Potential use of *Lentinus squarrosulus* mushroom as fermenting agent and source of natural antioxidant additive in livestock feed

Noorlidah Abdullah,* Ching-Ching Lau and Siti Marjiana Ismail

**Abstract**

**BACKGROUND:** Fermenting feed has gained a lot of popularity in recent years owing to its renowned benefits to the livestock and feed quality. In the current study, *Lentinus squarrosulus* mushroom mycelium was tested for its potential as a fermenting agent and source of natural antioxidant in the feed.

**RESULTS:** Phenolic content of methanolic and hot water extracts of the mycelium culture and its fermented maize ranged from 94.01 to 386.59 mg gallic acid equivalents g\(^{-1}\) extract while the DPPH radical scavenging, CUPRAC, reducing power (RPA) and \(\beta\)-carotene bleaching (BCB) antioxidant activity had EC\(_{50}\) values ranged from 15.30 to 120.3, 0.74 to 4.71, 1.86 to 13.5 and 0.01 to 0.21 mg mL\(^{-1}\), respectively. At 1.0 – 20.0 mg mL\(^{-1}\), the extracts retarded 21.02 – 55.35% of lipid deterioration. Pearson correlation analysis revealed the phenolic content of the extracts has moderate correlation with DPPH (\(r = 0.589\)) and RPA (\(r = 0.580\)), also a high correlation with BCB antioxidant activity (\(r = 0.872\)). In principal component analysis, DPPH, CUPRAC and RPA were seen to be clustered tightly together while BCB antioxidant activity was grouped with the phenolic content.

**CONCLUSION:** Overall, *L. squarrosulus* mycelium functioned as antioxidants via several mechanisms, involving either electron transfer or hydrogen transfer-based reactions suggesting it as a promising fermentation agent to enhance feed nutrition and the fermented maize as a valuable feed resource.

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**Keywords:** mushrooms; mycelium; submerged fermentation; solid substrate fermentation

**INTRODUCTION**

Rancidity is one of the major issues involving feed quality. Prolonged exposure to air, heat, sunlight and poor storage conditions are among the factors promoting oxidative rancidity in the feed. The drawbacks of long-term supply of deteriorated feed to animals are documented in several studies, which include low growth performance, weak immune system, increase mortality rate and producing animal-based products with lower nutritional quality.1–3 Moreover, oxidised feeds are considered to be toxic, hence this has provoked fears about the safety of food products derived from the affected animals.

Antioxidant supplementation is found as a practical solution to overcome this circumstance.4 Furthermore, antioxidant properties in the feed are perceived to be deposited in the muscle and cellular membranes upon consumption leading to the production of meat, milk and eggs with improved nutritional quality and oxidative stability.5 Besides, inclusion of antioxidants directly in the animal's diet was found to provide better meat quality compared to post-mortem application in processed meat.5 Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are widely used as antioxidant supplements but their potential carcinogenic effect is a health concern.6 Fermentation is an efficient and natural way that could augment antioxidative properties in the final product.7 Fermenting feed has gained a lot of popularity in recent years owing to the benefits of which includes an improvement of gut health8 and reduced pathogen infection in the animals,9 as well as increased feed digestibility resulting in enhanced animal feed intake and growth performance.10 Additionally, the performance of animals fed with fermented diets has characteristics similar to that of animals inoculated with antibiotic growth promoters. This makes it a good alternative to the use of antibiotics, which have fallen under scrutiny due to the potential risk of the emergence of drug-resistant bacteria over long-term administration.10

Bacteria and yeast are among the most common inoculants used in feed.7,10 Recently, mushroom mycelium has come into the limelight as a potential fermenting agent from natural origin. Examples of reported studies include fermentation of straw and wheat by *Trametes versicolor*11 and *Pleurotus ostreatus*,12 respectively. *Lentinus squarrosulus* used in the current study is a wild edible mushroom found in many parts of Asia, and contains high nutrients and medicinal properties.13,14 Moreover, its fermented product was found to produce a pleasant aroma which is liked by animals.15

