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Improving the researches on *Hericium erinaceus* for a better use of its medicinal properties in central nervous system

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Inside there was a world, and no one knew it until then.

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1. INTRODUCTION

1.1 The genus *Hericium* and its presence in Italy

The present PhD thesis deals with the wood-inhabiting macrofungus *Hericium erinaceus* (Bull.) Pers. related to some aspects of its numerous medicinal properties.

According to the main international databases, this species belongs to Fungi, Dikarya, Basidiomycota, Agaricomycotina, Agaricomycetes, Russullales, Hericiaceae, Hericium. (http://www.mycobank.org; http://www.indexfungorum.org).

Taxonomy and phylogeny of the genus *Hericium* Pers. have been intensely debated in recent years both as it regards its collocation within Agaricomycetes and the intra-genus discrimination of single species. Thus, *Hericium* is currently located in *Russulales*, as well as many other former "*Aphyllophorales*", on the basis of both synapomorphic characters (e.g. gloeocystidia) and molecular data (Larsson E and Larsson KH, 2003). Phylogenetically, *Hericium* is classified in the *Hericiaceae* within the russuloid linage, and very closely to *Laxitextum*, with stereoid basidiome (Larsson KH, 2007).

Hericium is represented in Europe, Italy included, by four species: *H. alpestre* Pers.; *H. cirrhatum* (Pers.) Nikol; *H. coralloides* (Scop.) Pers.; *H. erinaceus* (Bull.) Pers..

At a subspecific level, different *subspecies*, *varietates* and *formae* have been proposed and some nomenclatural confusion was consequently generated by time, particularly among conifericolous taxa. In order to reduce such a confusion, *H. flagellum* (Scop.) Pers. has been proposed to replace *H. alpestre sensu stricto* (www.indexfungorum.org) but this has not always been accepted (http://www.mycobank.org). In the present doctoral project it was decided to follow the indications of Mycobank database, therefore the name *H. alpestre* has been used.

All *Hericium* species are pileate, fleshy and typically hydnoid, white to cream coloured when fresh; hyphal system is monomitic (Stalpers, 1996). Spines have been proposed to have an important evolutionary effectiveness related to the increase in spore production; moreover spine morphology is an important discriminant in species identification. Namely, *H. coralloides* only has branched spines while *H. erinaceus* only has spines up to 4-5 cm long. Sporophore is imbricate and flattened in *H. cirrhatum*, whereas it is globose to branched in the other European species (Bernicchia and Gorjon, 2010; Pallua *et al.*, 2015).

Hericium species develop their sporophores on living or dead broadleaved trees, generally they are considered weakly parasitic or saprotrophic, causing a white-rot. The main hosts reported in Europe

are *Fagus sylvatica* and *Quercus* spp. as regards *H. erinaceus*, *H. cirrhatum* and *H. coralloides*. As a whole, *H. coralloides* and *H. erinaceus* are reported in Eurasia and North America, whereas the presence of *H. cirrhatum* in North America was suggested but not furtherly confirmed by molecular data (Harrison, 1984). The geographic distribution of *H. alpestre* is almost exclusively limited to South Europe and Caucasus, since this species appears strictly related to the South-European orophytes in *Abies alba* L. complex (Boddy *et al*., 2011; Das *et al*., 2013; Hallenberg *et al*., 2013).
At the end of the Introduction chapter, the description of each European species is reported, with particular emphasis on *H. erinaceus*; hereafter the dichotomous key proposed by Bernicchia and Gorjon (2010) for *Hericium* European species has been copied down.

"Key to the European species of *Hericium*"

1. Basidiome pileate, caps usually dimidiate, imbricate and flattened, hyphae non-amyloid, spores 3.5-4.5 x 2.5-3.5 µm, seemingly smooth at the light microscope……. ***H. cirrhatum***
1. Basidiome fleshy, globose to branched, rarely resupinate, hyphae somewhat amyloid, spores verrucose at the light microscope……2
2. Basidiome globose, unbranched, spines up to 4(-5) cm long, spores 5-6.5 x 4-5(-5.5) µm…… ***H. erinaceus***
2. Basidiome branched, more rarely resupinate, spines up to 2 cm long, spores variable…….3
3. Spines unbranched and distributed in tufts along the ramifications of the basidiome, usually terminal as well, spines up to 2 cm long, spores 5-6.5 x 4.5-5.5 µm…….. ***H. alpestre***
3. Spines branched, evenly distributed on the lower side of the basidiome, spines up to 1 cm long, spores 3.5-5 x 2.5-4 µm…… ***H. coralloides***

1.1.1 *Hericium alpestre*

The description is mainly based on the following references: Bernicchia and Gorjon (2010), Hallenberg (1983), Stalpers (1996), www.eol.org, www.gbif.org, www.iucn.ekoo.se, www.mycobank.org.

The sporophore is up to 30 cm across, fleshy, branched and even forms are known to be resupinate. Individual branches are covered with unbranched tufted and pendant spines; the spines are usually thick and long up to 1,5 - 2 cm. Hyphal system is monomitic, sparsely branched, with simple sept and clamps; hyphae are hyaline, and in some specimens amyloid. Gloeoplerous hyphae are present, with a yellowish, oily content, more or less sulfo-positive.

Basidiospores are broadly ellipsoid to subglobose, minutely verrucose, amyloid, 5-6,5 x 4,5-5,5 µm.

