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Diet supplemented with *Grifola gargal* mushroom enhances growth, lipid content, and nutrient retention of juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract This study examined the suitability of the edible mushroom *Grifola gargal* as a dietary supplement for juvenile rainbow trout (*Oncorhynchus mykiss*). Three treatments were established in triplicate using 50 fish (0.33 ± 0.01 g) held in 50-L containers. Treatments consisted of feeds (42–45% protein, ca. 18% lipid) supplemented with fruiting-bodies of *G. gargal* at 0 g kg^{-1} (control diet (CTRL)), 25 g kg^{-1} (GG25), or 100 g kg^{-1} (GG100). Fish were hand-fed to apparent satiation twice a day (except on Sundays) for 56 days. Feed intake and growth were recorded throughout the study, and fish body proximate composition and nutrient retention were assessed at the end of the trial. Fish given GG25 diet had better growth and feed utilization than those given the other feeds. Final body weight was 2.37 ± 0.04 g (CTRL), 4.07 ± 0.07 g (GG25), and 1.94 ± 0.06 g (GG100) and the thermal-unit growth coefficient increased significantly from 0.64 ± 0.01 in CTRL to 0.87 ± 0.01 in GG25. The feed efficiency and the protein efficiency ratio were best for fish fed GG25, and body lipid was 42.3 ± 2.6 g kg^{-1} in CTRL and 75.3 ± 1.5 g kg^{-1} in GG25 treatments. This coincided with a lower viscerosomatic index in the fish given GG25 than in those provided with the other feeds. These results suggest that dietary supplementation with *G. gargal* at 25 g kg^{-1} enhances growth and leads to improved feed utilization in small rainbow trout.

Keywords Edible mushrooms · Feed ingredients · Fungi · Nutrition · Salmonids

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Introduction

World aquaculture production volume increased at a mean rate of 8.6% per year during the last three decades. In 2012, aquaculture accounted for 42% of total fish production (FAO 2016). In order to further increase fish production and to reduce the environmental impacts of this activity, fish farmers should continue to search for alternative sources of affordable and high-quality products to improve aquafeeds (FAO 2012). There is a consensus among feed manufacturers and fish farmers that quality feed should not only ensure superior growth but also improve health (Kiron 2012). In this way, an adequate nutrition may contribute to reducing the use of chemicals and drugs, thus contributing to cost effective and environmentally friendly farming practices.

Mushrooms, mainly higher Basidiomycetes, have been highly appreciated as a source of healthy food and medicine for mankind for millennia, since they are a source of many bioactive compounds (Stamets and Zwickey 2014; Wasser 2014). These include proteins of high biological value (which supply all the essential amino acids for fish with high digestibility) although at a relatively low proportion (15–30%), a complex wall of polysaccharides (including β-glucans and chitin), high amounts of vitamins (B1, B2, B12, C, D, and E) and minerals (including zinc, copper, iodine, selenium, and iron), various antioxidant and phenolic compounds, 5′-nucleotides, and polyunsaturated fatty acids, among others (Kalac 2009; Wani et al. 2010; Ito et al. 2011; Cohen et al. 2014; Toledo et al. 2016).

There are previous studies about the use of edible mushrooms' fruiting-bodies, mycelia, or by-products as dietary supplement for different species of farmed fish with antioxidant, immunomodulation, and disease resistance improvement properties (Mostak et al. 2015; Bilen et al. 2016; Manayi et al. 2016; Van Doan et al. 2016). It has been demonstrated that β-glucans present in edible mushrooms like *Lentinula edodes* (Nikl et al. 1991; Djordjevic et al. 2009; Baba et al. 2015), *Ganoderma lucidum* (Yin et al. 2009; Chang et al. 2013; Liu et al. 2015), and *Pleurotus ostreatus* (Kamilya et al. 2006; Dobsíková et al. 2013) produce potent immunostimulant and prebiotic effects. However, only a slight improvement in fish growth parameters has been obtained by using different *Pleurotus* sp. extracts as dietary supplements (Mostak et al. 2015; Bilen et al. 2016). Additionally, there are several reports on successful replacement of small fractions of fish meal by mushrooms' mycelium or by-products without significant reduction of fish growth (Paripuranam et al. 2011; Katya et al. 2014; Muin et al. 2015; Sartori et al. 2015).

