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The fungal alternative: Insights on medicinal mushrooms-based protein-rich biomasses by submerged fermentation of agro-industrial by-products

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ABSTRACT

Among the alternative proteins aimed at replacing those of animal origin, fungal proteins stand out as a promising resource capable of meeting environmental, health, and ethical demands. Fungal biomasses, or mycoproteins, are indeed rich in proteins and other macro- and micronutrients, while low in fats. However, their production is an ongoing challenge. This study focused on submerged fermentation, a highly controllable process that can couple high yields and agro-industrial by-products exploitation as growth media.

Five medicinal mushrooms (*Ganoderma resinaceum*, *Pleurotus ostreatus*, *Cordyceps militaris*, *Pleurotus eryngii*, and *Lentinula edodes*) were tested for their biomass growth, protein content, and antioxidant properties on several agro-industrial by-product-based media. Among the experimental lines, the highest biomass production and protein content (51%) were achieved in *P. eryngii* grown in black solider fly (*Hermetia illucens*) exuviae-media. Concerning the antioxidant properties, the production of fungal extracts through microwave-assisted maceration was as performing as, if not better than, ethanolic extraction.

1. Introduction

Despite animal-derived proteins are characterised by high nutritional value because of their amino acid profiles and high digestibility, consumption of meat and its derivates have several drawbacks, such as health concerns (*e.g.* cardiovascular diseases, cancer inducing) and severe environmental problems, namely high-water consumption, production of greenhouse gases, deforestation, and species extinction (Ritchie, Rosado, & Roser, 2019). Throughout history, several other protein sources have been used, which today are acquiring greater importance in terms of environmental sustainability, such as plants (of which legumes first), seaweeds, algae, insects, and fungi (Kurek et al., 2022). Mushrooms, which belong to the kingdom *Fungi*, have been part of the culinary and medicinal tradition of many cultures around the world for a long time, and trades and globalisation sped up exchanges among those. Worldwide mushroom production has seen an ever-increasing production rate since early 2000. Indeed, it has grown nearly ten times as global cultivation area and five times as production in the last two decades, with China as global leader for both. Global mushroom industry was valued 63 bln USD in 2013, divided mainly in edible, medicinal and wild mushrooms (54%, 38%, and 8%, respectively) (Royse, Baars, & Tan, 2017). The term 'edible mushrooms' refers to the carpophores of several species of higher fungi, mostly belonging to the phylum Basidiomycota, both cultivated and wild. Among the cultivated, *Agaricus bisporus* (J.E. Lange) Imbach, *Lentinula edodes* (Berk.) Pegler, and *Pleurotus ostreatus* (Jacq.) P. Kumm. are the most renowned, and *Boletus edulis* Bull. for the wild ones.

From a nutritional perspective, mushrooms exhibit a comprehensive

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array of qualities that define them as a wholesome and well-rounded dietary choice. In terms of fat content, mushrooms are characterised by low-fat levels, which generally range from 1% to 16% on a dry weight (dw) basis. Notably, most of these fats consist of unsaturated fatty acids (*e.g.* linoleic and linolenic acid), up to 82.5% of total fat content (Pedneault, Angers, Gosselin, & Tweddell, 2008; Valverde, Hernández-Pérez, & Paredes-López, 2015). Coupled with the absence of cholesterol, mushrooms are highly suitable for diets destined to people affected by hyperlipidemia and dietary supplements.

Fungi are characterised by a wide range of protein content, up to 45% of the total dry composition, as reported by Finnigan et al. (Finnigan, Needham, & Abbott, 2017). Moreover, fermented fungal biomasses high in proteins, often referred as mycoproteins, are a promising source of essential amino acids (EAAs, nearly 42% in some fungal strains) able of positively trigger myogenesis, suggesting their potential suitability as an ingredient in the diet of athletes and individuals recovering muscle tone lost during their hospitalisation or as a result of illnesses (Monteyne et al., 2020). When it comes to edible mushrooms, their protein content exhibits significant variation. Indeed, a study by Valverde et al. reporting the proximate compositions of several edible mushrooms, shows protein contents ranging between 4.5% and 37.4% dw (Valverde et al., 2015). Additionally, research on energy intake has revealed that meals rich in mycoprotein have frequently resulted in reduced appetite and enhanced control over insulin and glucose levels in obese patients. However, it is important to note that this area of study is currently undergoing extensive investigation (Bottin et al., 2016). Mushrooms are also considered an 'high in fibre food' by the EU Commission (one third chitin, or poly-*N*-acetyl glucosamine, and two thirds β-glucans) and contain vitamins (B9, B12), minerals (Ca, P, Mn, Zn), and choline, while low in sodium (Derbyshire & Delange, 2021). Again, fibre content varies widely, ranging from 6.20% to 54.12% dw as Salamat et al. reported (Salamat, Shahid, & Najeeb, 2017).

Concerning the environmental sustainability, the production of fungi and edible mushrooms does not face the challenges associated with livestock farming and high protein-content plant crops, making them a promising candidate for a novel, protein-rich, and environmentally friendly food source. For instance, greenhouse gases production per kg of mycoprotein-based products is up to ten time lower than beef's (Majumder, Miatur, Saha, & Hossain, 2024) and requires a minor fraction of the lands needed to livestock and crop farming (Ritchie et al., 2019). Finally, ethic-driven lifestyles and religious beliefs must be considered in the modern globalised world. Indeed, fungi-based foods have shown the potential for ruling the vegetarian and vegan food market, and they are considered strongly Islam, Christianity, Judaism, and Buddhism friendly products, religions that are mainly widespread among the fastest growing populations in the world (Hashempour-Baltork, Khosravi-Darani, Hosseini, Farshi, & Reihani, 2020).

As well as the edible ones, medicinal mushrooms belong mostly to the phylum Basidiomycota and have been known for a long time. The first evidence of their use for curative purposes dates to the Neolithic (Grienke, Zöll, Peintner, & Rollinger, 2014), and evidence from different eras testifies to the use of these mushrooms in different times and cultures, such as in the Ancient Egypt, the Imperial Rome, and throughout the history of the Far East, until traditional Chinese medicine still in use today (El Sheikha & Hu, 2018). Their therapeutic properties are related to the classes of bioactive compounds and the fungal species under investigation. Among these compounds, polysaccharides, terpenes, phenols, and alkaloids are the more active and reported in literature (El Sheikha & Hu, 2018). Concerning their therapeutic value, medicinal mushrooms are under investigation for being potentially able to treat rheumatoid arthritis, cardiovascular and neurodegenerative diseases, stimulate or suppress the immune system and the DNA repair mechanisms, and then for several other applications (*e.g.* anticancer, antidiabetic, antiviral, antiaging, antiobesity, antimicrobial) (Gargano et al., 2017). Despite there is a plethora of mushrooms with proven bioactive compounds, 85% of the global mushroom production is focused on five genera, namely *Lentinula* Earle, *Pleurotus* (Fr.) P. Kumm. [*P. ostreatus*, *P. eryngii* (DC.) Quél., *P. djamor* (Rumph. ex Fr.) Boedijn, *P. pulmonarius* (Fr.) Quél., and *P. citrinopileatus* Singer], *Auricularia* Bull., *Agaricus* L. (*A. bisporus* and *A. brasiliensis* Wasser, M. Didukh, Amazonas & Stamets), and *Flammulina* P. Karst., (22%, 19%, 17%, 15%, 11%, respectively) (Royse, 2014). Other cultivated mushroom species, either medicinal or edible, are *Ganoderma lucidum* (Curtis) P. Karst., *Volvariella volvacea*, *Hericium erinaceus* (Bull.) Pers., *Grifola frondosa* (Dicks.) Gray, and *Trametes versicolor* (L.) Lloyd.