The distribution is restricted to Central and South Europe (Austria, Belgium, Bulgaria, Croatia, France, Germany, Poland, Czech Republic, Romania, Russia, Slovakia, Slovenia, Switzerland, The Netherlands, Ukraine), and also in Italy where it is reported in Emilia-Romagna, Toscana and Trentino Alto-Adige (Onofri, 2005).

Fallen trunks and stumps of *Abies alba* lying on the ground, but sometimes on still standing trees, are the substrata colonized.

According to "The Global Fungal Red List", *H. alpestre* is just considered "an appropriate fungus to assess" (www.iucn.ekoo.se).

- *H. alpestre* was recorded and collected during the samplings carried out for this PhD project.

Sporohores were detected in November 2017 and 2018 at "Parco Nazionale Foreste Casentinesi, Monte Falterona e Campigna", Arezzo, AR, Italy, thanks to dr. Annarosa Bernicchia's personal communication and the permission issued by the Park authority, and in October 2018 at Mt. Abetone, Pistoia, PT, Italy, thanks to the local Mycological Group.

The sporophores, collected from *A. alba* standing or lying dead trunk (fig.1), were processed at Mycological Laboratory of Department of Earth and Environmental Sciences (DSTA), University of Pavia (Italy), to isolate the strains as described in Cesaroni *et al*. (2019).

In Petri dishes containing 2% malt extract agar (MEA), the fungus showed the culture characteristics reported by Hallenberg (1983) but it grew very slowly (0,92mm/d) (fig. 2).

Fig. 1 (photos by V. Cesaroni ©): a) *H. alpestre* at Mt. Abetone (PT); b) *H. alpestre* at Parco Nazionale Foreste Casentinesi, Monte Falterona e Campigna (AR).

Fig. 2 (photo by V. Cesaroni ©): Petri dish (2% MEA) with *H. alpestre* mycelium isolated in pure culture.

1.1.2 *Hericium cirrhatum*

As reported by Bernicchia and Gorjon (2010), the sporophore is pileate, dimidiate to semicircular, fleshy, with imbricate growth, upper sterile surface more or less smooth to granulose or tomentose, cream coloured. Hymenophore is hydnoid with spines 1-2 cm long, yellowish cream; context fibrous, whitish.

The hyphal system is monomitic; hyphae with clamps, thin-walled, frequently ampulliform and variable in size, usually 4-8 µm wide, hyaline. Gloeocystidia cylindrical, sinuous, about 100 x 6-8 µm, with granular contents. Basidia clavate, 25-35 x 4-5 µm, with 4-sterigmata, with a basal clamp. Basidiospores are ellipsoid to subglobose, 3.5-4.5 x 2.5-3.5 µm, smooth at light microscope (rugose at SEM), hyaline, amyloid.

The species is widely distributed in Europe, mainly in Norway and Sweden, but also in Belarus, Belgium, Bulgaria, Denmark, Finland, France, Germany, Italy, Poland, Romania, Russia, Spain, The Netherlands, and Ukraine. It is recorded in the United Kingdom, Turkey, Australia and United States of America. In Italy, it is present in Basilicata, Calabria, Emilia-Romagna, Lombardia, Marche, Piemonte, Sicilia, Sardegna, Trentino Alto-Adige, and Veneto (Onofri, 2005).

It grows preferentially on *Fagus sylvatica* but other hardwood substrata such as *Betula* spp., *Quercus* spp. and *Acer* spp. can be colonized (Boddy *et al*., 2011).

Even if this species is more common than *H. alpestre* in Europe and in Italy too, it was never recorded during the samplings carried out for this PhD project.

1.1.3 *Hericium coralloides*

The description refers to the following literature datas: Bernicchia and Gorjon (2010), Hallenberg (1983), Stalpers (1996), www.eol.org, www.gbif.org, www.iucn.ekoo.se, www.mycobank.org.

Sporophore is fleshy, branched and even resupinate; individual branches are covered with spines; the spines are rather evenly distributed on the lower surface of the branches, like the teeth on a comb. There are specimens with thin and delicate branches as well as thick and short ones. The spines are usually thin and not more than 1 cm long. Hyphal system is monomitic, sparsely branched, simple septate with clamps, hyaline, in some specimens amyloid; gloeoplerous hyphae are present, with a yellowish, oily content, more or less sulfo-positive. Basidiospores are broadly ellipsoid to subglobose, minutely tuberculate, amyloid, 3,5-5 x 3-4 µm.

This species is widely distributed in Armenia, Bosnia and Herzegovina, Bulgaria, China, Croatia, Poland, Czech Republic, Denmark, Estonia, France, Germany, Greece, Iran, Italy, Japan, Korea, Latvia, Lituania, Macedonia, Netherlands, Norway, Philippines, Poland, Romania, Russia, Serbia, Slovakia, Spain, Sweden, Switzerland, Ukraine, and UK. In Italy it is present in Basilicata, Calabria, Emilia-Romagna, Lazio, Lombardia, Piemonte, Sardegna, Sicilia, Toscana, Trentino Alto-Adige and Veneto (Onofri, 2005).

The species develops its basidioma on standing or dead trunks of *Quercus* spp., Fagus spp., *Picea abies*, and *Pteridium aquilinum*.

According to "The Global Fungal Red List", *H. coralloides* has been included in the "Preliminary global red-list assessment" as LC (Least Concern).

- *H. coralloides* was recorded and collected during the samplings carried out for this PhD project.