Grifola gargal (Polyporales, higher Basidiomycetes) is an edible mushroom usually collected by native people in limited areas of the Andean-Patagonic *Nothofagus*-dominated forest of Argentina and Chile. *G. gargal* is suitable for commercial cultivation, and its fruiting-body composition has been previously described (Schmeda-Hirschmann et al. 1999; Postemsky et al. 2011; Harada et al. 2015a, b; Toledo et al. 2016). *G. gargal* antioxidant, antiinflammatory, and free radical scavenging properties have been demonstrated in several studies (Schmeda-Hirschmann et al. 1999; De Bruijn et al. 2008, 2009; Postemsky and Curvetto 2014, 2015). However, studies on the effects of this mushroom on in vitro or in vivo models remain scarce (Schmeda-Hirschmann et al. 1999; Postemsky et al. 2011; Harada et al. 2015a).

Due to its broad spectrum of bioactive compounds, the inclusion *G. gargal* fruiting-body as a dietary supplement could improve fish growth performance. In this study, we tested whether a dietary supplement based on *G. gargal* fruiting-body at two levels of inclusion (25 and 100 g kg^{-1}) benefits the growth performance of the rainbow trout *Oncorhynchus mykiss* and its nutritional composition.

Materials and methods

Mushroom collection and preparation

Mature fruiting-bodies of *G. gargal* were either collected in the field or bought from native gatherers at a local market of the Lanín National Park area, Argentina (Autumn 2015). After appropriate identification, fresh samples having good appearance were shredded, lyophilized, grounded to powder, mixed, and stored at −18 °C. The proximate composition was analyzed as described below and is detailed in Table 1.

Diet formulation

The basal diet was formulated in our laboratory to meet the nutrient requirements of rainbow trout according to Hardy (2002). The feed ingredients are listed in Table 2. White fish meal (crude protein 596 g kg^{-1}) and fish oil from Agustinier SA, Mar del Plata, Argentina, were supplied by a local fish feed manufacturer. Blood meal and hydrolyzed feather meal were purchased from Pollolin SA, Cipolletti, Argentina. The basal diet was used as control diet (CTRL). Experimental diets were prepared by replacing equal amounts of whole-wheat in the basal diet for *G. gargal* mushroom powder at two proportions: 25 g kg^{-1} (GG25) and 100 g kg^{-1} (GG100). All diets were designed to be isonitrogenous, isolipidic, and isoenergetic. To prepare the feed, all the ingredients were mixed thoroughly, hydrated to form a paste, and pelletized. Once dried in a hot air column, pellets were crumbled, sieved to obtain 0.7–1.2 mm particles, and stored at 4 °C until use. Feed proximate composition is summarized in Table 2.

Fish and experimental design

O. mykiss fry were obtained from the hatchery of *Centro de Ecología Aplicada de Neuquén* (CEAN), Junín de los Andes, Neuquén, Argentina. The trials were carried out in 50-L PVC containers supplied with constant freshwater flow from the Chimehuín River and maintained in a 14:10 light/dark cycle. During the experiment, water quality conditions were as follows: daily mean temperature 11.3 ± 1.7 °C (min. 8.1, max. 14.4 °C), pH 6.8 ± 0.2, conductivity 76 ± 19 μs cm^{-1}, and dissolved oxygen above 94%. A total of 450 fry were randomly distributed into 9 groups of 50 individuals (16.4 ± 0.2 g total biomass per container). An extra group of 50 fish was separated for the initial proximate composition analysis. The initial

Table 1 Proximate analysis of *Grifola gargal* fruiting-bodies (on dry weight basis)

Proximate composition (g kg^{-1})	
Crude protein (CP)[a]	102.7 (146.3)
Ether extract (EE)	42.2
Ash	54.7
Nitrogen-free extract (NFE)[b]	800.4 (756.8)
Total nitrogen (TN)	23.4

[a] CP = TN × 4.38, according to Danell and Eaker (1992). The values between parenthesis were calculated with the conversion factor 6.25.