According to the 2008/98/EC Waste Framework Directive of the European Parliament and of the Council, "waste means any substance or object which the holder discards or intends or is required to discard" (Art 3.1) while a by-product is "a substance or object, resulting from a production process, the primary aim of which is not the production of that item, may be regarded as not being waste (Art 5.1) (European Parliament, 2008). Thus, this distinction opens up the possibility of exploiting by-products from the agro-industrial sector to grow mushrooms at reduced costs and supporting the idea of circular economy, in particular by creating wealth from local 'discards'. Fungi can grow on many substrates, and in the last years researchers have been able to cultivate mushrooms on several agro-industrial by-products successfully (Supplementary Table 1). The loss of these by-products and food wastes, corresponding to 1.3 billion tons or 1 trillion USD (United Nations Environment Programme (UNEP), 2021), forms an immense pool of potential growth substrates, rich in nutrients and low cost, to support the fungal production thanks to microbial fermentations.

There are two main types of fermentation widely used, namely the solid-state fermentation (SSF) and the submerged fermentation (SmF) having complementary pros and cons. Indeed, SSF is characterised by higher product yield, lower energy and expenses requirement, very low moisture content (which implies a lower risk of contamination) and it uses solid by-products as growth substrate (Cerda et al., 2019). Historically SSF has been used to produce traditional fermented foods, while nowadays is used for many applications, such as hydrolytic enzymes production (cellulases and xylanases) and mycoprotein production (Mejias, Cerda, Barrena, Gea, & Sánchez, 2018; Stoffel et al., 2019). On the other hand, SSF requires long fermentation periods, growth medium is likely to be less homogenous and its parameters cannot be controlled as the SmF ones. The SmF, whose growth medium is characterised by more than 95% water content, allows higher control of the fermentation parameters and has been widely used to produce valuable products such as enzymes, pharmaceuticals and to treat liquid wastes. Despites this, SmF implies higher production costs, higher energy input and less productivity (Astolfi et al., 2011).

This study wants to provide a deeper insight on using agro-industrial by-products as growth substrate to produce protein-rich fungal biomasses (or mycoproteins). Among them, poorly investigated substrates were also taken into consideration, to better adhere to the paradigms of the UN's Sustainable Development Goals. In particular, to the best of the authors' knowledge, some of the investigated by-products (*i.e.* insects derived by-products) were never used to sustain microbial growth before. The fungi involved will be picked among the most notorious medicinal mushrooms, to understand whether fungal fermentation can produce biomasses having not only a high protein content, but functional compounds and properties as well. Contrariwise to traditional SSF, SmF has been poorly investigated to produce valuable fungal biomass characterised by high protein content and antioxidant activity, using substrates derived from the Circular Economy supply chain.

2. Materials and methods

2.1. Fungal strain selection

Five medicinal mushrooms were selected according to literature and preliminary studies about their ability of using complex carbon and nitrogen sources to achieve high biomass production yield, together

with high nutritional quality and nutraceutical properties: *Cordyceps militaris* (MUT 6392), *Ganoderma resinaceum* (MUT 3345), *Lentinula edodes* (MUT 6391), *Pleurotus eryngii* (MUT 6390) and *Pleurotus ostreatus* (MUT 2976). All the strains are preserved at Mycotheca Universitatis Taurinensis (MUT) in Turin (Italy).

2.2. Agro-industrial by-products used in submerged fermentation

In collaboration with local agro-industrial companies, seven by-products were used to design submerged liquid fermentation media. In particular, the by-products used for the experimentation included: *Hermetia illucens* (HiE) and *Tenebrio molitor* (TmE) exuviae coming from the rearing of black solider fly and mealworm for feed purposes, peels and seeds derived from tomato processing (TB), fourth rate production by-product (IVB, mainly constituted by potatoes, carrots, and other vegetables at a lower rate).

These by-products have acquired commercial interest due to their availability or novelty on the market, and are characterised by different dry matter, crude protein, and total fat content (HiE: 94.9%, 31.7%, 13.9% (Fricke, Saborowski, & Slater, 2024); TmE: 86.8%, 32.9%, 3.6% (Ravzanaadii, Kim, Choi, Hong, & Kim, 2012); TB: 66.6%, 20.9%, 14.1% (Silva et al., 2019), respectively).

Other than these by-products, three different cereals-derived vinasse syrups were added to this study, namely CBA, CBB and CBC, which are differently high in either carbon or nitrogen content (355 g/L [C] and 0.028 g/L [N], 311 g/L [C] and 0.032 g/L [N], 67 g/L [C] and 62.89 g/L [N], respectively).

All these by-products were used in different concentrations, either as unique sources of nutrients or in combinations, to establish twenty-two experimental lines. Potato Dextrose Broth (PDB) and Malt Extract (ME) were chosen as positive controls (Table 1), while Peptone was added in specific lines as additional nitrogen source (reported as 'N', Kjeldahl total nitrogen content 11–14%). All the chemicals were purchased from Sigma-Aldrich (Merck Group KGaA, Darmstadt, Germany).

2.3. Fungal pre-growth, inoculum, and fermentation conditions

The five fungi were pre-grown on malt extract agar plates, from which the biomasses were collected with sterile scalpels. Having the concentration of 1 cm^2 per mL, they were homogenised in deionised sterile water. Regardless the growth substrate and for both the following screenings, 5 mL of the inoculum were aliquoted in 500 mL flasks, having 300 mL of substrate each. Inoculated flasks were incubated for 21 days at 24 °C in stirred conditions (120 rpm), and all culture lines were performed in triplicates for statistical purposes, both in the following first and second screening.

2.4. First and second screening

The 24 experimental lines (Supplementary Table 2) underwent a first screening, to pick the most suitable ones in term of matrix concentration, general fermentation conditions and produced biomass (in grams of dried fungal biomass produced per L of media, or g/L). As representative of the five medicinal mushrooms, *G. resinaceum* (MUT 3345) was randomly chosen for this step and was inoculated in SmF conditions. End of fermentation biomasses were collected, sieved, lyophilised and stored at −20 °C.

From the first screening, nine out of twenty-four lines were chosen for the following step: ME (20 g/L), HiE (30 g/L for *G. resinaceum*, *P. eryngii*, *L. edodes* and *C. militaris*, 20 g/L for *P. ostreatus*), TB (30 g/L), IVB (30 g/L), CBA (20 g/L) + N (2 g/L), CBA (20 g/L) + CBC (2 g/L), PDB (24 g/L), TmE (10 g/L), CBA (20 g/L) + IVB (20 g/L). Despite no biomass production, 10 g/L TmE and 30 g/L TB were included because interesting due to the chosen fungi (entomopathogenic fungus *C. militaris*), their availability on the market and their peculiar nutritional composition (*i.e.* lycopene). In particular, because of the lightness of *T. molitor* exuviae, only the 10 g/L concentration was considered suitable for SmF. Then, all the five mushrooms of this study were grown in SmF conditions on these substrates to highlight the most productive mushroom-substrate combinations.

Table 1
Culture lines (22) and positive controls (2, in bold) used in the first screening.

N°	Culture media	N°	Culture media
1	**ME (20 g/L)**	13	IVB (20 g/L)
2	**PDB (24 g/L)**	14	IVB (30 g/L)
3	HiE (10 g/L)	15	CBA 20 g/L
4	HiE (20 g/L)	16	CBA (20 g/L) + N (2 g/L)
5	HiE (30 g/L)	17	CBA (20 g/L) + CBC (2 g/L)
6	TmE (10 g/L)	18	CBB (20 g/L)
7	TmE (20 g/L)	19	CBB (20 g/L) + N (2 g/L)
8	TmE (30 g/L)	20	CBB (20 g/L) + CBC (2 g/L)
9	TB (10 g/L)	21	CBC (10 g/L)
10	TB (20 g/L)	22	CBC (20 g/L)
11	TB (30 g/L)	23	CBC (30 g/L)
12	IVB (10 g/L)	24	CBA + IVB (both 20 g/L)

2.5. Fungal fermentation yield

After harvesting the wet fungal biomasses and their lyophilisation, fungal fermentation yields were determined by gravimetric analysis, particularly by weighing the total harvested lyophilised fungal biomasses and dividing them by the volume of growth media.

2.6. Chemical composition of fungal biomass

The nutritional values of the fungi grown in the second screening were evaluated by proximate analysis, according to the guidelines of the Association of Analytical Chemistry Officers (A.O.A.C). Specifically, the protein, ash, and lipid contents of the biomasses previously lyophilised, then dried in oven (60 °C, O/N) to measure and fully eliminate any moisture left, were measured as described by Gasco et al. (Gasco et al., 2016). Total carbohydrates were calculated based on the % of the other parameters, as described by Cohen et al. (Cohen et al., 2014).

