.** The properties of P. *putida* used in the present study

| *Pseudomonas* strain | Phosphate solubilizing | ACC-deaminase production | IAA production (mg $L^{-1}$) | Siderophore production (halo diameter/colony diameter) |
|---|---|---|---|---|
| *P. Putida* strain R156 | + | + | 9.8 | 1.9 |

ACC: 1-aminocyclopropane-1-carboxylic acid; IAA: Indole-3-acetic acid

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Before spawning, all casing soil supplement treatments were sterilized in an autoclave (121°C, 34.1 lb) for 15 minutes. The spawning of *A. bisporus* was performed at a rate of 5 g $kg^{-1}$ compost by layered seeding. Then, the substrates were inoculated with *P. putida* except for the non-inoculated treatment which was left without any bacteria (Kim et al., 2008). At the next step, the casing soil treated with either Fe chelate, soybean flour, or none (the control) was applied uniformly to form a 4-cm thickness layer and the ruffling operation was performed. At this moment, the compost was 25°C with pH 7.5 and 70% moisture and the casing soil had pH 7.4 with 73% moisture. After spawning, they were kept in a growth chamber at a relative humidity of 90±5%, a temperature of 24±1°C, and $CO_2$ content of 5000-6000 ppm in darkness until pinhead parts emerged (Remezan and Siah Sar, 2010). After two weeks, when mycelia grew inside the casing soil, the aeration (shocking) operation was performed at a humidity of 85±5%, $CO_2$ content of 800-1000 ppm, and a temperature of 17±1°C (Remezan and Siah Sar, 2010). Ruffles and temperature drop imposed a shock to trigger the reproductive phase. All mushroom-specific operations including irrigation were uniformly applied to all treatments.

At the end of the culture period, mushrooms were harvested before full maturity. So, 1 $m^2$ was harvested from each plot to measure the traits described below. The picked mushrooms were placed in paper pockets and their fresh weight was measured with a digital 0.01-g scale to record it as the fresh yield. Among the picked mushrooms, 20 were selected randomly to measure their height and cap diameter with a tape measure (Mamiro and Royse, 2008). Then, to determine their dry weight, the mushrooms were cut into thin layers and oven-dried at 70°C for 24 hours.

To find out ash content, 5 g of mushroom dry matter was weighed precisely and was placed in Chinese crucibles inside an electrical furnace at 550°C. After 8 hours, they were weighed. The following equation was used to estimate ash content (Vieyra, 2009):

$$\text{Ash content (\%)} = \frac{\text{Ash weight of mushrooms (g)}}{\text{Dry matter weight of mushrooms (g)}} \times 100$$

Dry matter (DM) content and biological efficiency (BE) of the mushrooms were calculated by the following equations (Kirbag and Akyuz, 2009):

$$\text{DM (\%)} = \frac{\text{Dry weight of mushrooms (g)}}{\text{Fresh weight of mushrooms (g)}} \times 100$$

$$\text{BE (\%)} = \frac{\text{Fresh weight of mushrooms (g)}}{\text{Total substrates used (g)}} \times 100$$

To measure the protein content of the mushrooms, nitrogen (N) content was first measured by the Kjeldahl method and then, protein content was estimated by the following equation (Han, 1999):

$$\text{Protein content (\%)} = \text{N} \times 4.38$$

Vitamin C was determined according to the method of Klein and Perry (1982). Mushroom mycelia (1 g) was extracted with 10 ml of 1% metaphosphoric acid for 45 minutes and filtered through a Whatman filter paper No. 1. The filtrate (1 ml) was mixed with 9 ml of 2,6-dichloroindophenol and the absorbance was measured at 515 nm.

The anti-oxidant capacity in mushroom samples was determined by measuring the decrease in absorbance at 517 nm by a UV–vis spectrophotometer (Model 5384, Agilent Technologies) due to the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical reduction, indicating the antioxidant activity of the compounds in a short time. It was obtained from the following equation (Sanchez-Moreno et al., 1998):

$$\text{Free radical scavenging activity} = \frac{(Ac - As)}{Ac} \times 100$$

where *Ac* is the absorbance of 100 µM methanolic DPPH only and *As* is the absorbance of the reaction mixture.

The lysine, methionine, and tryptophan contents were analyzed according to the method of Bosch et al., (2006) with some modifications. Mushroom powders (1 g) were shaken with 50 ml of 0.1 N hydrochloric acid (HCl) solution for 60 minutes and filtered. The purified filtrate was mixed with OPA (o-phthalaldehyde reagent), shaken to facilitate derivatization. Then, it was immediately injected onto the HPLC for assessment (Waters Alliance 2695). Each of the amino acids was quantified by the curve of the authentic amino acid.

All data were subjected to the analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC, USA V9.4) Software. In case the F test indicated statistical significance at $P<0.01$ or $P<0.05$, the least significant difference (LSD) was used to separate the means.