[b] NFE = total − moisture − CP − EE − ash

Table 2 Formulate and proximate composition of the experimental diets

Diets	CTRL	GG25	GG100
Ingredients (g kg^{-1})			
Fish meal	570	570	570
Fish oil	160	160	160
Whole-wheat	149	124	49
G. gargal fruiting-body	0	25	100
Blood meal	50	50	50
Feather meal	50	50	50
Vitamin and mineral premix[a]	10	10	10
Choline chloride	5.0	5.0	5.0
L-Lysine	4.0	4.0	4.0
L-Methionine	1.0	1.0	1.0
Ascorbic acid	1.0	1.0	1.0
Carboxymethylcellulose	20	20	20
Proximate composition (g kg^{-1})			
Moisture	58.5	51.6	53.1
Crude protein (CP)	423	430	452
Ether extract (EE)	180	180	182
Ash	164	161	163
Nitrogen-free extract (NFE)[b]	175	178	150
Gross energy (kJ g^{-1})[c]	20.1	20.3	20.4

[a] Vitamin and mineral premix contains (as g kg^{-1} premix) vitamin A, 2,400,000 IU; vitamin D3, 480,000 IU; vitamin E, 50,000 IU; thiamine, 2; riboflavin, 4; pyridoxine, 3; pantothenic acid, 8; biotin, 0.2; niacin, 30; folic acid, 1.2; cyanocobalamin, 0.006; ascorbic acid, 30; menadione, 1.6; sodium selenite, 0.06; potassium iodide, 0.35; cupric sulfate pentahydrate, 1; zinc sulfate heptahydrate, 14; manganese sulfate monohydrate, 10; ferrous sulfate, 20; cobalt chloride, 0.4

[b] NFE = total − moisture − CP − EE − ash

[c] Calculated on the basis of 23.6, 39.5, and 17.2 kJ g^{-1} of CP, EE, and NFE, respectively (NRC 1993)

mean body weight (IBW) was 0.33 ± 0.01 g. Three formulated diets were used for the experiment (CTRL, GG25, and GG100), following a randomized design of triplicates. Fish were hand-fed to apparent satiation twice a day (except on Sundays), at 10:00 and 17:00 h for 56 days (austral spring season, October to December). The weight of the feed delivered into each container was estimated weekly, in order to calculate feed intake (FI) at the end of the experiment. Special care was taken to ensure that fish had eaten all the feed. Throughout the experiment, besides food deprivation on every Sunday, once every 2 weeks, all the fish in each container were starved from Saturday 17 h to Monday 10 h, anesthetized with 100 ppm benzocaine, counted, and weighted to calculate total biomass. Dead fish were counted daily at feeding times in order to calculate percentage survival by container as 100 × final fish number / initial fish number.

Estimation of growth and feeding parameters

At the end of the experiment and 16 h after the last meal, all fish were weighed and counted to calculate the following parameters: Final body weight (FBW) was estimated by dividing total fish biomass in each container by the number of fish; thermal-unit growth coefficient (TGC) was calculated as $1000 \times (FBW^{0.209} - IBW^{0.209}) / (T \times D)$, where T is the mean daily temperature in degree Celsius (Dumas et al. 2007) and D is the number of days between measurements; feeding efficiency (FE) was calculated as $100 \times (FBW + M - IBW) / FI$ were FI

is the feed delivered between measurements (g) and M is the body weight of the dead fish. Daily feed intake (DFI) was calculated as 100 × FI × 2 / ((FBW + IBW) × D), and protein efficiency ratio (PER) was calculated as (FBW − IBW) / protein intake.

Sample collection, morphometric parameters, and liver glycogen analysis

Twenty fish per container (n = 3) were killed by benzocaine overdose and used for whole-body proximate composition analysis. Another five fish per container (n = 3) were randomly selected and killed by benzocaine overdose. These were individually weighed (g), fork length (L) was measured (cm), and their abdominal cavity was opened. The whole digestive tracts and livers were dissected and weighed (g) to calculate viscerosomatic index (VSI) as 100 × (viscera weight + perivisceral adipose tissue weight) / body weight and hepatosomatic index (HSI) as 100 × liver weight / body weight. Glycogen content was determined in liver tissue homogenates, according to the method described by Van Handel (1965) and referred to a glucose standard curve in meq Glu g^{-1} of fresh liver tissue. Fulton's condition factor (k) was calculated as 100 × fish weight / L^3.