2.7. Extracts preparation from fungal biomasses

Fungal extracts intended for the assessment of antioxidant capacity were prepared from the fungal biomass obtained during the second screening phase. Conventional hydroalcoholic fungal extracts were prepared according to Zhang et al. (Zhang, Lin, & Ye, 2018), but optimised for medicinal mushrooms. Briefly, after adding 1 mL of 70% (*v*/v) ethanol to 30 mg of dry fungal mycelium, samples were homogenised using Tissue Lyser (20.9 Hz, 2 min) and macerated for 2 days at 4 °C. The same protocol was applied using deionised water as solvent. After the maceration step, extracts were centrifuged (5000 rpm, 10 min, 25 °C) to separate the supernatant from solid residue. Extracts were then stored at −20 °C until subsequent analysis.

Microwave-Assisted Extraction (MAE) was performed with the same fungal biomasses, according to Cavalluzzi et al., (Cavalluzzi et al., 2022), still optimised for medicinal mushrooms. A closed-system MAE was carried out at constant temperature under continuous stirring condition in a Discover Benchmate Microwave Synthetizer by CEM Corporation© (Charlotte, NA, USA) equipped with the software Synergy™. Briefly, 30 mg of lyophilised mycelia powder per mL of solvent (deionised water or ethanol 70%) were irradiated using microwaves at 80 °C for 5 min. The solution was then filtered through Whatman (No. 1) filter paper and centrifuged (8000 rpm, 10 min, 25 °C). Finally, supernatant was transferred into Eppendorf tubes and stored at −20 °C until subsequent analysis.

2.8. Total polyphenolic content (TPC) and ferric reducing antioxidant power (FRAP)

Total phenolic content (TPC) of the abovementioned extracts was determined by the Folin-Ciocalteu assay, according to Singleton & Rossi (Singleton & Rossi, 1965). Briefly, 5 μL of Folin-Ciocolteu reagent was added to 10 μL of 20% (*w/v*) Sodium Carbonate, 10 μL of extracts, and 80 μL of water were aliquoted in 96-well, flat-bottom microtiter plates. After incubation for 20 min in the dark and room temperature, the absorbance was read at 725 nm using a spectrophotometer (TECAN Infinite M200, Austria). For TPC quantification, gallic acid (GA) was used as a reference standard in an external calibration curve ($y = 0.3538\times + 0.019$; R^2: 0.9876; LOD: $81.197{\cdot}10^{-3}$ mg/mL; LOQ: $246.054{\cdot}10^{-3}$ mg/mL). TPC was expressed as μg GA equivalent (GAE) per 100 g dw ± standard deviation (SD).

To measure reducing activity, FRAP assay was assessed using a modified method described by Mannino et al. (Mannino et al., 2020). Briefly, 300 mM sodium acetate (VWR International, Milan, Italy) buffer (pH 3.6) were mixed to 10 mM 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) (VWR International, Milan, Italy) and 20 mM FeCl3 (VWR International, Milan, Italy) in a 8:1:1 (*v/v/v*) ratio. The assay protocol was optimised to monitor the bathochromic shift using a microplate reader, incubating 170 μL of the FRAP reaction mixture to 30 μL of ethanolic extract. After shaking, the absorbance of each well was read at 593 nm after an incubation time of 20 min at room temperature and in the dark. For determining FRAP value, Trolox was used as a reference standard in an external calibration curve ($y = 5.2828\times + 0.0069$; R^2: 0.9991; LOD: $1.12{\cdot}10^{-3}$ mg/mL; LOQ: $3.38{\cdot}10^{-3}$ mg/mL). FRAP was then expressed as mmol Trolox equivalent (TE) per 100 g dw ± SD.

2.9. Statistical analysis

Normality and homoscedasticity of the data were assessed by Shapiro–Wilk and Levene's tests, respectively. Student's paired *t*-test was used to determine the significant differences between the samples. Statistical analyses were performed using the Microsoft Office suite (Microsoft Corporation, Redmond, WA, USA) and R v.4.3.0 (R Core Team, 2023).

3. Results and discussion

3.1. First screening – 1 fungus vs 24 culture lines

C. militaris, *G. resinaceum*, *L. edodes*, *P. eryngii*, and *P. ostreatus* are well known medicinal mushrooms, and the strains we have used in this study (MUT 6392, MUT 3345, MUT 6391, MUT 6390 and MUT 2976, respectively) performed differently depending on the SmF growth substrate, leading to high amount of biomass and high protein content.

The purpose of the first step of this study was to choose the most suitable concentrations and combinations of the different by-products, to shape the best growth media in term of fermentation conditions and fungal growth. For instance, although high matrix concentration implies higher nutrient content, its excess may lead to a poor oxygen transfer in the liquid media and stuck the flask in the advanced phase of fermentation, both problems that reduce the fermentative performance and biomass yield. For this purpose, *G. resinaceum* was grown in 15 media made from five different by-products (HiE, TmE, TB, IVB and CBC) at 10, 20 and 30 g/L. Moreover, CBA and CBB were tested as unique nourishment source or in combination with extra nitrogen sources (peptone or CBC), ME and PDB were used as positive control because generally regarded as standard mycological liquid media. Finally, CBA were coupled with IVB, for a total of 24 media. The first trial results concerned fungal biomass yield of *G. resinaceum* on these twenty-four SmF culture lines, after 21 days of growth (Supplementary Table 2).

Both the two standard media used as positive controls produced fungal biomass, and ME showed nearly a five-fold higher biomass production than PDB (15.98 ± 2.58 g/L and 3.6 ± 0.13 g/L, respectively). HiE and IVB performed similarly as growth substrate for *G. resinaceum*, reporting proportionally increasing biomass production in relation to substrate concentration (for 10, 20 and 30 g/L were 4.46 ± 0.01 g/L, 7.85 ± 0.23 g/L, 11.32 ± 0.78 g/L and 3.39 ± 0.18 g/L, 7.1 ± 0.05 g/L, 13.7 ± 0.44 g/L, respectively): this is quite an expected result because of the higher availability of nutrients in the media. Anyway, this is not a general rule, because the growth was negatively influenced by rising CBC concentration (for 10 g/L was 3.36 ± 0.6 g/L, while no fungal biomass grew in the 20 g/L and 30 g/L lines), potentially because of an excess of nitrogen or poor oxygen transfer due to its density. TmE and TB did support no biomass production either. Despite this, two experimental lines made of these by-products (TB 30 g/L and TmE 10 g/L) were selected for the second screening, to maintain a second plant-based media instead keeping only IVB, which is known for its availability and cost-effectiveness (Eslami, Carpentieri, Pataro, & Ferrari, 2023).

As well, a TmE line was kept to study the unexplored behavior of the entomopathogenic fungus *C. militaris* in both the insect-based liquid media in the following step of this study. To our knowledge, this is the first investigation exploiting insect exuviae to design liquid growth media aimed to biomass production, despite the insect-fungi association is well-known, and insect exuviae have been used as bait to capture zoosporic fungi (Pavgi & Singh, 1971).

CBA-based substrates resulted in a good carbon but not nitrogen source. Indeed, it only permitted fungal growth when combined with another nitrogen-rich source, such as peptone or CBC (0.36 ± 0 g/L, 5.47 ± 0.28 g/L, and 11.23 ± 0.57 g/L for the 10, 20 and 30 g/L lines). CBB appears to be unaffected by whether a nitrogen-rich source is added or not (5.73 ± 0.38 g/L, 6.49 ± 0.19 g/L, and 4.49 ± 0.02 g/L for the 10, 20, and 30 g/L lines). Nitrogen addition/depletion is fundamental in medium engineering because it can heavily influence either the biomass or metabolite production, as well as influencing the overall cost of the growth medium (Premalatha, Vijayalakshmi, Shanmugavel, & Rajakumar, 2023), and these results confirm that different nitrogen sources, and their different concentrations, should be taken into consideration while designing experiments involving medium engineering. Finally, CBA + IVB gave the best result overall, higher than the positive control ME (19.34 ± 4.7 g/L and 15.98 ± 2.58 g/L, respectively).