## 3. Results and Discussion

Mushroom number and yield were influenced by the interaction effect of SF × Fe × B (Table 4). When no Fe was applied ($Fe_0$), the highest mushroom number was obtained from $SF_1 \times B_1$, but under the application of Fe ($Fe_{500}$), no significant dif ference was observed between $SF_1 \times B_1$ and $SF_2 \times B_1$. Similarly, the non-inoculated mushrooms did not show a significant difference between $SF_{1.5}$ and $SF_3$, but these two treatments had more mushrooms than $SF_0$. Mushroom fresh yield responded

to the treatments just like how mushroom number did so that the maximum yield was obtained from the treatments that produced the most number of mushrooms. The application of soybean flour, especially $SF_{1.5}$, to the substrates treated with $Fe_{500}$ and *Pseudomonas* was related to a higher yield than the substrates just treated with *Pseudomonas*.

The increase in mushroom number and yield by increasing the application of soybean flour up to 0.5 kg $m^{-2}$ was also reported by Zied et al., (2011). They observed that when soybean powder was applied in excess to the mushroom need, the yield was decreased and the mushroom quality was lost sharply (Table 5). Jiang et al., (2011) and Carrasco et al., (2018) ascribed the effect of soybean powder overuse on yield loss to the rise of temperature immediately after the application of soybean powder resulting from the severe metabolic and enzymatic activity of the mushrooms.

We observed that when $SF_3$ was applied without bacteria, it reduced yield, but when it was accompanied with *P. putida*, no significant difference occurred with $SF_{1.5}$ because bacteria consume N content of soybean powder, thereby making it unavailable to the mushrooms and providing them with the nutrients with delay. Similar to our findings,

Mohammad and Sabaa (2013) found that supplements can be made more efficient by inducing a delay in their availability versus their immediate availability. They demonstrated the positive role of *P. putida* and *Rhodopseudomonas palustris* in impeding the availability of nutrients. Kim et al., (2008) employed *Pseudomonas* strain P7014 and observed that the growth of mushroom mycelia was increased by 1.6 folds. Also, primordia were formed earlier and the total number of days for mushroom growth was decreased versus their non-inoculated counterparts

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**Table 4**. Analysis of variance for the effects of Fe, bacteria and soy-flour on mushroom number, fresh yield, biological efficiency, dry matter ratio, ash content, protein content and protein yield

| S.O.V | df | Mean square (MS) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Mushroom | Mushroom | Biological | Dry matter | Ash | Protein | Protein |
| Replication | 3 | 50634** | 0.036ns | 0.004ns | 0.395 ns | 3.09ns | 30.5ns | 1.43* |
| Iron (Fe) | 1 | 6509187** | 0.185** | 1208** | 2.62** | 1.072ns | 0.634ns | 0.028ns |
| Pseudomonas (B) | 1 | 263835** | 134** | 2935** | 0.189 ns | 4.38* | 58.0** | 19.6** |
| Soy-flour (SF) | 2 | 56961** | 20.4** | 445** | 0.6718** | 19.3** | 294** | 12.1** |
| Fe × B | 1 | 92.5ns | 23.4** | 513** | 1.989** | 1.78 ns | 345 ** | 5.11** |
| SF × Fe | 2 | 53905** | 3.67** | 78.6** | 0.117ns | 0.465ns | 265** | 7.19** |
| SF× B | 2 | 11705ns | 1.41** | 27.1** | 1.822** | 0.708ns | 21.7ns | 1.32ns |
| SF × Fe × B | 2 | 46628** | 2.99** | 69.6** | 2.247** | 0.831** | 87.6** | 4.93** |
| Error | 33 | 5486 | 0.028 | 0.599 | 0.185 | 0.943 | 14.2 | 0.469 |
| CV (%) | | 6.510 | 6.92 | 11.9 | 8.280 | 6.620 | 13.4 | 13.7 |

** $P$ <0.01, * $P$ <0.05, ns not significant.

**Table 5.** Means comparison for the interactive effect of Fe × bacteria × soy-flour on mushroom number, fresh yield, biological efficiency, dry matter ratio, protein content, and protein yield