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Many biochemical changes may occur during the fermentation process which affects the bioactivity of the final product. The biological analysis of fermented feed in terms of microbial properties and nutritional values has been widely discussed. The high levels of lactic acid bacteria with low pH and Enterobacteria counts are deemed as the main factors responsible for the enhanced fermented feed quality. Nevertheless, antioxidant could also play a role in protecting the feed against spoilage and improving the animal’s performance. However, to the best of our knowledge, there is no report on the antioxidant potential of *L. squarrosulus* mycelium and its fermented product tested based on several mechanisms of action. Therefore, the objective of this work was to evaluate the antioxidant efficacy of *L. squarrosulus* mycelium and the potential of its fermented product (maize as a model) as dietary antioxidant in livestock feed.

**Materials and Methods**

**Chemicals**

2,2-Diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, ferrous sulfate, β-carotene, linoleic acid, neocuproine, copper(II) chloride dihydrate, gallic acid and BHA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin–Ciocalteu phenol reagent, ammonium acetate, sodium phosphate monobasic and sodium phosphate dibasic were purchased from Merck (Darmstadt, Germany). Thiobarbituric acid was obtained from AppliChem (Darmstadt, Germany). All chemicals and reagents were of analytical grade.

**Preparation of Lentinus squarrosulus inoculum**

*L. squarrosulus* culture was authenticated by morphological and molecular methods by experts in the Mushroom Research Centre, University of Malaya, Malaysia. The mycelium was cultured on GYMP agar consisting of glucose (1.5%), yeast extract (0.8%), malt extract (0.8%) and peptone (0.8%) (w/v). The culture was deposited in the Mushroom Research Centre culture collection, University of Malaya, and assigned a culture code (KUM 50016).

**Sample preparation of methanol and hot water extract from Lentinus squarrosulus mycelium broth**

*L. squarrosulus* mycelium broth (LSMB) was acquired by submerged fermentation. Ten Erlenmeyer flasks (250 mL) containing 50 mL of GYMP liquid media were autoclaved at 121 °C for 15 min followed by cooling to 25 °C. Each flask was inoculated with five 9-mm diameter mycelia plugs cut from the periphery of a 7-day-old colony. After 14 days incubation at 25 °C, the grains and mycelia were crushed and lyophilised. Hot water and methanol extraction was carried out following the method as described in the section above. The extracts obtained were designated as ‘*L. squarrosulus* fermented maize methanolic’ (LSFM-M) and ‘*L. squarrosulus* fermented maize hot water’ (LSFM-HW), respectively.

**DPPH radical scavenging assay**

Scavenging effect of the test samples against DPPH radicals were measured according to the method of Daker et al. Test solution (0.1 mL) was added to 3.9 mL of a 0.06 mmol L⁻¹ DPPH–methanol solution, and the absorbance at 517 nm was recorded at 0, 1, 2 and every 15 min thereafter until the reaction reached a plateau (against a methanol blank). BHA was used as the positive control. The scavenging activity of DPPH was calculated as: % radical scavenging activity = \[1 - \left( \frac{A_c}{A_r} \right) \times 100\], where \(A_c\) is the absorbance of the reaction mixture and \(A_r\) is the absorbance of 0.06 mmol L⁻¹ methanolic DPPH. The EC₅₀ (mg mL⁻¹) value was the effective concentration at which 50% of the DPPH radicals were scavenged and was obtained by interpolation from the linear regression analysis.

**Cupric ion reducing antioxidant capacity (CUPRAC)**

CUPRAC was determined according to the method of Apak et al. Test samples (1 mL) were added to a mixture containing 1 mL each 10 mmol L⁻¹ copper(II), 7.5 mmol L⁻¹ neocuproine and 1 mol L⁻¹ ammonium acetate buffer (pH 7.0). The mixture was reacted at room temperature for 30 min and absorbance at 450 nm recorded against a reagent blank. BHA was used as the positive control. The EC₅₀ (mg mL⁻¹) value was the effective concentration at which the absorbance was 0.5 for cupric ion reducing capacity and was obtained by interpolation from the linear regression analysis.