One sporohore was detected in October 2016 at "Località Buonconvento" (Sovicille, Siena) thanks to a personal communication of a local Mycological Group. The sporophore, collected from a dead standing trunk of *Fagus* (fig. 3), was processed at Mycological Laboratory of Department of Earth and Environmental Sciences (DSTA), University of Pavia (Italy), to isolate the strain as described in Cesaroni *et al*. (2019).

In Petri dishes containing 2% malt extract agar (MEA), the fungus showed culture characteristics reported by Hallenberg (1983) but it grew very slowly (1,33 mm/d) (fig. 4).

Fig. 3 (photo by V. Cesaroni ©): sporophore of *H. coralloides* in the surruondings of Siena.

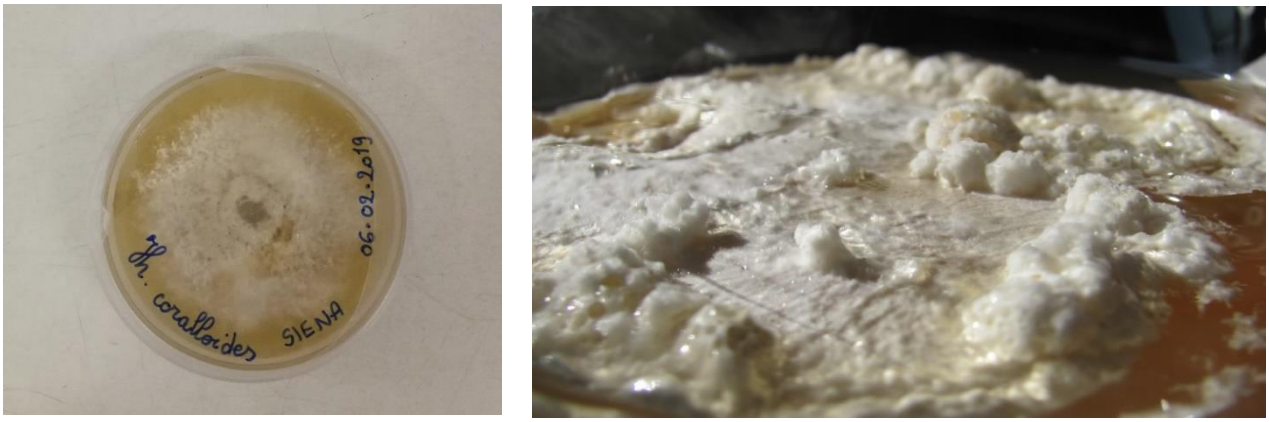

Fig. 4 (photos by V. Cesaroni ©): a) Petri dish (2% MEA) with *H. coralloides* mycelium isolated in pure culture; b) mycelium of *H. coralloides* on culture broth (2% ME).

The wild type sporophore and its isolate were analyzed by HPLC-ESI-MS/MS method at the laboratory of Mass Spectrometry of the Centro Grandi Strumenti of the University of Pavia (Italy). The preliminary data (not shown) revealed that erinacine A was detected by the chromatographic traces in the *H. coralloides* mycelium at a retention time of 10.42 min.; while the UV chromatographic trace of the sporophore did not reveal the presence of both hericenone C and D, detected as standard at the retention time of 42.91 min and 44.53 min. In the same UV chromatographic trace, other peaks are present: by comparison with literature data (Arnone *et al.*, 1995; Kobayashi *et al.*, 2018), the peaks detected at the retention time of 45.36, 46.27, 46.50 and 47.65 mins were attributed to hericenes D, A, B and C. These data could be confirmed when standard molecules will be available.

1.1.4 *Hericium erinaceus*

Bulliard, the first taxonomist that described this species in 1797, having found a strong resemblance to the hedgehog (in Latin *erinaceus*), chose the appellation "*erinaceus*" for this *Hericium* (Fig. 5). Thanks to its particular morphological aspect and the wide geographical distribution, many common names have been attributed to this fungus: the German name is Igel-Stachelbart; some common names in the UK are Bearded Hedgehog or Hedgehog Mushroom; in Japan is known as Yamabushitake (Yamabushi means mountain priest); in China the most common name is Houtou, which means monkey head. In other parts of the worlds it is also called pompom mushrooms, bear's head, white beard and so on. Nowadays the most popular common name is Lion's mane mushroom.