Based on the results and observations of the first step, the following nine growth media were chosen for the second step: HiE, TB, IVB (30 g/L), CBA + N, CBA + CBC (both 20 g/L + 2 g/L), CBA + IVB (20 g/L + 20 g/L), TmE (10 g/L), ME (20 g/L) and PDB (24 g/L).

3.2. Second screening – 5 fungi vs 9 culture lines

The second screening results demonstrated that in SmF most of fungi used were able to use agro-industrial by-products as nutrient source. In several cases they reached, or exceeded, biomass production obtained using standard commercial culture media, ME and PDB (Fig. 1 and Supplementary Table 3).

Among the growth media assessed, only IVB and CBA + IVB led to significant biomass yields for all the medicinal mushrooms in this study. Compared to the other by-product-based media, which gave one or more no biomass production, IVB alone yields were 17.8 ± 1.0 g/L for *P. eryngii*, 15.4 ± 1.1 g/L for *P. ostreatus* (the highest observed for this strain) 14.3 ± 0.4 g/L for *C. militaris*, 13.6 ± 0.4 g/L for *G. resinaceum* and 7.6 ± 1.3 g/L for L. *edodes*. CBA + IVB performed similar or even better than IVB alone, suggesting that the additional carbon source helped the strains growth: 23.6 ± 1.0 g/L for *P. eryngii* (the third highest yield overall), 19.3 ± 0.3 g/L for *G. resinaceum* (the highest for this strain) 13.4 ± 5 g/L for *P. ostreatus*, 13 ± 2 g/L for *C. militaris* and 10.9 ± 2.7 g/L for *L. edodes*. Noteworthy, CBA + IVB was the only media yielding more than 10 g/L of biomass for all the fungi in the work. Such results are higher than ones obtained on five fungal strains grown in SmF using a culture media based on potato peels (*Solanum tuberosum*),

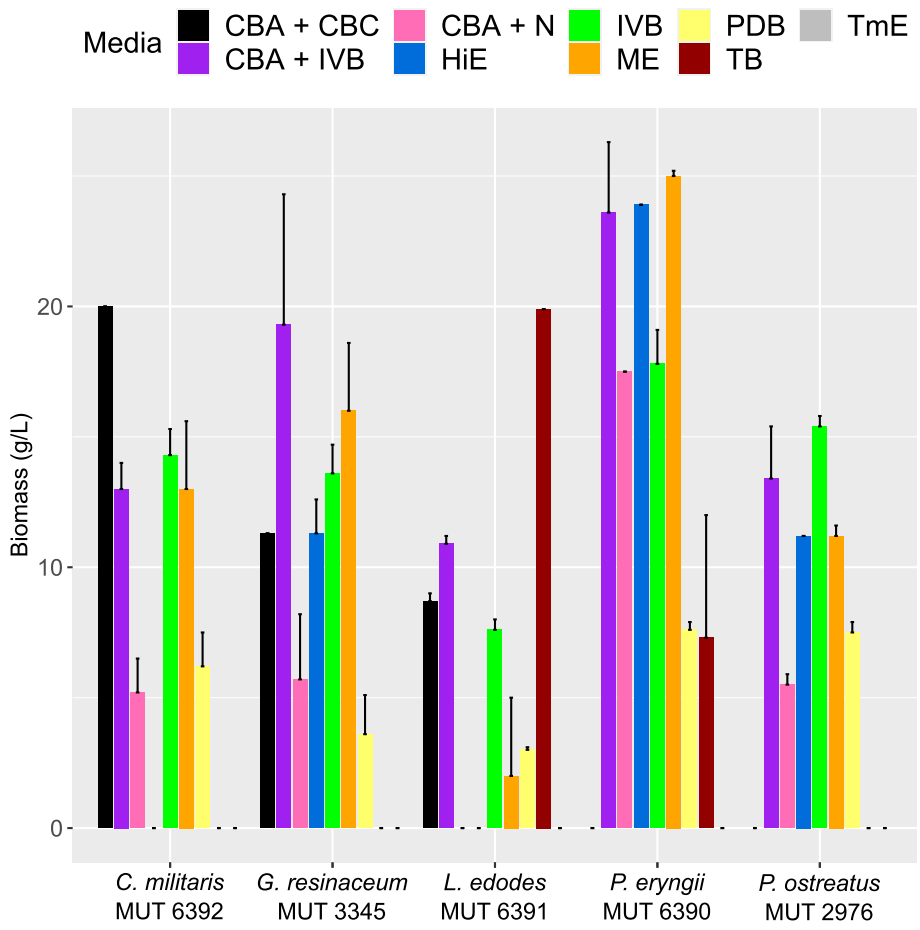

Fig. 1. Second Screening Biomass Production - Biomass production (g/L d.w.) in the second screening, in which nine media selected from the first screening were used to sustain the growth of five fungal strains. ME, HiE, TB, IVB, CBA + N, CBA+ CBC, PDB, TmE, and CBA + IVB stand for Malt Extract, *Hermetia illucens* exuviae, Tomato By-product, fourth rate vegetable discards, cereal-derived vinasse A supplemented with N (peptone), cereal-derived vinasse A supplemented with cereal-derived vinasse C, Potato Dextrose Broth, *Tenebrio molitor* exuviae, and cereal-derived vinasse A supplemented with fourth rate vegetable discards as growth media, respectively.

peptone, and dextrose. In such work, *Ganoderma lucidum* and *Pleurotus spp.* yielded around 8 g/L and 14 g/L, respectively (Mshandete & Mgonja, 2009). ME and PDB, the two positive control media, gave positive biomass yields for all the mushrooms either, but PDB was always lower than IVB and CBA + IVB, while ME being clearly better than IVB only when supporting *P. eryngii* growth (25 ± 2.6 g/L and 17.8 ± 1.0 g/L, respectively).

HiE performed brilliantly as growth media for *P. eryngii*, *G. resinaceum,* and *P. ostreatus*: 23.9 ± 0.9 g/L (second highest yield overall, first if not considering the control ME), 11.3 ± 1.0 g/L and 11.2 ± 1.3 g/L, respectively. On the other hand, *L. edodes* and *C. militaris* did not grow in HiE. It is interesting that the latter, an entomopathogenic fungi, did not grow on this substrate, as well did not in TmE (in which no fungi grew). Literature does not report data about fungi grown on insect exuviae in SmF conditions and therefore, this work is the first which tested fungal ability to grow on insect exuviae. *C. militaris* inability to grow on HiE and inability of all fungi to grow on TmE should be more investigated and it may depend on several factors such as absence of nutrients or presence of culture inhibitors, as shown by Canteri de Souza et al. *(*Canteri de Souza, Custódio Caloni, Wilson, & Sergio Almeida, 2018*)*.

TB was not a good culture substrate for most of the fungi tested, except for L. *edodes*, whose substrate offered the best condition for biomass production and production rate was 19.9 ± 4.7 g/L. In general, *L. edodes* has been the least performing fungus, not growing on three out of nine media (HiE, CBA + N and TmE) and usually recording the lowest yields for the others (particularly in the control ME, 2 ± 0.2 g/L). The inability of three out of five mushrooms (*G. resinaceum, P. ostreatus* and *C. militaris*) to grow on TB remains unexplained in this study, therefore requiring further deeper investigations.

CBA (high carbon content) was used combined with N (peptone) or CBC, both high in nitrogen. Fungi showed preferences for the additional nitrogen source. In fact, CBA + N supported the growth of more fungi, but with lower yields (5.7 ± 0.3 g/L for *G. resinaceum*, 5.5 ± 2.5 g/L for *P. ostreatus*, 5.2 ± 0.4 g/L for *C. militaris*, 17.5 ± 1.3 g/L for *P. eryngii*, none for L. *edodes*), opposite of CBA + CBC (11.3 ± 0.3 g/L for *G. resinaceum*, 20 ± 1.4 g/L for *C. militaris*, 8.7 ± 1.5 g/L for *L. edodes*). It is interesting to notice that the two *Pleurotus* did not grow on CBA + CBC, thus CBC composition and potential *Pleurotus*-specific antinutritional compounds should be investigated. Moreover *C. militaris* reached its highest yield on this substrate, result supported by similar work, in which molasses was used as culture media for its relative *Cordyceps sinensis* (Cha et al., 2007).