Proximate composition and nutrient retention

Chemical analysis of formulated diets, *G. gargal* fruiting-body, and fish whole-body were performed by standard procedures according to AOAC (1990). Total nitrogen (TN) was determined using the semi-micro-Kjeldahl method and crude protein (CP) was estimated using 6.25 × TN. For mushroom analysis, a conversion factor of 4.38 was used according to Danell and Eaker (1992). However, since the composition of *G. gargal* fruiting-body could differ from that of the species studied by these authors, we also calculated CP with 6.25 as the conversion factor and included this value between parenthesis in Table 1. Ether extract (EE) was measured gravimetrically following sample extraction of 1 g in petroleum ether, using a Soxhlet apparatus. Moisture and ash were measured gravimetrically, the former after drying in an oven at 105 °C for 3 h and the latter by combustion in a muffle at 550 °C for 6 h. Nitrogen-free extract (NFE) was calculated by difference (total − CP − EE − ash − moisture content). To estimate the gross energy of each formulated diet, the corresponding CP, EE and NFE were multiplied by 23.6, 39.5, and 17.2 kJ g^{-1}, respectively (NRC 1993). Nutrient retention rates for CP, EE, and ash were calculated as the difference between nutrient content at end of the experiment minus initial content and as a percentage of nutrients offered in the diet during the experiment (Glencross et al. 2007).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). When appropriate, normal distribution and homogeneity of variance were checked by Kolmogorov-Smirnov's and Bartlett's tests, respectively. One-way ANOVA and Tukey's post hoc comparisons were applied to identify differences among groups. Two-way ANOVA with repeated measures and Bonferroni's post-hoc comparisons were applied to identify differences in growth between diets throughout time. We considered a value of $p < 0.05$ as statistically significant (Zar 1999).

Results

Growth performance and morphometric parameters

Differences in growth among diets along time are shown in Fig. 1. Two-way ANOVA showed a statistically significant interaction of diet × day in the fish's mean weight ($p < 0.0001$). GG25 showed the highest growth rate and was significantly different from GG100 ($p < 0.05$) from day 14 onwards and from the CTRL group ($p < 0.001$) from day 28 onwards. Since day 28, the fish's weight of GG100 was significantly lower than that of CTRL ($p < 0.001$). At day 56, FBW values obtained with the different diets were 2.37 ± 0.04 g (CTRL), 4.07 ± 0.07 g (GG25), and 1.94 ± 0.06 g (GG100). The TGC calculated for GG25 (0.87 ± 0.01) was significantly higher ($p < 0.0001$) than those obtained for CTRL (0.64 ± 0.01) and GG100 (0.56 ± 0.01).

No significant differences among diets were detected in fish survival percentage and Fulton's *k* (Table 3). The VSI obtained for GG25 (13.7 ± 0.3%) was significantly lower than those for CTRL and GG100, both near 16% ($p < 0.01$; Table 3). The HSI obtained for GG supplemented diets (1.48 ± 0.03% for GG25 and 1.65 ± 0.04% for GG100) were both significantly lower than that obtained for CTRL (1.92 ± 0.03%; $p < 0.001$; Table 3). Liver glycogen was significantly higher ($p < 0.05$) in GG25 (13.4 ± 2.7 meq Glu g^{-1}) than in CTRL and GG100 (4.3 ± 1.1 and 4.3 ± 1.8, respectively; Table 3).

Feeding performance, proximate analysis, and nutrient retention ratio

All the evaluated feeding parameters (FE, DFI, and PER) were significantly different among diets (Table 3). FE shows significant differences ($p < 0.05$) among groups with values of 94.2 ± 1.2, 64.5 ± 0.7, and 58.0 ± 1.6% for GG25, CTRL, and GG100 respectively. The DFI value was lowest (3.23 ± 0.03%) for GG25 and was significantly different ($p < 0.001$) from both CTRL (4.21 ± 0.06%) and GG100 (4.39 ± 0.15%). The highest value of PER was 2.19 for the GG25 group and was significantly different ($p < 0.01$) from both CTRL (1.52) and GG100 (1.34).