**Reducing power activity (RPA)**

The RPA of test samples was determined as described by Abdullah et al., where test samples (1 mL) were mixed with 2.5 mL 0.2 mol L⁻¹ phosphate buffer (pH 6.6) and 2.5 mL potassium ferricyanide (1%), incubated at 50 °C for 20 min. Then, 2.5 mL of TCA (10%) was added and the mixture centrifuged at 10000 × g for 10 min. Supernatant (2.5 mL) was mixed with 0.5 mL ferric chloride (0.1%) and absorbance measured at 700 nm. BHA was used as the positive control. The EC₅₀ (mg mL⁻¹) value was the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from the linear regression analysis.

**β-Carotene bleaching (BCB) antioxidant assay**

The procedure was carried out as described by Abdullah et al. β-Carotene (2 mg) was dissolved in 10 mL chloroform and mixed with 40 μL linoleic acid and 400 μL Tween 80. One millilitre was pipetted into a 100 mL round-bottom flask, the chloroform evaporated under vacuum and 100 mL distilled water added with vigorous shaking. Aliquots of this emulsion (4.8 mL) were transferred into tubes containing test samples (0.2 mL) and the zero time absorbance measured immediately at 470 nm. The tubes were incubated at 50 °C for 2 h and another reading taken. Distilled water was used as the control and a blank (no β-carotene) was prepared for background subtraction. Degradation rate (DR) was calculated as: \[DR = \ln(A_{t=0}/A_{t=120}) \times 1/120\], where \(A_{t=0}\) is the absorbance of the reaction mixture at time 0 min and \(A_{t=120}\) is the
The absorbance of the reaction mixture after 120 min. The antioxidant activity was calculated using the following formula: antioxidant activity = (DR_control − DR_sample)/DR_control × 100.

The EC50 (mg mL−1) value was the effective concentration at which 50% of the β-carotene oxidation was inhibited.

**Anti-lipid peroxidation (ALP) effect**

The ability of test samples to inhibit lipid peroxidation was determined according to the method of Daker et al. The reaction mixture contained 1 mL of egg yolk emulsified with 0.1 mol L−1 phosphate buffer, pH 7.4 (final concentration of 25 g L−1) and 100 μL of 1 mmol L−1 Fe2+. After the addition of test sample, the mixture was incubated at 37 °C for 1 h. Then, 0.5 mL of freshly prepared 15% trichloroacetic acid and 1.0 mL 1% thiobarbituric acid were added to the mixture. The reaction tubes were further incubated in a boiling water bath for 10 min, cooled to room temperature and centrifuged at 3500 × g for 10 min to remove precipitated proteins. The formation of thiobarbituric acid reactive substances (TBARS) in 100 μL of supernatant was measured at 532 nm against the buffered egg yolk/Fe2+ (control). The % inhibition was calculated by: % inhibition = (1 − A1/A2) × 100, where A1 is the absorbance of the sample and A2 is the absorbance of the control.

**Estimation of total phenolic content (TPC)**

The TPC of the test samples was estimated by the Folin–Ciocalteu colorimetric assay as described by Abdullah et al. Briefly, 250 μL sample was added to 250 μL 10% Folín–Ciocalteu phenolic reagent and incubated at room temperature for 3 min. The mixture was incubated for another hour after the addition of 500 μL 10% sodium carbonate solution. The absorbance of the mixture was read at 750 nm and the phenol content determined by comparing the absorbance values of samples to a gallic acid standard curve. The concentration of total phenols was expressed in mg gallic acid equivalent per gram of test sample (mg GAE g−1 extract).