Additionally, the role of substrate has been demonstrated as the key to modulate proteins, lipids, carbohydrates, and ash production of each fungal strain, as shown in several papers published in literature (Zurbano, Bellere, & Savilla, 2017).

As interesting remarks regarding one fungal strain, *P. eryngii* has been the best performing fungus overall, displaying the highest biomass production on six out of nine media. In particular, *P. eryngii* grown in ME, HiE and CBA + IVB are the only three combinations which broke the 20 g/L biomass yield value among all the tested fungi.

3.3. Proximate analysis of fungal biomass

The proximate analysis of fungal biomasses was carried out only when the productivity was over a threshold limit value of 5 g/L, and it was conducted in duplicate using 10 g of lyophilised, then successively dried, fungal biomass (Fig. 2 and Supplementary Table 4).

First notable finding of this study is the high inter- and intraspecific diversity, either in biomass yield or protein content dry weight, related with the used growth media. In fact, the same mushroom, *Pleurotus eryngii*, gave the lowest and highest protein content among all the experimental lines assessed, ranging 5.385 ÷ 51.025 g/100 g (grown in CBA + IVB and HiE, respectively). Concerning the growth substrates, HiE gave the three highest raw protein contents (40.95% in *G. resinaceum* and *P. ostreatus*, 51.03% in *P. eryngii*) but produced no biomass for *C. militaris* and L. *edodes*. These percentages endorse the use of mushrooms as a valid alternative protein source. In fact, *P. eryngii* grown in HiE has similar protein content to beef, chicken, and eggs (51.5, 51.3, and 52.6 g/100 g dw, respectively), while higher than pork (40.7 g/100 g dw) and other plant-based protein sources such as soybean and beans (39.9 g/100 g dw and 26.7 g/100 g dw, respectively). Only seafood and algae can be characterised by significantly higher protein contents (tuna 74.8 g/100 g dw, mussels 61.3 g/100 g dw, and the cyanobacterium *Arthrospira platensis* or spirulina 65.4 g/100 g dw). However, the latter is used as a dietary supplement, not as food itself (Parodi et al., 2018).

As abovementioned, TmE did produce no biomass in all the experimental lines. To our knowledge, this is the first study in literature which uses insect exuviae as growth substrate for fungi: although they are both exuviae-based, the difference between the performances of these two substrates outlines once again how important is the right choice of fungal strain-substrate combination. Moreover, the inability of the entomopathogenic *C. militaris* to grow in these media strengthen the idea that insects are an interesting but complex and still unpredictable growth substrate for fungal growth, therefore worthy of further study.

It is interesting that, in the lines that proceeded in this phase, the lowest protein contents were always retrieved from CBA + IVB (8.86% for *G. resinaceum*, 11.22% for *P. ostreatus*, 6.85% for *C. militaris*, 5.39% for *P. eryngii*, 10.38% for L. *edodes*), although it was the only substrate that worked with all the mushrooms with IVB, which gave the second lowest response in general. Despite their broad versatility as growth substrate for the tested mushrooms, the protein content of IVB and CBA + IVB did not reach the average protein content of common meats (Parodi et al., 2018), while CBA + IVB contents were always slightly lower than IVB ones. Given these results, we suggest the use of plant-derived by-products to grow very different mushrooms on the same medium or for early screenings, but they need further optimisation if taken to more advanced stages of work.

Joining the data of biomass production and protein content, raw protein yield per growth substrate liter was calculated for each experimental line which underwent the second step. This is fundamental on an industrial point of view because high protein contents need to be coupled with high biomass yield, otherwise the process may become economically unsustainable. As shown in Fig. 3, the more promising growth substrate is among the less explored so far, the *Hermetia illucens* exuviae. When it supported fungal growth, it has been the only substrate to yield more than 4 g/L of protein: for *G. resinaceum* 4.63 ± 0.409 g/L, for *P. ostreatus* 4.59 ± 0.532 g/L, and for *P. eryngii* 12.19 ± 0.459 g/L. Assuming no change in the latter yield during a hypothetical industrial

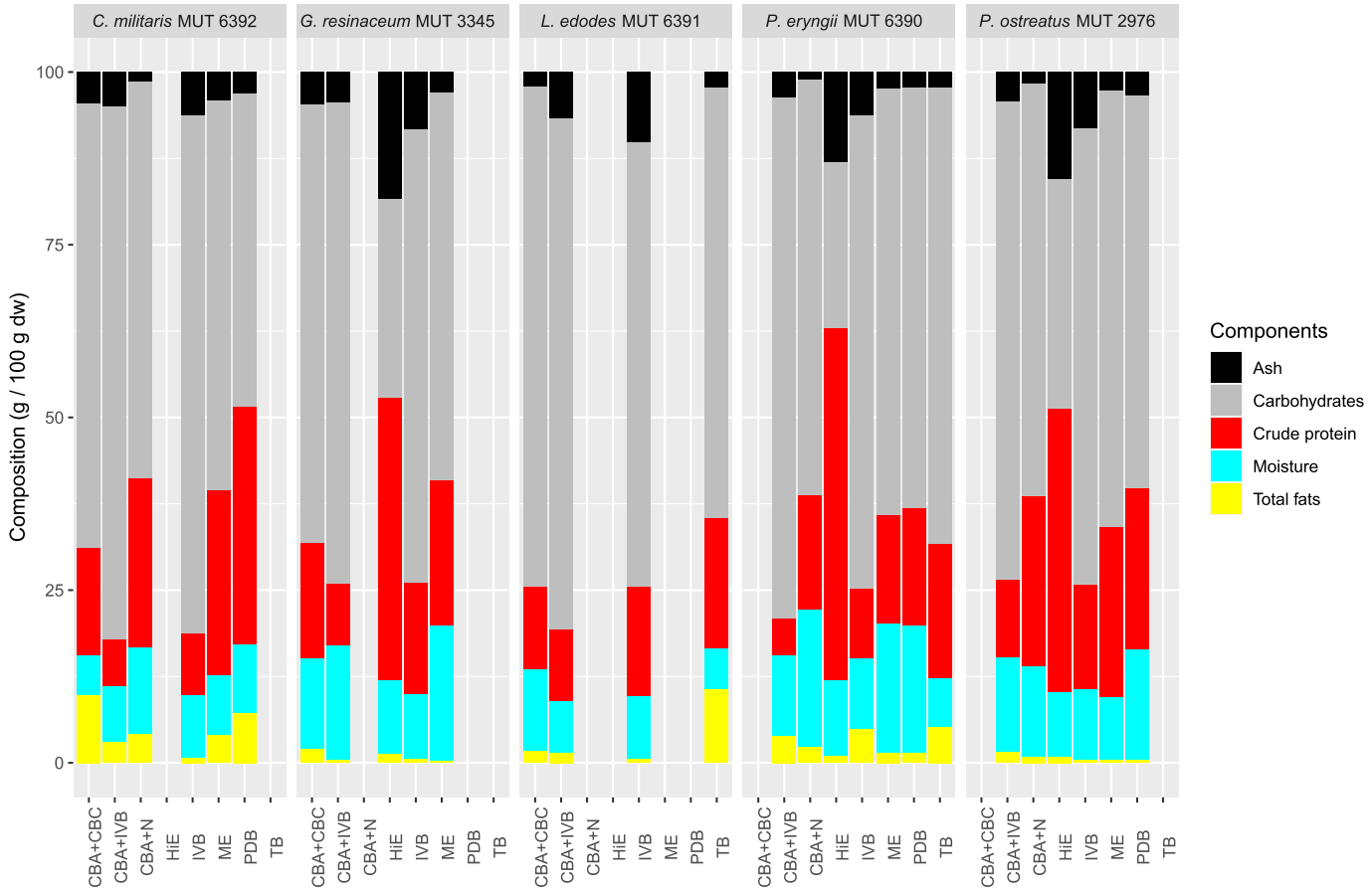

Fig. 2. Proximate analysis of the five mushrooms - Proximate analysis of the fungal biomasses harvested in the second screening (g per 100 g of lyophilised and dried sample). Gr, Po, Cm, Pe, and Le stand for *G. resinaceum* (MUT 3345), *P. ostreatus* (MUT 2976), *C. militaris* (MUT 6392), *P. eryngii* (MUT 6390) and *L. edodes* (MUT 6391) respectively.