| Fe | B | SF | Mushroom number $m^{-2}$ | Mushroom yield (kg $m^{-2}$) | Biological efficiency (%) | Dry matter ratio (%) | Protein content (%) | Protein Yield (kg $m^{-2}$) |
|---|---|---|---|---|---|---|---|---|
| | | $SF_0$ | 807c | 17.03c | 84.50b | 5.38ab | 23.52c | 4.267c |
| | $B_1$ | $SF_{1.5}$ | 1041a | 20.36a | 95.06a | 5.46a | 34.18a | 6.486a |
| | | $SF_3$ | 904b | 18.03b | 84.03b | 4.79bc | 34.93a | 6.245a |
| $Fe_0$ | | $SF_0$ (control) | 796c | 17.09c | 69.56d | 5.04abc | 19.83d | 3.393d |
| | $B_0$ | $SF_{1.5}$ | 891b | 18.00b | 86.02b | 4.493c | 24.01c | 4.422c |
| | | $SF_3$ | 844b | 18.10b | 79.80c | 5.04abc | 30.66b | 5.216b |
| | | $SF_0$ | 826ab | 16.073b | 70.36b | 5.428a-c | 26.20ab | 4.921abc |
| | $B_1$ | $SF_{1.5}$ | 836a | 20.181a | 87.14a | 5.235bc | 24.08b | 4.874abc |
| $Fe_{500}$ | | $SF_3$ | 862a | 20.178a | 87.13a | 5.102c | 28.15ab | 5.701a |
| | | $SF_0$ | 711b | 16.587b | 67.96b | 5.534ab | 27.5ab | 4.630bc |
| | $B_0$ | $SF_{1.5}$ | 751ab | 17.354b | 71.33b | 5.176bc | 31.190a | 5.425ab |
| | | $SF_3$ | 761ab | 19.150a | 73.91b | 5.618a | 26.13b | 4.184c |

$B_0$: inoculation, $B_1$ : non- inoculation;
$Fe_0$: non-use of Fe, $Fe_{500}$: use of 500 mg Fe $L^{-1}$;
$SF_0$, $SF_{1.5}$ and $SF_3$: use of soy-flour as 0, 1.5% and 3% proportion of compost dry weight, respectively.

Furthermore, inoculation with this bacteria increased mushroom weight and accelerated tillering and primordia formation. In a study on the use of non-sulfur bacteria, Vieira and Pecchia (2018) showed that the application of 5000 mL of suspension containing $3.3 \times 10^9$ viable cells $mL^{-1}$ on each block increased mushroom yield by 39.53%, but mushroom DM and protein content did not differ from the control significantly. The highest concentration of this bacteria suspension exhibited the highest mushroom yield of 14.33 kg $m^{-2}$

About biological efficiency, ANOVA showed that the interaction effect of SF × Fe × B was significant for biological efficiency (Table 4). The application of soybean flour to the non-inoculated plots that were not treated with Fe increased this trait by 8.4% for $SF_{1.5}$ and 0.63% for $SF_3$ when compared to $SF_0$ (the control), but inoculation with *Pseudomonas* in the absence of Fe increased biological efficiency by 19.6% and 5.9% in the treatments of $SF_{1.5}$ and $SF_3$ versus $SF_0$, respectively. Fe application in the absence of *Pseudomonas* lessened the impact of soybean flour so that there was not a significant difference between different levels of SF.

But, when it was applied in the presence of *Pseudomonas*, although the two levels of $SF_{1.5}$ and $SF_3$ did not differ significantly, their biological efficiency was significantly higher than that of $SF_0$ by 9.7%. Overall, the highest biological efficiency was obtained from the treatment of $Fe_0 + B_1 + S_{1.5}$ (Table 5). The results indicate that Fe application decreases biological efficiency, which was ascribed to its impact on reducing the activity of substrate bacteria by Carrasco et al., (2019) who found that higher rates of Fe decreased the synthesis of organic acids by bacteria on the one hand and reduced bacteria-host symbiosis on the other.

Thus, the decreased impact of soybean flour in the absence of *Pseudomonas* may be related to the retardation of soybean flour decomposition. However, by the activities it has in the rhizosphere *Pseudomonas* application contributes to the decomposition of soybean flour and makes it available to mushrooms gradually. This finding is consistent with the report of Colauto et al., (2016).

Dry matter percentage was affected by the interaction effect of SF × Fe × P (Table 4). In substrates not treated with Fe ($Fe_0$), the highest dry matter percentage was obtained from $SF_{1.5}$ inoculated with *Pseudomonas* ($B_1 + SF_{1.5}$) although it did not differ from some other treatments significantly. However, it increased dry matter percentage by 7.41% versus the treatment of $B_0 + SF_0$. In Fe-treated casing soils ($Fe_{500}$), the highest dry matter percentage was observed in the treatment of $B_0 + SF_3$ whose dry matter percentage was 11.9% higher than that of the control (Table 5). Ash content was influenced by the main effects of *Pseudomonas* and soybean flour as well (Table 4).