**Statistical analysis**

Antioxidant analyses and estimation of TPC were carried out in triplicate (n = 3) and the results reported as mean ± standard deviation. The Pearson correlation test was used to calculate the correlations between the five antioxidant assays and TPC of LSMB and LSF M extracts. Differences of P < 0.05 were considered statistically significant and P < 0.01 was taken to be statistically highly significant. Principal component analysis (PCA) was employed to study clusters of the antioxidant assays and TPC. The data analysed by PCA was displayed as bi-plot. All the data were analysed using SPSS 22.0 package (SPSS Inc., Chicago, IL, USA).

**RESULTS AND DISCUSSION**

**Evaluation of the antioxidant potentials in the extracts**

Antioxidant compounds may act in vivo through different mechanisms which include termination of chain initiation by radical scavenging, chelating of metal ions and elimination of peroxides. As such, a single antioxidant assay was inadequate to provide an overview of the total antioxidant capacity of the samples. In the present study, antioxidant activities of LSMB and LSF M (extracted with methanol and hot water) were evaluated for DPPH radical scavenging activity, CUPRAC, RPA, BCB antioxidant and ALP activity.

**DPPH radical scavenging assay**

The DPPH radical scavenging assay measured the neutralising ability of extracts toward DPPH free radicals by transferring an electron to DPPH where decolourisation of the solution was inversely correlated with the radical scavenging activity of the extracts. The result obtained in Fig. 1 showed the methanolic extract has a higher DPPH antiradical activity compared to its water extract. This is in agreement with the finding that the DPPH reagent has a high affinity toward lipophilic antioxidants as opposed to hydrophilic ones. At 10–50 mg mL−1, the scavenging abilities of LSMB-M and LSMB-HW were 34.4–92.1% and 23.6–92.3%, respectively, with EC50 values of 15.30 and 18.23 mg mL−1, respectively. When the L. squarrosulus was used as an inoculant in feedstuff, the fermented maize produced lower scavenging activity. At 10–50 mg mL−1, LSFM-M and LSF M-HW scavenged the DPPH radical at 9.9–53.4% and 1.0–19.3%, respectively, with EC50 values of 45.78 and 120.3 mg mL−1, respectively. A study by Miyamoto and co-workers showed oral administration of fermented feed increased DPPH antiradical activity in the animal products, although the feed itself had little of its activity. Our previous investigation revealed L. squarrosulus fermented maize has 1.5 times better DPPH antiradical effect than its unfermented counterpart. Hence, the true effect of administering L. squarrosulus fermented feed to the production of animal products with antiradical activity warrants further investigation.

**CUPRAC and RPA assays**

The CUPRAC and RPA assay represented the capability of antioxidants to reduce cupric [Cu(II)] and ferric [Fe(III)] ions to cuprous [Cu(I)] and ferrous [Fe(II)] ions, respectively. The amount of reduced ions formed is monitored spectrophotometrically, where a higher absorbance value indicated higher antioxidant activity.

Presently, CUPRAC assay is not commonly used among researchers working on antioxidant studies, and little has been published on the evaluation of cupric ion-reducing ability of mushroom mycelia. However, this antioxidant assay claimed to
offer many advantages over other electron-transfer based methods which include: the working pH of the method is close to physiological (i.e. pH 7.0), it can be used with hydrophilic and lipophilic antioxidants, the selective oxidation of antioxidant compounds without affecting sugars and citric acid commonly found in foodstuffs and the capacity to assay SH-bearing antioxidants.19

In the current study, the absorbance values recorded by LSMB-M and LSMB-HW were 0.04–1.09 and 0.09–1.30, respectively, when tested at 0.1–2.0 mg mL\(^{-1}\) while its simulation as animal feedstuff (LSFM-M and LSFM-HW) had absorbance values of 0.07–0.91 ± 0.02 and 0.03–0.20, respectively, (Fig. 2A). This was comparable to the values of hot water extracts from 14 culinary mushroom species which had absorbances ranging from 0.137 to 1.058.20