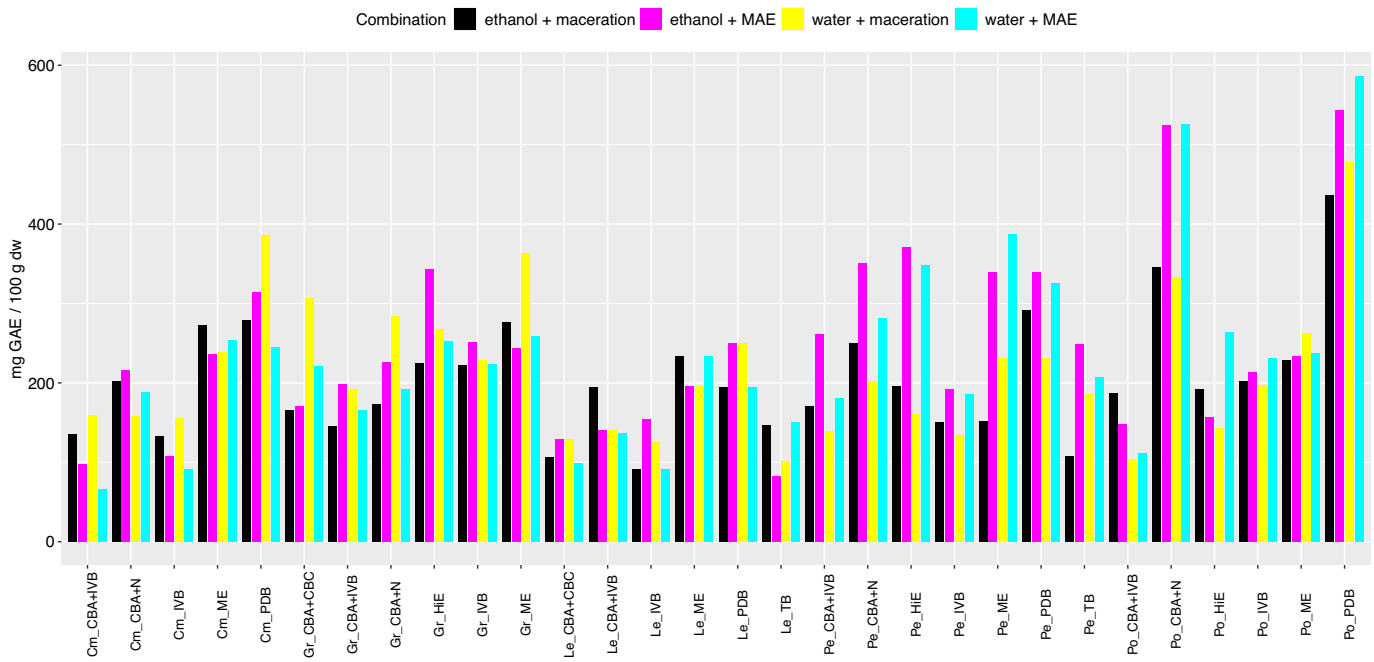

Fig. 3. Protein yield per media volume - Raw protein content per media volume (g/L). Its calculation was done by multiplying the components percentages from the proximal analysis per biomass yields (Supplementary Table 6).

scale-up, 82.03 L of water and 2.46 kg of *H. illucens* exuviae would be required to produce 1 kg of *P. eryngii* raw protein. Given this protein content, and that 15,497 L of water are required to produce 1 kg of beef meat (Schlink, Nguyen, & Viljoen, 2010), the water required to produce 1 kg beef protein would be 30,091 L, roughly 366 times more than the *P. eryngii* used during this study.

Although there is still a lot to understand about media engineering, the exuviae potential and the settings for fungal fermentations, we consider these data as a promising success. In fact, achieving such low water consumption without a proper optimization phase sparks hope for further reducing water consumption in protein production, then highlighting mycoproteins as a viable, desirable, and sustainable alternative protein source.

Finally, carbohydrate content ranged 23.96 to 77.08% (in *P. eryngii* and *C. militaris,* respectively), in agreement with average carbohydrates content in several common edible/medicinal mushrooms (Cohen et al., 2014), while the ether extract/total fat ranged from 0.315% to 10.63% (in *P. ostreatus* and L. *edodes,* respectively), and the ash content varied from a minimal value of 1225 g/100 g for *P. eryngii* grown in CBA + N to 18.325 g/100 g for *G. resinaceum* grown in HiE.

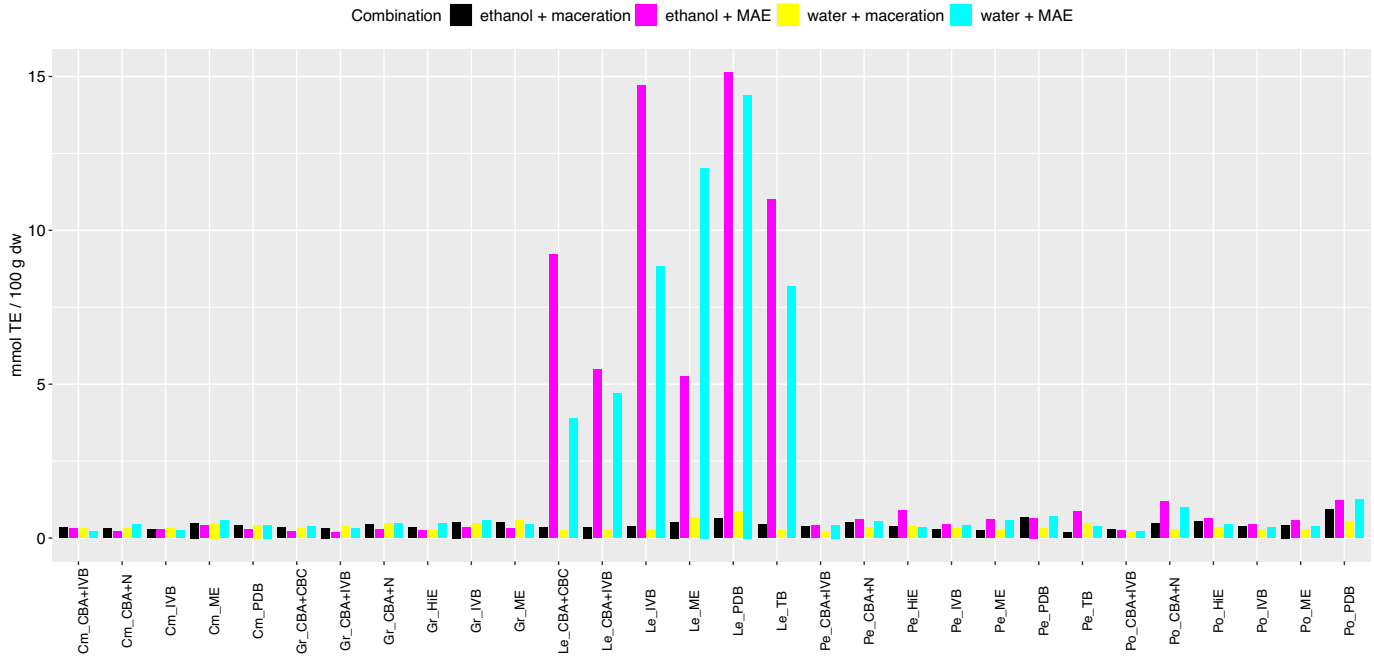

Fig. 4. TPC of the experimental lines - Total phenolic content (TPC) measured using Folin-Ciocalteau assay. Data are reported in mg GAE (gallic acid equivalent) per 100 g dw of fungal biomass on the y-axis. On the x-axis are reported the initials of the five fungal strains coupled with the growth media.