The inoculated treatment had 8.46% higher ash content than the non-inoculated treatment (Figure 1).. Among the soy-flour levels, $SF_{1.5}$ increased ash content by 11.2% versus $SF_0$ significantly, but $SF_3$ did not differ from $SF_0$ significantly (Figure 2). Kertesz and Thai (2018), who studied some bacterial and fungal strains, reported that the growth of Agaricus and Pleurotus fungal species can mostly be induced by the bacteria from the genera *Bacillus*, *Pseudomonas*, and *Bradyrhizobium.*

During the growth of the mushrooms, these microorganisms are active in soil, substrate, casing soil, or host and improve yield, shorten production period, and enhance dry matter percentage. An increase in dry matter results in an increase in ash content, and improving the growth conditions for mushrooms facilitates the availability of nutrients, especially N, thereby increasing dry matter (Vos et al., 2017). The higher dry matter and ash contents of mushrooms following the application of soybean flour and *P. putida* was attributed by Mohammad and Sabaa (2013) to the increased uptake of phosphates (P), N adjustment potential, and the ability to promote mycelium growth.

According to the results of ANOVA, the interaction effect of SF × Fe × B was significant for protein content and yield (Table 4). The comparison of means revealed that the maximum mushroom protein content was obtained from the soil treated with $B_1 + SF_3$ but not enriched with Fe, whereas, among the Fe-enriched soils, the one treated with $B_0 + SF_{1.5}$ exhibited the highest protein content although its protein content was 9.3% lower than that of $B_1 + SF_3$.

Among both Fe-enriched and non-enriched soils, the highest protein yield was related to the treatment of $B_1 + SF_3$. However, there was not a significant difference between $B_1 + SF_3$ and $B_1 + SF_{1.5}$ when they were applied to non-enriched soil. The application of $Fe_{500}$ alone ($Fe_{500} + B_0 + SF_0$) or in combination with *P. putida* ($Fe_{500} + B_1 + SF_0$) increased protein content by 29.5% and 23.1% versus the control ($Fe_0 + B_0 + SF_0$; Table 5). So, it can be said that soybean flour and *Pseudomonas* have a positive relationship with protein content, but Fe has a negative relationship with it. Although there are reports as to the positive impact of Fe on protein and yield of mushrooms, some have also reported its negative relationship with some supplements including wheat bran and cottonseed powder.

Carrasco et al., (2018) concluded that there was a positive significant relationship between protein content and amino acids, which is in agreement with our findings as to the effect of P. *putida* and soybean powder on the content of protein and amino acids. Methionine and tryptophan contents were influenced by the interaction effect of SF × B, but lysine was impacted only by the main effects of B and FS significantly (Table 6).

**Table 6**. Analysis of variance of Fe, bacteria and soy-flour effects on content of vitamin c, carbohydrate, tryptophan, lysine, methionine and antioxidant capacity of mushroom

| S.O.V | df | Mean square (MS) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Methionine | Lysine | Tryptophan | Carbohydrate content | Vitamin C | Anti-oxidant capacity |
| Replication | 3 | 6.40** | 0.022ns | 1.31** | 718** | 12.3** | 9.89** |
| Iron (Fe) | 1 | 0.012ns | 1.671ns | 0.309ns | 30.1ns | 0.047ns | 0.039ns |
| Pseudomonas(B) | 1 | 1.79** | 4.161** | 1.30** | 57.6** | 3.35** | 18.5** |
| Soy-flour (SF) | 2 | 3.09** | 6.67** | 1.732** | 294** | 0.297* | 0.018ns |
| Fe × B | 1 | 0.129ns | 0.135ns | 0.178ns | 346** | 0.211ns | 4.19** |
| SF × Fe | 2 | 0.032ns | 0.093ns | 0.228ns | 265** | 0.055ns | 0.030ns |
| SF× B | 2 | 0.142* | 0.089ns | 0.718** | 21.7ns | 0.367* | 0.347ns |
| SF × Fe × B | 2 | 0.005ns | 0.016ns | 0.109ns | 7.6ns | 0.0084ns | 0.594ns |
| Error | 33 | 0.044 | 0.282 | 0.130 | 13.27 | 0.091 | 0.223 |
| CV (%) | | 10.9 | 11.5 | 37.1 | 5.18 | 8.52 | 13.6 |

** $P$ <0.01, * $P$ <0.05, ns not significant.

Vieira and Pecchia (2018) ascribed the effect of *Pseudomonas* on increasing the mushroom protein content to the enhancement of substrate quality by shortening the composting process, facilitating the lignocellulose degradation, and the synergistic effect on mycelium growth by the release of nutrients.

Under both inoculation ($B_1$) and non-inoculation ($B_0$) conditions, $SF_3$ was related to the highest amounts of methionine and tryptophan although, under non-inoculation conditions, no statistically significant difference was observed between $SF_{1.5}$ and $SF_3$ so that under inoculation conditions, $SF_3$ exhibited 23.5% higher methionine content than $SF_{1.5}$ whereas it was 9.02% under non-inoculation conditions. This increase in tryptophan content was 34.1% under non-inoculation conditions and 15.7% under inoculation conditions (Figures 3 and 4).