As shown in Fig. 2B, the RPA of LSMB-M and LSMB-HW has EC\(_{50}\) of 3.29 and 4.45, respectively. This was comparable with EC\(_{50}\) values of methanolic (6.89 mg mL\(^{-1}\)) and hot water extract (3.97 mg mL\(^{-1}\)) from Agrocybe cylindracea mycelium.25,26 A higher RPA was recorded by Ganoderma tsugae and Armillaria mellea mycelia with EC\(_{50}\) values lower than 1.00 mg mL\(^{-1}\).27–29 LSFM-M has a substantially high RPA where at 0.1–1.5 mg mL\(^{-1}\); it had absorbances of 0.033–0.406 and an EC\(_{50}\) of 1.86 mg mL\(^{-1}\). This value is higher compared to yeast fermented soybean curd and bacteria fermented peanut meal hydrolysate (both had EC\(_{50}\) > 2.0 mg mL\(^{-1}\)).20 Compared to LSFM-M, its water extract counterpart has lower RPA with absorbances of 0.002–0.06.

Overall, LSFM-M exhibited only a slightly lower CUPRAC activity than LSMB-M but LSFM-HW showed a four-fold lower activity than LSMB-HW while LSFM-N exhibited a two-fold higher RPA than LSMB-M and LSFM-HW had a three-fold lower activity than LSMB-HW. This may suggest that most of the organic soluble antioxidants which are active cupric and ferric ion reducer in L. squarrosulus mycelium remained active following the solid state fermentation process as opposed to its water soluble counterpart. There might be some structural modifications of the components in maize due to enzymes liberated by L. squarrosulus mycelium during fermentation that resulted in the variations in the reducing capacity of the cupric and ferric ions. Numerous reports have been published on the transformation of antioxidant properties and phytochemical composition in fermented solid based products.16,31

Copper ions are chemically more reactive than iron ions, resulting in faster kinetics in redox reactions.19 The LSFM in the present study was high in both CUPRAC and RPA, suggesting its high possibility to counteract the action of oxidants, hence has prolonged shelf life. This criterion is important as feed tends to be exposed to poor storage conditions that promote oxidation.

**BCB antioxidant assay**

\(\beta\)-Carotene is extremely susceptible to free-radical mediated oxidation. The BCB antioxidant method is based on the discoloration of oxidised \(\beta\)-carotene due to the attack of linoleate free radicals in the system. Samples without antioxidants had a rapid decrease in absorbance, while in the presence of antioxidant, the colour was retained for a longer time.31 The antioxidant activity was expressed as % inhibition of discoloration relative to the control.

Referring to the result in Fig. 3, \(\beta\)-carotene in the system underwent rapid discoloration in the presence of low concentrations of extracts, while at a higher concentration (∼0.10 mg mL\(^{-1}\)), the \(\beta\)-carotene colour was retained after the 2 h of incubation. LSMB extracts showed high protective effect to hinder the extent of \(\beta\)-carotene bleaching, where LSMB-M and LSMB-HW at 0.01 mg mL\(^{-1}\) showed 37.6% and 49.03% BCB antioxidant activity, respectively, and at 0.5 mg mL\(^{-1}\), the % inhibition increased to 91.61% and 89.85%, respectively. The extracts had EC\(_{50}\) values of 0.03 and 0.01 mg mL\(^{-1}\), respectively. Its fermented product showed a slightly lower activity where the % inhibition of LSFM-M and LSFM-HW increased from 17.31% to 60.95% and 19.67% to 56.16%, respectively with EC\(_{50}\) values recorded at 0.21 and 0.19 mg mL\(^{-1}\), respectively. Both LSMB and LSFM extracts in the present study had a higher BCB antioxidant activity compared to extracts of 24 mushroom species mycelia reported by Asatiani and colleagues where most of the investigated species have EC\(_{50}\) > 2.0 mg mL\(^{-1}\).32

The presence of antioxidants involved in inhibiting the oxidation of \(\beta\)-carotene can reduce the extent of \(\beta\)-carotene destruction in feed ingredients, hence ensuring a sufficient supply of the compound to the animals. In addition to its role as a vitamin A precursor, \(\beta\)-carotene is important to the animal’s health by enhancing their immunity and increases their reproductive efficiency.33 This result is consistent with a report concerning the study on tomato. It was found that the loss of BCB antioxidant and DPPH antiradical capacity in the fruit due to the skin and seed removal were also accompanied by a significant decrease in their \(\beta\)-carotene content.34