3.4. Total phenolic content and ferric reducing antioxidant power

Medicinal mushrooms have been used throughout history for their nutraceutical and curative purposes, and modern science have identified several classes of compounds responsible for these properties. In particular, (poly)phenols have extensively studied for their antioxidant anticarcinogenic, antimutagenic, and anti-inflammatory effects, and their global market value is expected to grow from 4.13 to 6.05 bln USD in the period 2021–2028, particularly boosted by the COVID-19 pandemic (Fortune Business Insights, 2021). Because of these reasons, producing a mycoprotein rich in both proteins and antioxidants would redefine and improve the concept of meat alternatives, providing a functional alternative protein source.

Under our experimental conditions, TPC and FRAP results were highly species- and media-specific. Concerning the experimental lines, TPC (Fig. 4 and Supplementary Table 5) ranged from 65.498 (*C. militaris* in CBA + IVB, using MAE and water as solvent) to 586.415 mg GAE/100 g dw (*P. ostreatus* in PDB, using MAE and water as solvent), highlighting strong differences among the fungal species (*C. militaris* grown in the same conditions of the abovementioned *P. ostreatus* yielded (194.651 mg GAE/100 g dw, three-fold less). However, the average TPC remained relatively consistent, with values ranging from 156.881 ± 52.409 to 286.995 ± 148.516 mg GAE/100 g dw (in L. *edodes* and *P. ostreatus,* respectively).

Despite observing a similar level of variability, most of the TPC reported in this study are notably lower, and in many cases not easily comparable, to those found in the existing literature, often differing by one or two orders of magnitude. For instance, ethanolic extracts of *P. ostreatus* grown on barley in SSF by (Ianni et al., 2021) reached 423.7 * 10^2 mg GAE/100 g dw, while its conspecific best performing line in this study recorded 586.4 mg GAE/100 g dw. It has to be stressed that all the experimental lines of this work underwent no optimisation process, thus there still is a potential high margin of improvement regarding the TPC.

Completely different trend was observed for the antioxidant power. While four out of five mushrooms exhibited similar TPC, *L. edodes* (MUT 6391) showed significantly higher values when FRAP assay was performed (Fig. 5 and Supplementary Table 5). Excluding L. *edodes*, these values ranged from 0.175 (observed in *P. ostreatus* grown in CBA + IVB and using maceration with water as solvent) to 1.266 mg GAE/100 g dw (*P. ostreatus* in PDB, using MAE with water as solvent). In particular, this data underscores how the antioxidant power of the same strain can be highly influenced by growth media and extraction technique. As well, their average FRAP were comparable, ranging from 0.351 ± 0.095 to 0.538 ± 0.332 mmol TE/100 g dw (in *C. militaris* and *P. ostreatus,* respectively). As stated above, among all the mushrooms assessed in this work, the highest FRAP results were obtained for L. *edodes*. Indeed, despite having TPC similar to those of the other four mushrooms, *L. edodes* exhibited an average antioxidant power approximately 10-fold higher than the others (4.920 ± 5.376 mmol TE/100 g dw), with a maximum FRAP value equal to 15.136 mmol TE/100 g recorded for PDB, using MAE and ethanol as solvent. Although there were less data available or comparable about FRAP levels for the five mushrooms involved in this work, a similar range of values has been observed between our results and those found in literature. Though, the discrepancy between L. *edodes* TPC and FRAP values is interesting, the first comparable to the other mushrooms', while the latter much higher. In fact, there is usually a strong correlation between the phenolic content and the antioxidant capabilities of fungal biomasses (Azieana, Zainon, Noriham, & Rohana, 2017), which has not been observed in this case. This work does not provide an explanation for this phenomenon, but further studies will investigate how could have happened such. GC–MS analysis may shed a light on how the chemical composition of the medicinal mushrooms can affect their capabilities, given that several chemicals can synergically exert higher antioxidant power than when considered alone, as reported by Pereira et al. (Pereira, Arruda, de Morais, Eberlin, & Pastore, 2018).

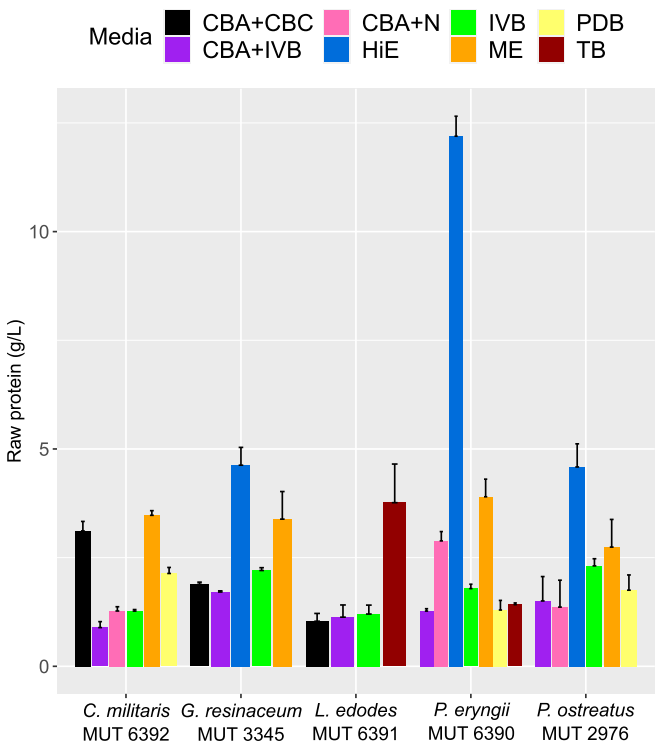

Fig. 5. FRAP of the experimental lines - Ferric-Reducing antioxidant power (FRAP). Data are reported in mmol TE (Trolox equivalent) per 100 g dw of fungal biomass on the y-axis. On the x-axis are reported the initials of the five fungal strains coupled with the growth media.

Concerning the FRAP assay, the recovery and comparison of data relating to antioxidant power is often hindered by the use of different units of measurement and reference substances (*i.e.* Trolox, Fe^{2+}, $FeSO_4$, and Ascorbic Acid equivalents). Thus, our investigation highlights the importance of establishing consensus on a single chemical standard, especially within scientific fields such as mycology, to both facilitate more accurate comparison and understanding scientific data.

3.5. Microwave-assisted extraction vs traditional maceration

Finally, we have assessed the extraction yield of two techniques, the microwave-assisted extraction (MAE) and the maceration extraction, using both water and ethanol 70% (*v*/v) as solvents. Although many new techniques to extract different fungal compounds are arising in recent years (Leong, Yang, & Chang, 2021), traditional maceration is still largely used as method to retrieve (poly)phenols from medicinal mushrooms. However, maceration is a time and solvent consuming technique, usually requiring up to 24–48 h. Because of these limitations and aiming for higher extraction efficiency, MAE is rapidly gaining popularity due to its fast extraction rates, lasting few hours at max (Özyürek, Bener, Güçlü, & Apak, 2014). As solvents instead, ethanol and methanol are the most frequently used for this purpose, thanks to the relatively hydrophilic nature of (poly)phenols, which makes them soluble in polar organic solvent. However, methanol is highly toxic, highly volatile, inflammable, and non-renewable. Thus, green solvents have been chosen to carry out the extractions (Prat et al., 2016). Furthermore, microwave-assisted extraction (MAE) has been evaluated as a green and innovative extraction technique, in comparison with maceration, with the aim of shortening extraction time, reducing solvent consumption, increasing extraction rate, and lowering energy consumption (Milani et al., 2020).

In the search for optimal experimental MAE conditions, the effect of

temperature, solid/liquid ratio, and solvent hydrophilicity degree on both total phenolic content and antioxidant properties of extracts have been investigated: the extractions have been carried out at three temperatures (60 °C, 80 °C, and 100 °C), with 30 mg/mL, 90 mg/mL, and 150 mg/mL as solid/liquid ratio, and using different green solvents (water, 70% ethanol, 70% isopropanol, absolute ethanol, isopropanol, and 2-methyl tetrahydrofuran, listed in order of decreasing hydrophilicity).

Preliminary results (data not shown) indicated that working at 80 °C, with a solid/liquid ratio of 30 mg/mL, and using either water or 70% ethanol as solvents, the highest efficiency could be obtained. These experimental conditions have been applied for all subsequent microwave-assisted extractions, and both water and 70% ethanol have also been used in macerations to finally compare the two extraction methods.