Lysine was also 11.8% higher in mushrooms obtained from inoculation with *P. putida* than in those with no inoculation background (Figure 5). The application of soybean flour increased lysine content significantly as well so that $SF_{1.5}$ and $SF_3$ increased this trait by 11.8% and 20.5% versus $SF_0$ (Figure 6). The application of protein-containing supplements during spawning has a well-proven role in increasing amino acids of mushrooms (Kertesz and Thai, 2018). According to Colak et al., (2007), lysine and methionine were decreased by as high as 49.1% in substrates with lower N content and the application of *Pseudomonas* to these substrates can further aggravate this decrease to 77.3%.

Colauto et al., (2016) state that the increased activity of bacteria in media with a high C/N ratio makes N unavailable to mycelia and not only impede harvest but also impairs fresh mushroom yield and quality. On the other hand, Chang and Wasser (2017) and Vieira and Pecchia (2018) argue that a low C/N ratio in mushroom substrate increases its temperature and disturbs microbial activity and mycelium growth. Wang et al., (2000) and Bonati et al., (2004) have found that substrate N affect mushroom protein content so that mushrooms with a higher protein content can be derived from substrates with a higher N content. Sing and Jain (2016) state that mushrooms may have high contents of minerals that exist in their growth media. Thus, the response of mushrooms to Fe application and the concentration of micronutrients in the mushroom highly depends on the planting method and the mineral contents of the compost (Spaulding and Beelman. 2003).

Data in Table 6 show that the interaction of Fe × SF was significant for mushroom carbohydrate content. When no inoculation was performed ($B_0$), $SF_0$ outperformed $SF_3$ and $SF_{1.5}$ significantly whilst in the treatments inoculated with *P. putida* ($B_1$), the highest carbohydrate content was obtained from $SF_3$ (Figure 7). The comparison of means for Fe × SF revealed that under no Fe application ($Fe_0$), the different levels of soybean flour did not differ significantly, but when Fe was applied ($Fe_{500}$), the difference between the soybean flour levels was significant, and maximum carbohydrate content was related to $SF_3$ (Figure 8).

Consistent with our findings, Chen et al., (2000), Jurak et al., (2014), and Chang and Wasser (2017) reported a reverse relationship between protein content and mushroom carbohydrate content and concluded that protein-rich supplements have a negligible or reducing impact on the carbohydrate content. But, Zied et al., (2011) disclosed that unlike wheat barn and cottonseed meal, soybean flour increased mushroom carbohydrate and protein contents significantly versus the control and other supplements.

In our study as well, the maximum carbohydrate content was obtained from $SF_3$ when it was applied along with Fe or *P. putida*, but when $SF_3$ was used alone, no significant increase was observed in carbohydrate content when compared to $SF_0$. Therefore, the results reveal that the enhancement of mushroom carbohydrate content by a supplement like soybean flour requires the cooperation of Fe or *P. putida*.