**ALP effect**

This method is based on the ability of the antioxidants to inhibit ferrous-induced peroxidation of egg yolk phospholipids, with...
the production of malondialdehyde as the hallmark of the peroxidation process. At 1.0–20.0 mg mL\(^{-1}\), LSMB-M and LSMB-HW have retarded 21.02–43.86% and 27.19–48.46% of lipid deterioration, respectively (Fig. 4). The fermented maize has shown better activity with 23.36–33.42% and 48.49–55.35% inhibited by LSFM-M and LSFM-HW, respectively. Furthermore, \(L.\ squarrosulus\) fermented maize was documented to have 1.6 times better ALP activity with 23.36–33.42% and 48.49–55.35% inhibited by mycelium broth (LSMB) and \(L.\ squarrosulus\) fermented maize (LSFM). Data expressed as mean ± standard deviation \((n = 3)\). The EC\(_{50}\) (mg mL\(^{-1}\)) value was the effective concentration at which 50% of BCB activity was inhibited. Butylated hydroxyanisole (BHA) was used as a positive control.

Figure 3. Inhibition (% of \(\beta\)-carotene bleaching (BCB) activity of methanolic (M) and hot water extracts (HW) of \(L.\ squarrosulus\) mycelium broth (LSMB) and \(L.\ squarrosulus\) fermented maize (LSFM). Data expressed as mean ± standard deviation \((n = 3)\). The EC\(_{50}\) (mg mL\(^{-1}\)) value was the effective concentration at which 50% of BCB activity was inhibited. Butylated hydroxyanisole (BHA) was used as a positive control.

Figure 4. Anti-lipid peroxidation abilities of methanolic (M) and hot water extracts (HW) of \(L.\ squarrosulus\) mycelium broth (LSMB) and \(L.\ squarrosulus\) fermented maize (LSFM). Data expressed as mean ± standard deviation \((n = 3)\). Butylated hydroxyanisole (BHA) was used as a positive control.

Inter-relationship of antioxidant activities and TPC in the LSMB and LSFM extracts

Phenolic compounds have been frequently reported as bioactive components associated with antioxidant properties. Regression analysis was performed for the correlations among antioxidant assays and TPC in the extracts from LSMB and LSFM (Table 1). Apparently, the result of TPC in the current study has moderated correlation with DPPH \((r = 0.589)\) and RPA \((r = 0.580)\), also a high correlation with BCB antioxidant activity \((r = 0.872)\). This is in agreement with previous studies which indicated that phenolic substances generally has a better correlation with DPPH antiradical activity, ferric reducing antioxidant assay\(^{38}\) and BCB antioxidant activity.\(^{37}\) On the other hand, there was no significant correlation between TPC and their ability to reduce cupric ions. It has been suggested that not all of the phenolic compounds are active cupric ion reductor.\(^{34}\) Therefore, the type of phenolic compounds present in the extract is more influential to the CUPRAC activity than their total content. It is noteworthy that there are but the deposition of its ALP compound in the muscle and cellular membranes upon consumption can increase oxidative and colour stability of animal products, hence extending the retail display life.