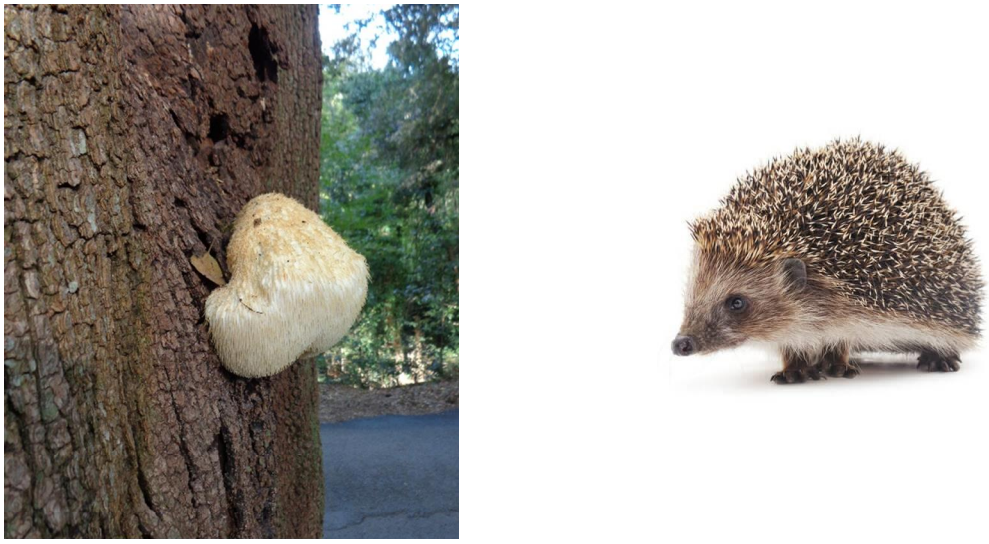

Fig. 5: *Hericium erinaceus* wild type sporophore found in the surroundings of Siena (Italy) (photo by V. Cesaroni©) and an hedgehog.

H. erinaceus is characterized by a conspicuous pileate, fleshy, hydnoid and globose sporophore, consisting of numerous single, typically long, dangling, fleshy spines, which are at first white, becoming yellowish, and then brownish with age. It is morphologically close related to *Climacodon*, but the latter differs in having inamyloid basidiospores (Bernicchia and Gorjon, 2010). The upper sterile surface is more or less hirsute to tomentose and cream coloured. The hymenophore is hydnoid with spines 1-4(5) cm long, yellowish cream, in a fibrous and whitish context, pendent, arranged in a beard-like manner. The hyphal system is monomitic, hyaline, clamps are present; hyphae with thick and amyloid walls, frequently ampullate and variable in size (6-10/15 µm wide). Gloeocystidia with granular content are present, they are variable in shape and size (3-)5-13 µm, often protruding into the hymenium. Basidia are clavate, 25-40 x 5-8 µm, with 4-sterigmata and a

basal clamp. Basidiospores are short ellipsoid to subglobose, 5-6.5 x 4-5.5 µm, minutely verrucose, thick-walled, more or less hyaline, amyloid (Harrison, 1973; Stalpers, 1996; Stamets, 2005; Bernicchia and Gorjon, 2010; Thongbai *et al.*, 2015).

H. erinaceus is widely distributed in the Northern hemisphere (fig. 6); in particular, it has been recorded in Armenia, Australia, Austria, Belgium, Bosnia and Herzegovina, Bulgaria, China, Colombia, Costa Rica, Croatia, Czech Republic, Denmark, France, Georgia, Germany, Greece, Hungary, India, Italy, Japan, Korea, Luxemburg, Macedonia, Mexico, Mongolia, Nepal, Netherlands, Norway, Pakistan, Poland, Portugal, Romania, Russia, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, Ukraine, UK, USA. In Italy, it is present in Basilicata, Calabria, Emilia-Romagna, Lazio, Liguria, Lombardia, Molise, Piemonte, Sardegna, Sicilia, Toscana, Umbria and Veneto (Onofri, 2005; Bernicchia and Gorjon, 2010).

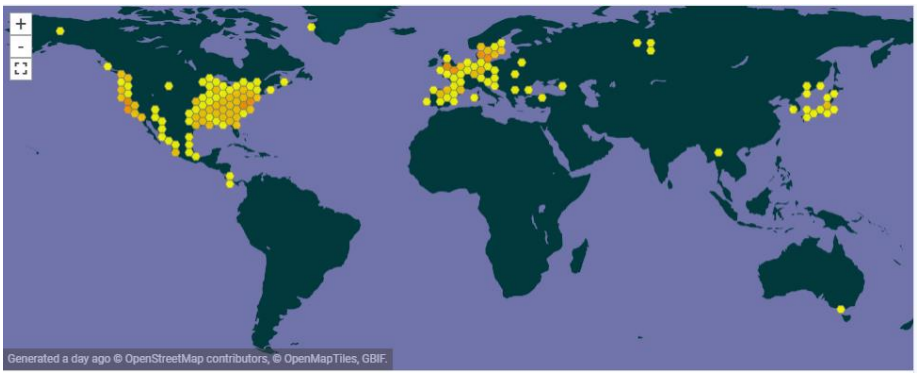

Fig. 6: geographic distribution of *H.erinaceus* around the world (www.gbif.org).

The species is considered a saprotroph or a weak parasite on old deciduous and sclerophylous trees: mainly *Fagus sylvatica* and *Quercus* spp.; occasionally it can grow on *Betula*, *Fraxinus*, *Juglans*, *Malus*, *Ailanthus* and *Sorbus* (Fraiture and Otto, 2015).

In Europe, it prefers beech and oak wood (http://iucn.ekoo.se): it grows mainly on *Fagus* in the UK, Denmark and Poland, on *Fagus* and *Quercus* spp. in Austria, the Czech Republic and in Slovakia; almost exclusively on *Quercus* in Hungary (Kunca and Čiliak, 2017). In North America, most frequently it habits oak and less frequently beech, and occasionally other trees (Boddy *et al.* 2011). It grows both in preserved and in regularly managed forests but almost always on old trees. However, it was reported also on young living beech trees (15-20 cm in diameter) in Krasnodar territory in Russia (http://iucn.ekoo.se).

In Italy it occurs mainly along the Apennines mountain chain, in Sicily and Sardinia; it grows on *Fagus sylvatica* and *Quercus ilex*, *Q. petraea*, *Q. pubescens* (Venturella *et al*., 2016; Savino *et al*., 2017; Girometta *et al*., 2018; Cesaroni *et al*., 2019; Siniscalco *et al*., 2019).