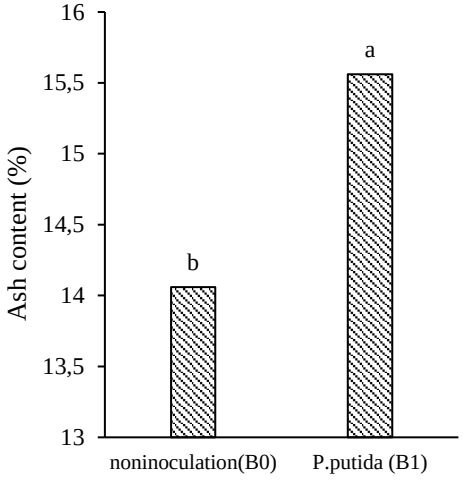


**Figure 1**. Mean comparison of bacteria main effect on ash content

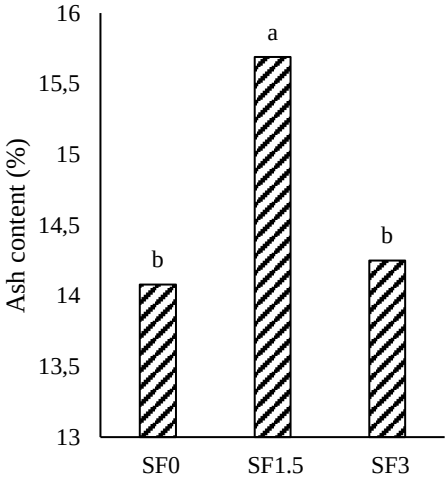


**Figure 2**. Mean comparison of soy-flour main effect on ash content

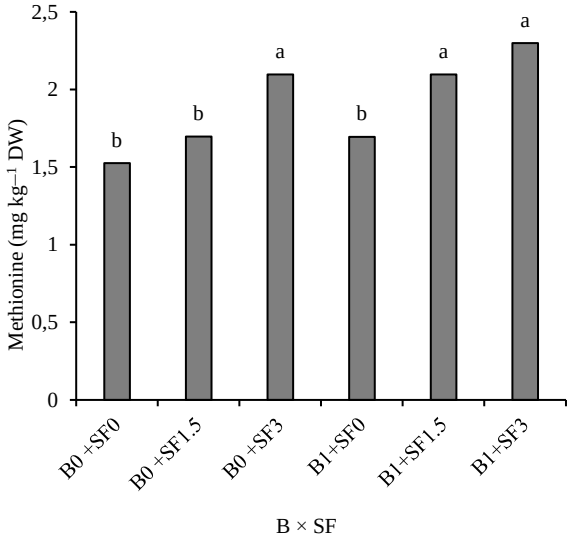


**Figure 3**. Mean comparison of interaction effect of SF× B on methionine content

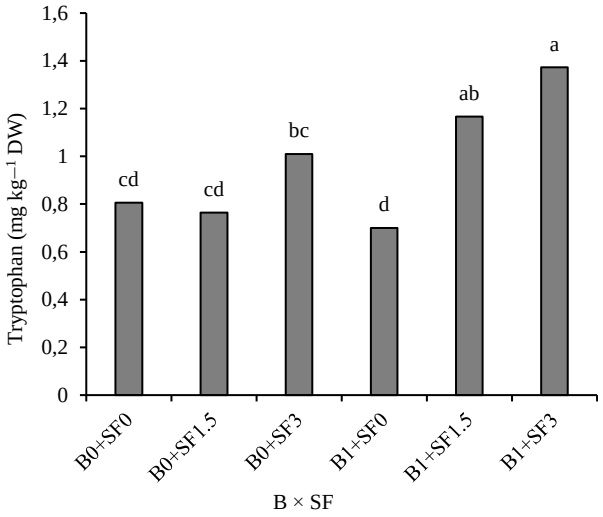


**Figure 4**. Mean comparison of interaction effect of SF× B on tryptophan content

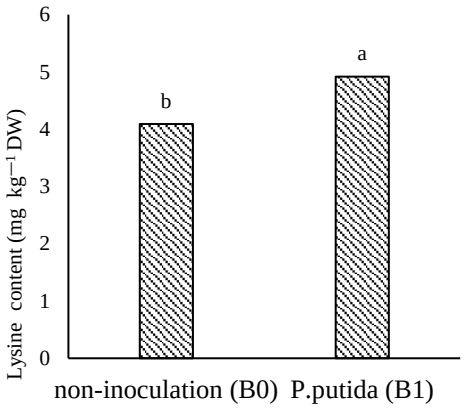


**Figure 5**. Mean comparison of bacteria main effect on lysine content

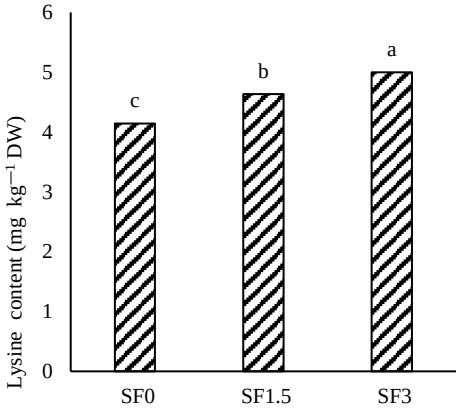


**Figure 6**. Mean comparison of soy-flour main effect on lysine content

Antioxidant capacity was affected by the interactions of B × Fe and B × SF (Table 6). The comparison of means for B × Fe showed that under both inoculated ($B_0$) and non-inoculated ($B_1$) conditions, $Fe_{500}$ had higher antioxidant capacity than $Fe_0$, the only difference being that it was 10.32% higher in $Fe_{500}$ than in $Fe_0$ under non-inoculated conditions whereas it was 19.44% higher under inoculated conditions (Figure 9). Based on the comparison of means for B × SF, when no inoculation was applied, higher soybean flour rate was related to higher antioxidant capacity so that $FS_{1.5}$ had 12.8% higher and $FS_3$ had 22.4% higher antioxidant capacity than the control, respectively.

In the plots inoculated with *P. putida*, there was not a significant difference between $FS_{1.5}$ and $FS_3$, but their antioxidant capacities were significantly higher than $FS_0$. It was revealed that the antioxidant capacity in $FS_3$ was increased by 22.4% in the non-inoculated plots and by 39.8% in the inoculated plots. (Figure 10). Ebadi et al., (2012) reported that among IAA producing bacteria, ACC deaminase synthesizing bacteria, phosphate solubilizing bacteria, and siderophore producing bacteria, the highest yield was related to the ACC deaminase synthesizing bacteria with 12% increase in mushroom fresh weight as compared to the control. However, they showed that the highest dry matter level, the most number of mushrooms, and the highest protein content were related to the bacteria that had these features at a moderate level.