Estimation of TPC

The Folin–Ciocalteu method was used to estimate the TPC of LSMB and LSFM extracts based on the reducing power of phenolic hydroxyl groups.\(^{21}\) Referring to the result in Fig. 5, LSMB-M and LSMB-HW had a TPC of 163.42 and 386.59 mg GAE g\(^{-1}\) extract, respectively, while LSFM-M and LSFM-HW contained 122.63 and 94.01 mg GAE g\(^{-1}\) extract, respectively. These were substantially higher than the amount reported for other mushrooms mycelia. Hot water extracts of \(G.\ tsugae\), \(A.\ cylindracea\) and \(A.\ mellea\) mycelial cultures contain 41.03, 27.28 and 30.90 mg GAE g\(^{-1}\) extract, respectively, and methanolic extracts have 35.60, 15.55 and 27.10 mg GAE g\(^{-1}\) extract, respectively,\(^{25–29}\) while methanolic and hot water extracts of corncob fermented by \(G.\ sinensis\) contained 24.0 and 35.3 mg GAE g\(^{-1}\) extract, respectively.\(^{35}\) Our previous investigation has revealed \(L.\ squarrosulus\) fermentation increases the TPC in maize by 1.3 times,\(^{24}\) suggesting that it has enormous potential to enhance the nutritional and antioxidant properties of the feed.

Figure 5. Total phenolic content (TPC) of methanolic and hot water extracts of \(L.\ squarrosulus\) mycelium broth (LSMB) and \(L.\ squarrosulus\) fermented maize (LSFM). TPC values are designated as mg gallic acid equivalents in 1 g extract (mg GAE g\(^{-1}\) extract) and expressed as mean ± standard deviation \((n = 3)\). Butylated hydroxyanisole (BHA) was used as a positive control.

<table>
<thead>
<tr>
<th>Concentration (mg mL(^{-1}))</th>
<th>Methanol (M)</th>
<th>Hot Water (HW)</th>
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<tbody>
<tr>
<td>0.1</td>
<td>LSMB</td>
<td>LSFM</td>
</tr>
<tr>
<td>0.2</td>
<td>LSMB</td>
<td>LSFM</td>
</tr>
<tr>
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<td>LSMB</td>
<td>LSFM</td>
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<tr>
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<td>LSMB</td>
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<td>LSMB</td>
<td>LSFM</td>
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</tr>
<tr>
<td>1.0</td>
<td>LSMB</td>
<td>LSFM</td>
</tr>
</tbody>
</table>

**Table 1. Total phenolic content (mg GAE g\(^{-1}\) extract**

- TPC of BHA = 630.04 ± 21.78 mg GAE g\(^{-1}\) extract
- TPC values are designated as mg gallic acid equivalents in 1 g extract (mg GAE g\(^{-1}\) extract) and expressed as mean ± standard deviation \((n = 3)\).
- Butylated hydroxyanisole (BHA) was used as a positive control.

**Figure 3.** Inhibition (%) of \(\beta\)-carotene bleaching (BCB) activity of methanolic (M) and hot water extracts (HW) of \(L.\ squarrosulus\) mycelium broth (LSMB) and \(L.\ squarrosulus\) fermented maize (LSFM). Data expressed as mean ± standard deviation \((n = 3)\). The EC\(_{50}\) (mg mL\(^{-1}\)) value was the effective concentration at which 50% of BCB activity was inhibited. Butylated hydroxyanisole (BHA) was used as a positive control.

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**Table 1. Total phenolic content (mg GAE g\(^{-1}\) extract**

- TPC of BHA = 630.04 ± 21.78 mg GAE g\(^{-1}\) extract
- TPC values are designated as mg gallic acid equivalents in 1 g extract (mg GAE g\(^{-1}\) extract) and expressed as mean ± standard deviation \((n = 3)\).
- Butylated hydroxyanisole (BHA) was used as a positive control.
Table 1. Correlation coefficients (r) between TPC and antioxidant potential of extracts from *Lentinus squarrosulus* mycelial broth and *L. squarrosulus* fermented maize

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TPC</th>
<th>ALP</th>
<th>BCB</th>
<th>RPA</th>
<th>CUPRAC</th>
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<tbody>
<tr>
<td>DPPH</td>
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<td>0.067</td>
<td>0.654*</td>
<td>0.980**</td>
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<td>CUPRAC</td>
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<td>0.513</td>
<td>0.997**</td>
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<td>0.253</td>
<td>0.556</td>
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<tr>
<td>BCB</td>
<td>0.872**</td>
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<tr>
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<td>0.147</td>
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<td>–</td>
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</table>

*Correlation is significant at P < 0.05.
**Correlation is significant at P < 0.01.