The whole-body proximate composition analysis showed significant differences in CP, EE and moisture content among groups (Table 4). The CP value obtained for GG25 (142.9 ± 1.0 g kg^{-1} body weight) was significantly higher ($p < 0.005$) than the values obtained

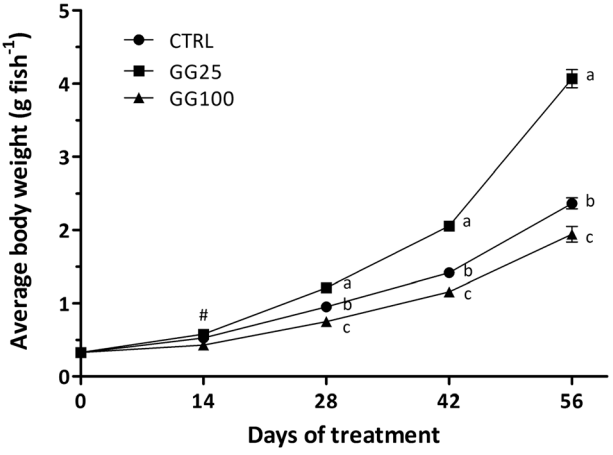

Fig. 1 Growth curve of *Oncorhynchus mykiss* fed with *Grifola gargal* supplemented (GG25-GG100) or basal (CTRL) diets, during 8 weeks. The total biomass of three containers per diet was evaluated at different days and the average body weight calculated. Values are expressed as mean ± SD of three replicates. *Number sign* indicates a significant difference between GG25 and GG100 ($p < 0.05$). *Different letters* at same days indicate significant differences among diets ($p < 0.001$)

Table 3 Morphometric parameters, feeding performance, and liver glycogen analysis of *O. mykiss* fed with *G. gargal* supplemented (GG25-GG100) or basal (CTRL) diets, during 8 weeks

	CTRL	GG25	GG100	ANOVA *p*
Survival %	98.7 ± 0.7	98.7 ± 0.7	98.0 ± 1.2	0.824
Fulton's *k*	1.20 ± 0.03	1.24 ± 0.02	1.22 ± 0.01	0.515
VSI %	15.9 ± 0.6 a	13.7 ± 0.3 b	16.1 ± 0.1 a	<0.01
HSI %	1.92 ± 0.03 a	1.48 ± 0.03 b	1.65 ± 0.04 c	<0.001
Glycogen[a]	4.3 ± 1.1 a	13.4 ± 2.7 b	4.3 ± 1.8 a	<0.05
FE %	64.5 ± 0.7 a	94.2 ± 1.2 b	58.0 ± 1.6 c	<0.0001
DFI %	4.21 ± 0.06 a	3.23 ± 0.03 b	4.39 ± 0.15 a	<0.0005
PER	1.52 ± 0.02 a	2.19 ± 0.03 b	1.34 ± 0.03 c	<0.0001

Data are presented as mean values ± SEM of three replicates. Different lowercase letters in the same row represent significant differences among groups by Tukey's test ($p < 0.05$)

VSI viscerosomatic index, *HSI* hepatosomatic index, *FE* feeding efficiency, *DFI* daily feed intake, *PER* protein efficiency ratio.

[a] meq Glu g^{-1} of wet liver

for CTRL (133.9 ± 2.5) and GG100 (131.0 ± 1.3). EE for GG25 was 75.3 ± 1.5 g kg^{-1} and was significantly higher than the values of 42.3 ± 2.6 and 43.0 ± 2.1 g kg^{-1} obtained for CTRL and GG100, respectively ($p < 0.001$).

The nutrient retention ratios for CP, EE, and ash were significantly different among groups. EE retention for GG25 (41.8 ± 0.4%) was significantly higher ($p < 0.001$) than those obtained for CTRL (16.1 ± 0.7%) and GG100 (15.6 ± 1.1%). CP and ash retention also resulted significantly higher when GG25 diet was administered ($p < 0.05$). These results are shown in Table 4.

Discussion

Our results show that the administration of 25 g kg^{-1} of the mushroom *G. gargal* as a dietary supplement for juvenile rainbow trout produces an outright improvement of growth performance, lipid content, and nutrient retention. This is the first study on fish to show such effects using mushroom's whole fruiting-body. It is important to notice that the TGC value obtained for fish fed with CTRL diet lies within the range considered normal for *O. mykiss* smaller than 20 g (Dumas et al. 2007).