Sporophores are usually fruiting from September to December (or over winter and spring in warmer climates), and only rarely there are two 'flushes' of fruiting from a single location on a tree within a year. The fungus is characterized by intermittent fruiting on occupied trees over several decades and this often continues for many years after the branch or trunk falls to the ground (https://www.mushroomexpert.com/hericium_erinaceus.html).

H. erinaceus is included in the list of 21 fungal indicator species of the conservation value of European beech forests (Christensen et al., 2004) and is red-listed as well as protected by law in many European countries (Dahlberg and Croneborg, 2003; Fraiture and Otto, 2015), according to "The Global Fungal Red List" (http://iucn.ekoo.se).

A part of the European localities of the species is situated in the area of National Parks and nature reserves (e.g., in Slovakia and Poland): this increases conservation effectiveness through habitat protection.

The occurrence of the species strongly depends on the presence of a proper substrate - mainly old and large beech or oak trees, large diameter dead wood, and rather more humid and mild climate. Of particular concern in this respect is the removal of older wood, particularly trunks and main branches of beech in areas with known fruiting populations of the fungus, because of the extended period required for their natural regeneration and replacement. Further research on the establishment phase of the fungus is required to help inform decision-making regarding which and how many trees are best left 'for the fungus' where woodland is being managed for the dual aims of forestry and biodiversity.

- Samplings of *H. erinaceus* were performed during this PhD project.

The first *H. erinaceus* sporophore and its strain (H.e.1) were respectively sampled and isolated in 2013; so, this sample was available since the beginning of the PhD project for analytical analyses.

Other sporohores were detected in October 2016 (strain H.e.2) in the surroundings of Siena at Colle Val d'Elsa (Italy); in October and November 2017 and in October 2018 (strain H.e.3 and H.e.4) in the surroundings of Siena (Italy),

thanks to personal communications of local Mycological Groups.

The sporophores, collected on living trees of *Q. ilex* (fig.7), were consequently processed at Mycological Laboratory of Department of Earth and Environmental Sciences (DSTA), University of Pavia (Italy), to isolate the strains as described in Cesaroni *et al*. (2019).

Fig. 7 (photos by V. Cesaroni ©): on the left H.e.1 and on the right H.e.2 sporophores sampled in the surroundings of Siena (Italy).

The strains grew in Petri dishes (MEA 2%) as reported by Stalpers (1978) and Stamets (2005): mycelium was whitish, forming triangular zones of collected rhizomorphs, radiating from the dense center section. The mat was downy or downy-farinaceous, remaining so or becoming thinly cottony. As cultures age, the mycelia became yellow to distinctly pinkish or greyish.

Each strain showed also peculiar morphological characteristics: H.e.1 had a transversal growth into the culture medium similar to H.e.3 that appeared more cottony; H.e.2 showed a negative geotropism generating various primordia from the mycelium; H.e.4 had a transversal growth, producing red-brown metabolites, probably phenols, so the cultural medium became darker (fig. 8).

Till now, only the strains H.e.1 and H.e.2 were analyzed by HPLC-ESI-MS/MS method at the laboratory of Mass Spectrometry of the Centro Grandi Strumenti of the University of Pavia, Italy. Data were published in Ratto *et al*. (2019) and Corana *et al*. (2019).

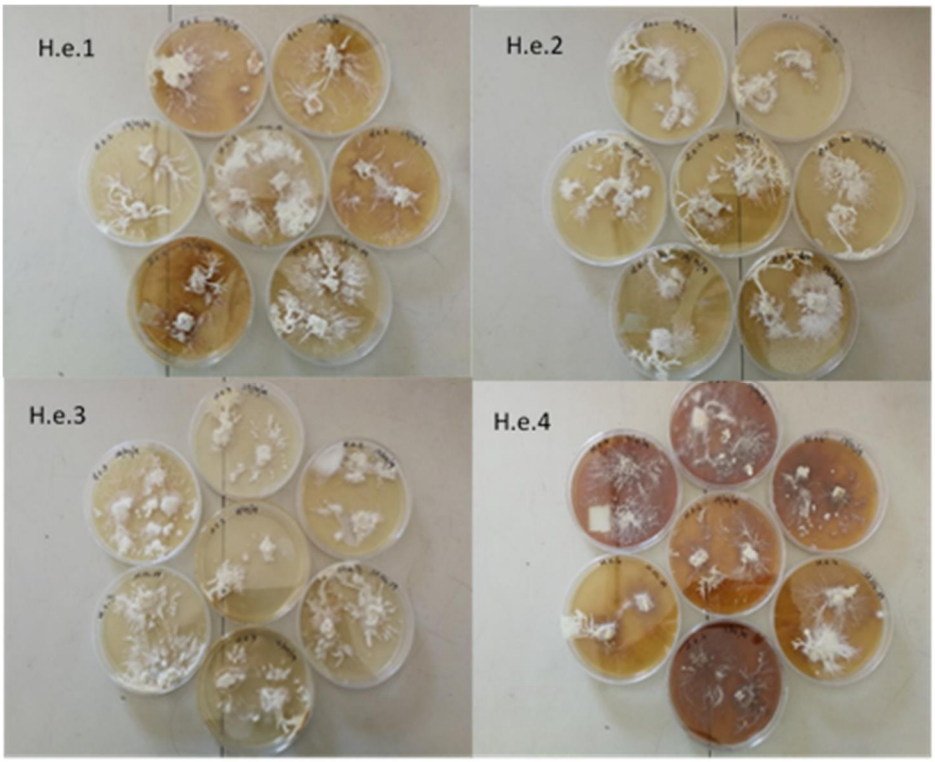

Fig. 8 (photos by V. Cesaroni ©): Petri dishes of different strains of *H. erinaceus* collected in Tuscany (Italy).