Singh et al., (2000), on the other hand, reported that among 34 isolates of *Pseudomonas fluorescens* in the casing soil, two isolates increased nodule-like structures and the yield of *A. bisporus* significantly and shortened the fruiting part development period by 7 days. These isolates have a biocontrolling effect on different pathogenic fungi and increase yield by controlling their pathogenicity. The difference in results for *A. bisporus* may arise from the difference in physical and chemical characteristics of the applied casing soil and/or the studied mushroom origin. This difference in results for the inoculation with growth-promoting bacteria is mainly attributed to the diversity in plant type and species, soil composition, the presence of indigenous microorganisms, soil moisture, and inadequate understanding of mechanisms by which growth-promoting bacteria influence plant growth. The interaction effect of B × SF was significant on vitamin C content (Table 6).

Based on the comparison of means for B × SF, the application of soybean flour under no inoculation conditions ($B_0$) increased vitamin C content by 28.5% in $SF_{1.5}$ and 20.12% in $SF_3$. Under inoculation with *P. putida*, $SF_{1.5}$ enhanced vitamin C content by 15.4% and 46.2% versus $B_1 + SF_0$ and $B_1 + SF_3$, respectively whilst $SF_3$ did not differ from $B_1 + SF_0$ significantly although it resulted in 31.3% higher vitamin C content than $B_0 + SF_0$ (Figure 11). Most studies have reported an increased level of vitamin C by 21.4% in mushrooms treated with soybean flour (Golestani et al., 2014; Nurudeen et al., 2014; Carrasco et al., 2018), which is in agreement with our findings. But, this increase reached 46% when it was accompanied by inoculation with *P. putida*, implying a synergy between bacteria and soybean flour.

Zied et al., (2011) reported synergy between *Pseudomonas* and organic N sources as well. Mishra et al., (2013) state that ascorbic acid is a powerful secondary antioxidant that reduces the oxidized form of α-tocopherol and N plays a key role in vitamin C accumulation. The role of microorganisms, e.g. *Pseudomonas*, in increasing vitamin C has been reported by Zarenejad et al., (2012) and Siyoum et al., (2015), too.