Table 4 Whole-body proximate composition analysis and nutrient retention rates of *O. mykiss* fed with *G. gargal* supplemented (GG25-GG100) or basal (CTRL) diets, during 8 weeks

	Initial	CTRL	GG25	GG100	ANOVA *p*
Moisture (g kg^{-1})	840.0	793.0 ± 8.0 a	752.7 ± 1.3 b	794.3 ± 2.8 a	<0.001
CP (g kg^{-1})	110.0	133.9 ± 2.5 a	142.9 ± 1.0 b	131.0 ± 1.3 a	<0.005
EE (g kg^{-1})	25.9	42.3 ± 2.6 a	75.3 ± 1.5 b	43.0 ± 2.1 a	<0.0001
Ash (g kg^{-1})	14.1	24.1 ± 1.3	23.9 ± 1.0	23.1 ± 0.5	0.6922
CP retention (%)		21.0 ± 0.1 a	31.9 ± 0.3 b	18.2 ± 0.5 c	<0.0001
EE retention (%)		16.1 ± 0.7 a	41.8 ± 0.4 b	15.6 ± 1.1 a	<0.0001
Ash retention (%)		9.6 ± 0.3 a	14.5 ± 0.3 b	9.3 ± 0.2 a	<0.0001

Data are presented as mean values ± SEM of three replicates. Different lowercase letters in the same row represent significant differences among groups by Tukey's test ($p < 0.05$)

CP crude protein, *EE* ether extract

Besides the changes observed in growth, dietary supplementation with *G. gargal* at 25 g kg^{-1} induces modifications in the trout's whole-body composition. The main change was an increment in the total lipid content (assessed as EE, from 42.3 g kg^{-1} in CTRL to 75.3 g kg^{-1} in GG25). This increase coincides with a significant reduction of the viscerosomatic index, suggesting that the increase in lipid accumulation due to this treatment occurs mainly in the carcass. We have also observed an important increase in liver glycogen in fish fed with the GG25 diet. Liver glycogen represents an important energy reserve, which could be mobilized to meet increased energy demands under stressful conditions, such as handling, food deprivation, or transfer to sea water (Morales et al. 1990; Soengas et al. 1991; Vijayan and Moon 1992).

In contrast to GG25, GG100 diet has a negative effect on rainbow trout growth rate, which suggests that the supplementation dose of 100 g kg^{-1} is excessive. However, we have not detected any significant changes in fish survival nor in Fulton's *k*, which allows us to presume the lack of acute toxic effects with this dose. In this sense, Postemsky et al. (2011) have reported that *G. gargal* from different sources has no evident toxic or genotoxic effects. The daily feed intake (DFI) is similar between GG100 and CTRL, which suggest that the *G. gargal* fruiting-body's characteristic strong anise-almond odor (Rajchenberg 2002; Harada et al. 2015a) did not affect feed palatability.

Fish fed with *G. gargal* show significantly lower hepatosomatic index (HSI) than CTRL fish. This suggests a positive effect of *G. gargal* supplements on liver metabolism, since HSI correlates positively with vitamin E deficiency in *O. mykiss* (Pearce et al. 2003; Puangkaew et al. 2005) and with hepatic toxicity in other fish (Chien and Hwang 2001; Wolf and Wolfe 2005; Bervoets et al. 2009).

The inclusion of *G. gargal* at 25 g kg^{-1} contributes to improve fish feeding parameters. DFI was reduced from 4.2% in the CTRL to 3.2% in GG25, which means economizing foodstuffs. Feeding efficiency was significantly increased, although this result should be considered with care since the CTRL value was lower than the expected (Woynarovich et al. 2011). An improvement in feed utilization in the GG25 group was also observed in nutrient retention rates and protein efficiency ratio (PER). Particularly, the increased PER suggests that this level of *G. gargal* supplementation improves protein assimilation (Hoffman and Falvo 2004).

In conclusion, dietary supplementation with *G. gargal* fruiting-body at 25 g kg^{-1} improves growth performance, lipid content, and feed utilization parameters, which are of great importance for the fish farming industry. These findings contribute to build on a cornerstone of aquaculture research, which is the search of new ingredients and dietary supplements to enhance nutritional quality and to position edible mushrooms as suitable candidates.

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