1.1.5 References

- Arnone A, Cardillo R, Nasini G, De Pava OV. 1994. Secondary mold metabolites: Part 46. Hericenes A-C and erinapyrone C, new metabolites produced by the fungus Hericium erinaceus. J. Nat. Prod., 57:602–606.
- Bernicchia A, Gorjón SP. 2010. Fungi Europaei - Corticiaceae s.l.. Ed. Candusso, Alassio (SV, Italy):315-319.
- Boddy L, Crockatt ME, Ainsworth AM. 2011. Ecology of *Hericium cirrhatum, H. coralloides* and *H. erinaceus* in the UK. Fungal ecology, 4:163-173.
- Cesaroni V, Brusoni M, Cusaro CM, Girometta C, Perini C, Picco AM, Rossi P, Salerni E, Savino E. 2019. Phylogenetic Comparison between Italian and Worldwide Hericium Species (Agaricomycetes). Int J Med Mushrooms. 24;3511:1-14.
- Corana F, Cesaroni V, Mannucci B, Baiguera RM, Picco AM, Savino E, Ratto D, Perini C, Kawagishi H, Girometta CE, Rossi P. 2019. Array of Metabolites in Italian Hericium erinaceus Mycelium, Primordium, and Sporophore. Molecules, 2019(24),3511:1-14.
- Christensen M, Heilmann-Clausen J, Walleyn R, Adamcik S. 2004. Wood-inhabiting fungi as indicators of nature value in European beech forests; ed. Marchetti M., Monitoring and indicators of forest biodiversity in Europe – From ideas to operationality. EFI Proceedings. 51.
- Dahlberg A, Croneborg H. 2003. 33 threatened fungi in Europe. Complementary and revised information on candidates for listing in Appendix 1 of the Bern Convention. Swedish Environmental Protection Agency (EPA) and European Council for the Conservation of Fungi (ECCF). 82.
- Das K, Stalpers JA, Stielow JB. 2013. Two new species of hydnoid-fungi from India. IMA Fungus. 4(2):359–69.
- Fraiture A, Otto P. 2015. Distribution, ecology and status of 51 macromycetes in Europe - Results of the ECCF Mapping Programme; ed. Scripta Botanica Belgica. 53:247.
- Girometta C, Bernicchia A, Brusoni M, Cesaroni V, Corana F, Cusaro CM, Guglielminetti ML, Mannucci B, Kawagishi H, Perini C, Picco AM, Rossi P, Salerni E, Savino E. 2018. Preliminary characterization of Hericium species collected in Toscana (Italy). State of the World's Fungi Symposium Royal Botanic Gardens, Kew. Poster.
- Hallenberg N. 1983. Hericium coralloides and H. alpestre (Basidiomycetes) in Europe. Mycotaxon, XVIII(1):181 – 189.

- Hallenberg N, Nilsson RH, Robledo G. 2013. Species complexes in *Hericium* (Russulales, Agaricomycota) and a new species - *Hericium rajchenbergii* - from southern South America. Mycol Prog. 12:413–20.
- Harrison KA. 1984. Creolophus in North America. Mycologia 76(6):1121-1123.
- Kobayashi S, Hamada Y, Yasumoto T, Hashino Y, Masuyama A, Nagai K. 2018. Total syntheses and endoplasmic reticulum stress suppressive activities of hericenes A-C and their derivatives. Tetrahedron Lett. 59:1733–1736.
- Kunca V, Ciliak M. 2017. Habitat preferences of *Hericium erinaceus* in Slovakia. Fungal Ecology. 27:189-192.
- Larsson E, Larsson KH. 2003. Phylogenetic relationships of Russuloid Basidiomycetes with emphasis on Aphyllophoralean taxa. Mycologia, 95(6): 1037-1065.
- Larsson KH. 2007. Re-thinking the classification of corticoid fungi. Mycol. Res. 111(9):1040-1063.
- Onofri S. (coordinated by) 2005. Checklist of Italian fungi. Ed. Delfino C., Sassari (SS, Italy):162-163.
- Pallua JD., Kuhn V, Pallua AF, Pfaller K, Pallua AK, Recheis W, Pöder R. 2015. Application of micro-computed tomography to microstructure studies of the medicinal fungus *Hericium coralloides*. Mycologia, 107(1):227–38.
- Ratto D, Corana F, Mannucci B, Priori EC, Cobelli, F, Roda E, Ferrari B, Occhinegro A, Di Iorio C, De Luca F, Cesaroni V, Girometta C, Bottone MG, Savino E, Kawagishi H, Rossi P. 2019. Hericium erinaceus improves recognition memory and induces hippocampal and cerebellar neurogenesis in frail mice during aging. Nutrients. 2019(11):1-20.
- Savino E, Girometta C, Cesaroni V, Rodolfi M, Perini C, Angelini C, Picco AM. 2017. Conservation of biodiversity of *Hericium erinaceus* in Italy. IMMC9, Palermo. Poster.
- Siniscalco C, Bianco PM, Floccia F, Campana L. 2019. La Flora Micologica delle principali essenze vegetali italiane. Il contributo storico del Centro di Eccellenza ISPRA presso il GMEM- AMB ai piani di gestione degli habitat di interesse comunitario. ISPRA, Manuali e linee guida n. 187/2019.
- Stalpers JA. 1978. Identification of wood-inhabiting Aphyllophorales in pure culture. Studies in Mycology. 16:1-248.
- Stalpers JA. 1996. The Aphyllophoraceous fungi II. Keys to the species of the Hericiales. Studies in Mycology. 40:1-185.