Given the retrieved data, we analysed the influence of the two solvents and the two extraction techniques on the phenolic content and the antioxidant power.

There was no statistical difference in TPC (mg GAE/100 g dw) using water (mean = 206.9, SD = 76.2) or ethanol (mean = 207.6, SD = 71.6) as solvents ($t = 0.1257$, df = 57, p-value = 0.9004). As well, there was no statistical difference in TPC (mg GAE/100 g dw) using MAE (mean = 214.8, SD = 76.2) or maceration (mean = 201.8, SD = 69.1) as extraction technique ($t = -0.96682$, df = 55, p-value = 0.3379) (Fig. 6A).

Things changed when performing the same analysis on FRAP values. In fact, because of their extreme nature, *L. edodes* FRAP measurements were treated as outliers and removed in this analysis, to be treated separately, so as not to weigh too much in the overall analysis, leading to misinterpretations on the four mushroom performances. Therefore, concerning the other four mushrooms, there was no statistical difference in FRAP (mmol TE/100 g dw) using water (mean = 0.394, SD = 0.137) or ethanol 70% (mean = 0.427, SD = 0.172) as solvents ($t = 1.3559$, df = 53, p-value = 0.1809). However, there was statistical difference in FRAP (mmol TE /100 g dw) using MAE (mean = 0.484, SD = 0.261) or maceration (mean = 0.385, SD = 0.117) as extraction technique ($t = -2.4101$, df = 49, p-value = 0.01975) (Fig. 6B), with MAE leading to an average 25.7% higher antioxidant activity of its extracts.

Lentinula edodes extractions, which have been excluded from the analysis above, led to unexpectedly high antioxidant activity, particularly for MAE ones. According to literature, this is an interesting and unique data, because only one work reports similar, but lower, antioxidant activity than L. *edodes* (MUT 6391) average one (3. 583 mmol TE/100 g and 4.92 mmol TE/100 g) (Radzki, Slawinska, Jablonska-Rys, & Michalak-Majewska, 2016). Overall, the highest FRAP level recorded by this study was L. edodes grown on PBD, using MAE and ethanol as extraction technique and solvent, respectively (15.14 mmol TE/100 g).

Concerning to the *P. ostreatus* grown on *H. illucens* exuviae-based media which overshadowed the other combinations because of its biomass yield and protein content, it gave a mean TPC of 189.0 mg GAE/100 g dw (max 263.4 mg GAE/100 g dw) and a mean FRAP of 0.493 mmol TE/100 g dw (max 0.640 mmol TE/100 g dw). These results are lower than the literature ones (see Supplementary Information), but as considered earlier, a proper optimisation process might enhance its (poly)phenol content, thus its antioxidant value.

These results have huge environmental and financial implications. MAE has already proven being effective, or even more efficient, at extracting antioxidants such as polyphenols from mushrooms, using water as solvent either (Xiaokang et al., 2020). The capacity of reducing extraction time, energy demand and environmental impact due to the protocol and chemicals involved is an important step ahead to shape a greener generation of extraction techniques. This work poses itself in this topic, suggesting how pursuing both high protein content and (poly) phenolic content is an achievable goal, although it needs more effort and resources that the ones involved in this study.

Moreover, our findings highlight the great potential that mushrooms have as novel functional foods. As mentioned above, the rising food and protein demand will require new and efficient solutions, and mushrooms

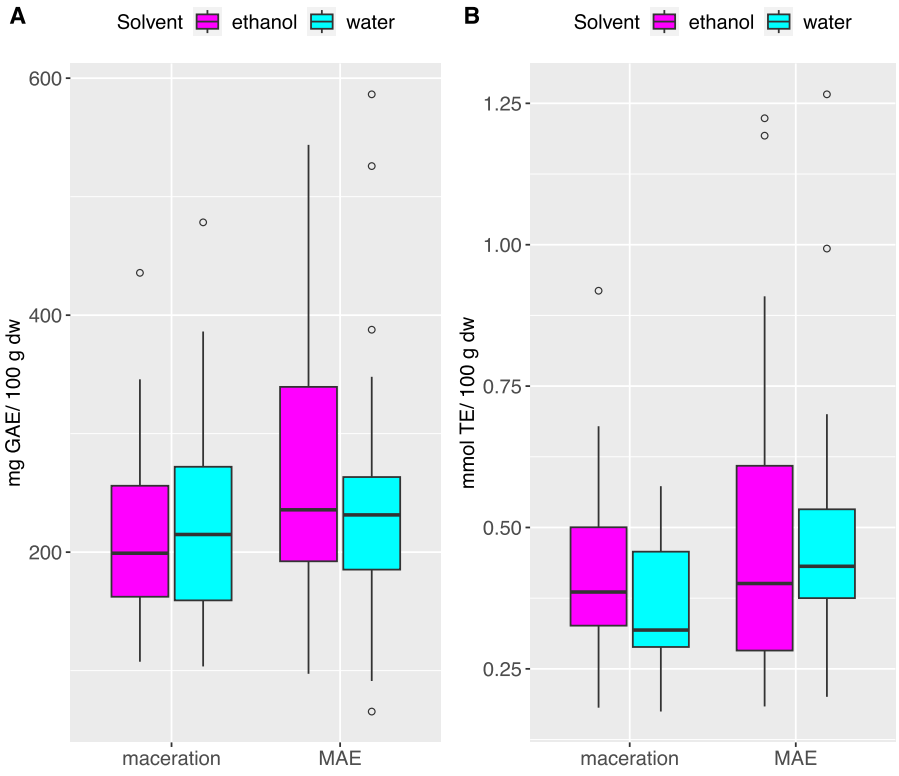

Fig. 6. Extraction techniques and solvents comparison - Total Phenolic Content (TPC, 6 A) and Ferric-Reducing Antioxidant Power (FRAP, 6B) of the fungal extracts using ethanol 70% or water as solvents, traditional maceration or Matrix-Assisted Extraction (MAE) as extraction techniques.

may represent a valid and environmentally friendly solution. To date, only one fungi-based protein product has been fully developed and commercialised by Marlow Foods (UK), Quorn®, which is constituted by *Fusarium venenatum* biomass. Thus, the markets are either eager and open to new protein-rich solutions, and as this study has shown, producing mushrooms using agro-industrial by-products is not only feasible, but able to achieve protein contents way higher than the traditional sources, reasonably with lower environmental impact.

4. Conclusions

Submerged fermentation is a powerful tool to grow Medicinal Mushrooms using by-products as growth media. Among the tested strains, *P. eryngii* (MUT 6390) grown in *H. illucens exuviae* liquid media gave both the highest biomass yield and protein content, 23.09 g/L and 51.03%, respectively. This is the first work that assessed the growth of mushrooms in an insect-based medium. Concerning the total phenolic content and FRAP, the highest values recoded were 586.415 mg GAE/100 g dw (*P. ostreatus*) and 15.14 mmol TE/100 g (*L. edodes*), both grown in Potato Dextrose Broth and extracted using Microwave-Assisted Extraction (MAE). This technique performed equally, or even better, than traditional maceration in terms of antioxidant activities found in their respective extracts, allowing considerable saving of time and energy. Therefore, in comparison with maceration, MAE is an advisable technique for fungal biomass extraction.

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CRediT authorship contribution statement

Davide Ferrero: Writing – review & editing, Writing – original draft, Visualization, Validation. **Elisa Moscato:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Federica Spina:** Supervision, Methodology, Investigation, Data curation, Conceptualization. **Maria Maddalena Cavalluzzi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Natalie Rotondo:** Investigation, Formal analysis. **Sara Bellezza Oddon:** Formal analysis, Data curation. **Maria Letizia Gargano:** Methodology, Formal analysis, Data curation, Conceptualization. **Giuseppe Venturella:** Writing – review & editing, Resources, Conceptualization. **Giovanni Lentini:** Writing – review & editing, Supervision. **Cinzia Margherita Bertea:** Writing – review & editing, Formal analysis. **Laura Gasco:** Writing – review & editing, Formal analysis. **Giovanna Cristina Varese:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Giovanna Cristina Varese reports financial support was provided by European Commission. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103721.

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