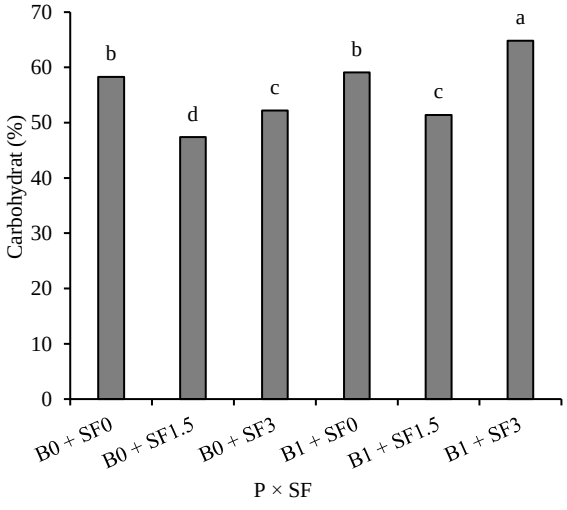


**Figure 7**. Mean comparison of P × SF on carbohydrate content of mushroom

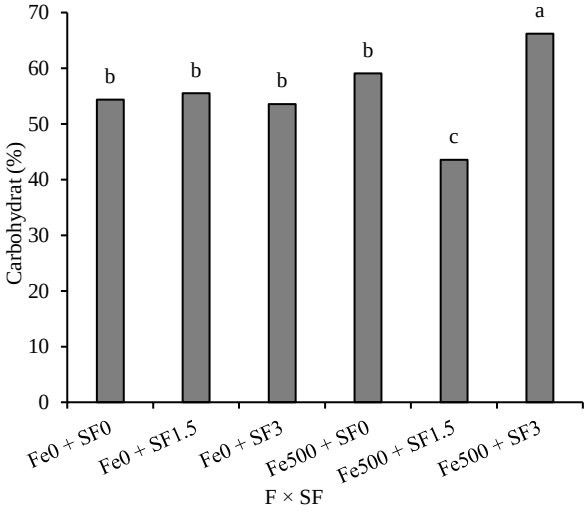


**Figure 8**. Mean comparison of F × SF on carbohydrate content of mushroom

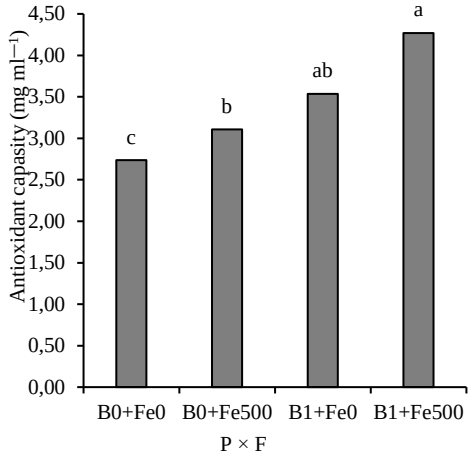


**Figure 9**. Mean comparison of P × F on total antioxidant capacity of mushroom

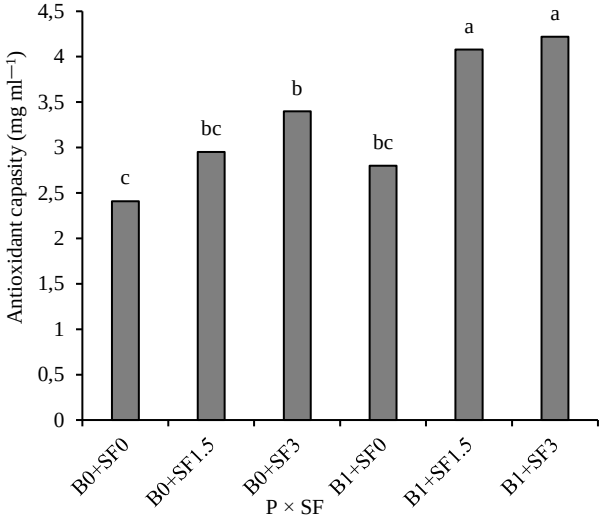


**Figure 10**. Mean comparison of P × SF on total antioxidant capacity of mushroom

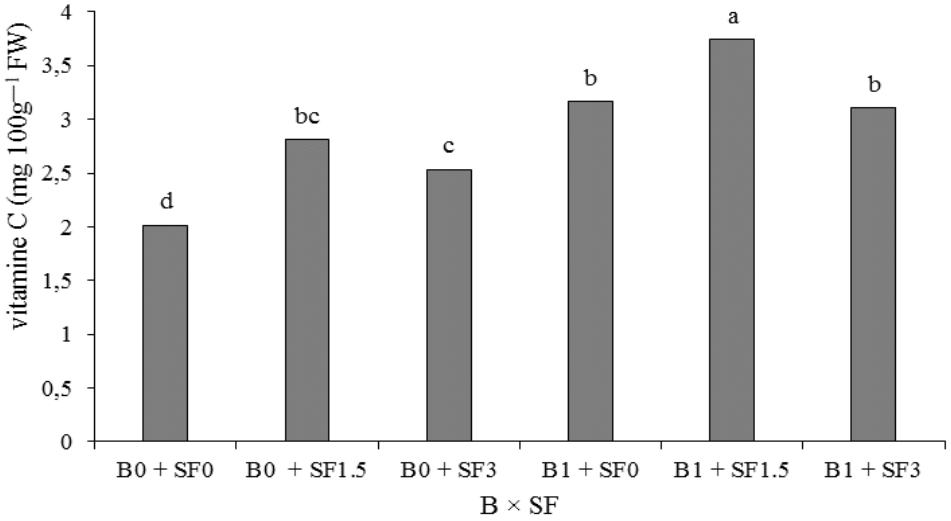


**Figure 11**. Mean comparison of P × SF on vitamin C content of mushroom

## 4. Conclusions

In the present work, the interaction effect of soybean flour, iron-chelate, and *P. putida*, as a source of N, Fe, and plant growth-promoting bacteria, respectively, were evaluated for improved quality traits and yield of edible mushroom. We found that the application of soybean flour supplement at a rate of 1.5% of compost dry weight ($SF_{1.5}$) increased most recorded traits, especially dry matter percentage, fresh yield, and biological efficiency when compared to $SF_3$.

The positive effect of $SF_{1.5}$ was found to be more profound in the presence of *P. putida* so that their synergic relationship was evident in most traits. However, Fe application ($Fe_{500}$) in the treatments did not differ from their Fe-excluded counterparts in terms of most traits, except for carbohydrate content and antioxidant capacity. The maximum mushroom number, fresh yield, protein, protein yield, biological yield, and vitamin C were obtained from $SF_{1.5}$ + *P. putida*. So, to improve yield and qualitative traits of mushrooms, it is recommended to apply 1.5% soybean flour along with *P. putida*. It is also recommended to address the effect of dual inoculation with *P. putida*-Arbuscular mycorrhiza and different integrated levels of soybean flour and meal on yield and nutrient uptake of edible mushrooms in future studies.

## Authors' Contribution

Abdul Karim Kashi designed and directed this project. Reza Salehi Mohammadi and Fereshteh Makneli performed these experiments. Ahmad Khaliqi and Reza Salehi Mohammadi analyzed the data and interpreted the results. Abdul Karim Kashi and Fereshteh Maknali wrote the manuscript.

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