- Stamets P. 2005. Notes on nutritional properties of culinary-medicinal mushrooms. International Journal of Medicinal Mushrooms. 7(1-2):103-110.
- Thongbai B, Rapior S, Hyde KD, Wittstein K, Stadler M. 2015. *Hericium erinaceus*, an amazing medicinal mushroom. Mycological Progress. 14(91):1-23.
- Venturella G, Gargano ML. 2016. Problematiche fitopatologiche degli alberi monumentali. 1° Convegno Nazionale alberi monumentali. Proceedings.

Sitography:

- http://www.mycobank.org
- http://www.indexfungorum.org
- https://eol.org
- https://www.gbif.org
- http://iucn.ekoo.se
- https://www.mushroomexpert.com/hericium_erinaceus.html.

1.2 Medicinal properties of *Hericium erinaceus*

Out of approximately 15.000 known species of mushrooms, 2000 are edible for humans and about 650 of them display potential therapeutic and healthy values (Rai *et al*., 2005).

Hericium erinaceus is listed as one of the "Four Famous Dishes" in China, where it is mostly consumed as food. The taste is usually described as a shrimp or lobster; the flavor and the odor belong to a mixture of chemical aromatic compounds. *H. erinaceus* contains, for 100 g of dried mushrooms, 22.3 g of proteins, 3.5 g of unsaturated fatty acids, 57 g of carbohydrates, and 22.3 g of fibers (Wang *et al*., 2014, Jiang *et al*., 2014).

It is known that a variety of constituents from *H. erinaceus* could have beneficial activities such as anti-tumor, immune-modulating, antihyperglucemic and antihypercholesterolemic, antimicrobial, antioxidant, antiaging and, in particular, neuroprotective activities (fig. 1).

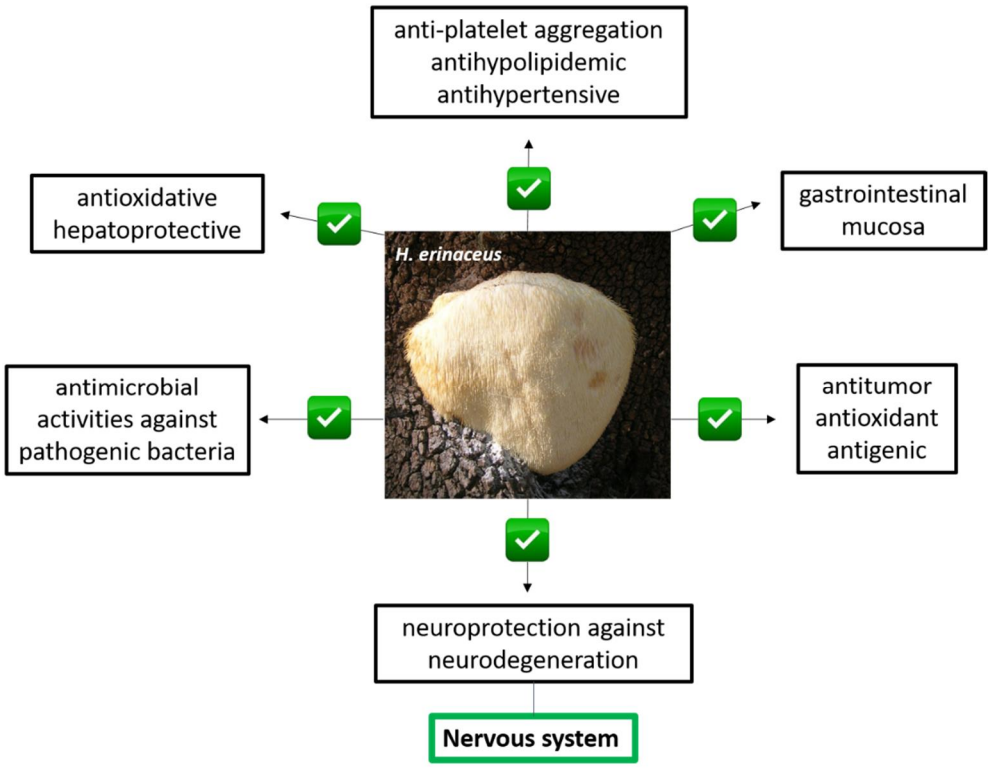

Fig 1 (drawing by V. Cesaroni): effects of *H. erinaceus* on human health.

The effects of *H. erinaceus* on central nervous system (CNS) have attracted great interest for its potential neuroprotective action on neurodegenerative diseases such as Alzheimer's, Parkinson's, dementia, depression, anxiety and cognitive impairment. Diseases of the aging nervous system such as Parkinson's, Alzheimer's and stroke are increasingly global public health problem.

H. erinaceus contains an array of metabolites, comprising more than 70 among secondary metabolites, structurally different and isolated from both, the mycelium and the sporophore (Friedman *et al*., 2015).