**Correlation is significant at P < 0.01.

Significant (P < 0.01) correlations between DPPH-RPA (r = 0.980) and DPPH-CUPRAC (r = 0.977) methods used to determine the antioxidant capacity of the studied LSMB and LSFM samples. Similar correlation coefficients (r = 0.8199 – 0.9999) between the three antioxidant assays were found in the rapeseed samples.38

The results of the LSMB and LSFM extracts were subjected to PCA using SPSS 22.0 package. Principal component analysis revealed the presence of two components with eigenvalues exceeding 1, which explained a total of 87.39% of the variance, with Component 1 contributing 65.59% and Component 2 contributing 21.78% (Fig. 6). Oblimin rotation was performed to aid in the interpretation of these two components. The result obtained was consistent with the Pearson correlation test in Table 1. DPPH, CUPRAC and RPA were seen being clustered tightly together while BCB antioxidant activity was being grouped with TPC. Based on reaction mechanisms involved, antioxidant capacity assays can be divided into two major groups, i.e. those involving electron transfer reactions and others based on hydrogen transfer reactions.21 Hence, this may explain the phenomenon of classification illustrated in Fig. 6 as DPPH, CUPRAC and RPA antioxidant assays are electron transfer based method while BCB antioxidant activity and ALP are hydrogen atom transfer based method. TPC utilises both electron transfer and hydrogen atom transfer based reaction mechanism. The close proximity of TPC with BCB antioxidant activity in the PCA bi-plot suggests TPC from LSMB and LSFM may act predominantly on hydrogen transfer based method.

Referring to the Pearson regression and PCA analysis, ALP assay was seen to have low association with the other antioxidant capacity assays. However, they may provide synergistic effect on the overall ALP activity of the feed. For instance, it has been well recognised that transition metal ion such as iron is an important catalyst for the generation of free radicals that initiate lipid peroxidation process. Hence, effective anti-radical agents and antioxidants with reducing capability to reduce the oxidants may afford protection against the generation of reactive oxygen species, thereby inhibiting peroxidation in the feed.

Several studies have documented a positive correlation between culture conditions and the antioxidant properties of mushroom mycelia,39,40 and a comprehensive overview of the production of phenolic compounds using different solid substrate fermentation conditions is available.31 Geese fed with fermented feed incubated at different time duration have shown variations on

![Figure 6](https://example.com/fig6.png)

**Figure 6.** Principal component analysis (PCA) loading plot of total phenolic content (TPC) and antioxidant potential of extracts from *L. squarrosulus* mycelium broth and its fermented maize. Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging; CUPRAC, cupric ion reducing antioxidant capacity; RPA, reducing power activity; BCB, β-carotene bleaching antioxidant activity; ALP, anti-lipid peroxidation antioxidant assay.
the growth performance and oxidative stability of the organs.61 Therefore, the antioxidant properties of maize fermented by L. squarrosulus mycelium may be further improved by optimising culture conditions for carbon and nitrogen source, temperature, moisture content, pH, aeration and growth time.

CONCLUSION
Although the positive control (BHA) had significantly higher activity than LSMB and LSFM extracts in all the antioxidant assays, it can be used as a feed additive at milligram levels only due to its carcinogenic potential in high doses, while mushroom mycelia is a natural product that could be consumed at gram levels as a feed. The total antioxidant capacity of feed may rely not only on the effect of the most potent antioxidants, but rather on the combined synergy action of all the antioxidants present in the diet. Besides, there is a growing consensus among researchers that using a combination of antioxidants may be more effective than single compounds on a long-term basis. The ability of LSMB and LSFM extracts in the current study to function as antioxidants via several mechanisms makes it a good source of antioxidant compounds. The present work had proven LSMB as a valuable fermentation agent, hence it can be commercialised as a starting culture for feed fermentation. Future work can be performed in vivo to verify the true effect of LSFM in the biological system before applying them to commercial animal feed and feed ingredients.

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