These bioactive metabolites could be classified mainly into (Kawagishi *et al*., 1994; Mizuno *et al*., 1992; Thongbai *et al*., 2015):

a) high molecular weight compounds, such as polysaccharides,
b) low molecular weight compounds, such as terpenoids.

Polysaccharides (xylans, glucoxylans, heteroxyloglucans, galactoxyloglucans) constitute about 20% of the total biomass of the mushroom (26.63% and 18.71% in sporophore and mycelium, respectively) and are mainly present in the cell walls (Sokol *et al*., 2016). They are implicated, as major bioactive components, in a wide range of properties such as anti-microbial, anti-diabetic, anti-hypertension, anti-tumor and in immune-modulation (Khan *et al*., 2013).

In figure 2 are reported the biological properties and the commercial typology of uses of polysaccharides extracted from *H. erinaceus* (He *et al*., 2017).

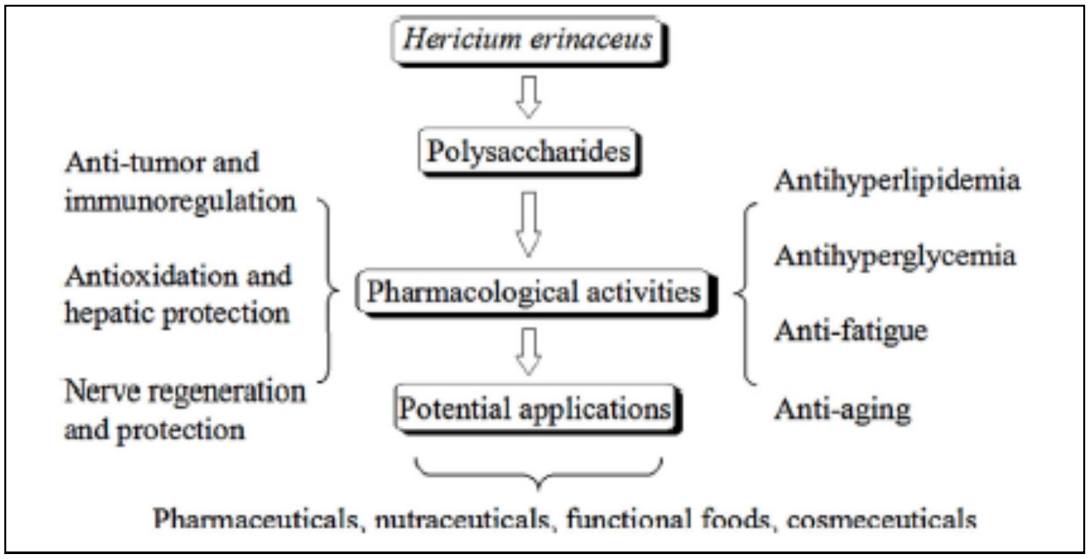

Fig. 2: biological properties and commercial typology of uses of polysaccharides extracted from *H. erinaceus* (He *et al*., 2017).

The low molecular weight compounds comprise a large number of terpenoids, secondary metabolites isolated from *H. erinaceus* sporophore and mycelium.

Hericenones, a benzyl alchol derivatives with simple fatty acids, isolated from the sporophore (fig. 3), and erinacines, a cyathane-type diterpenoids isolated from the mycelium, that include 20 members of 24 diterpenoids (fig. 4), are the most representative components of the low molecular weight compounds (Thongbai *et al*., 2015).

Different biological activity of single hericenones depends on the chain length and on the presence of double bonds in fatty acids.

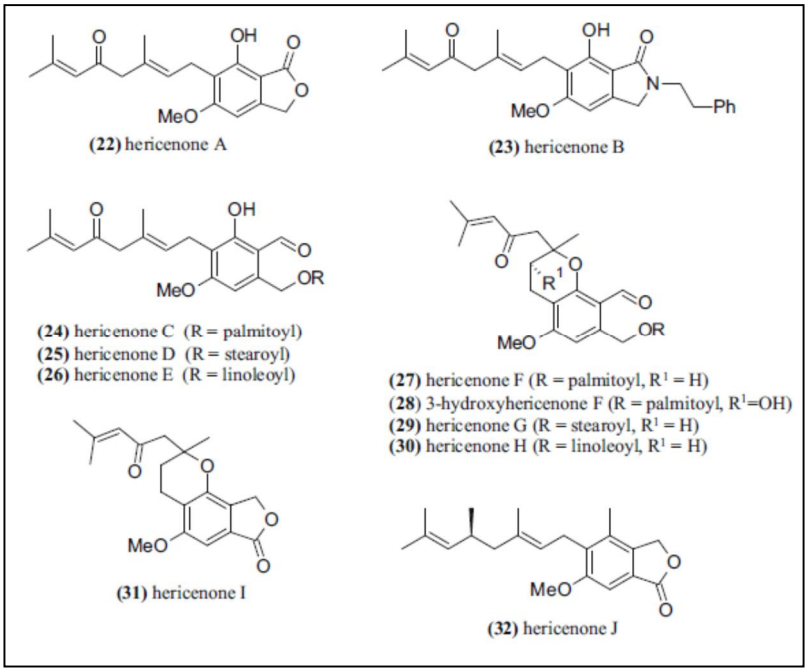

Fig. 3: chemical structures of hericenones (Thongbai *et al*., 2015).

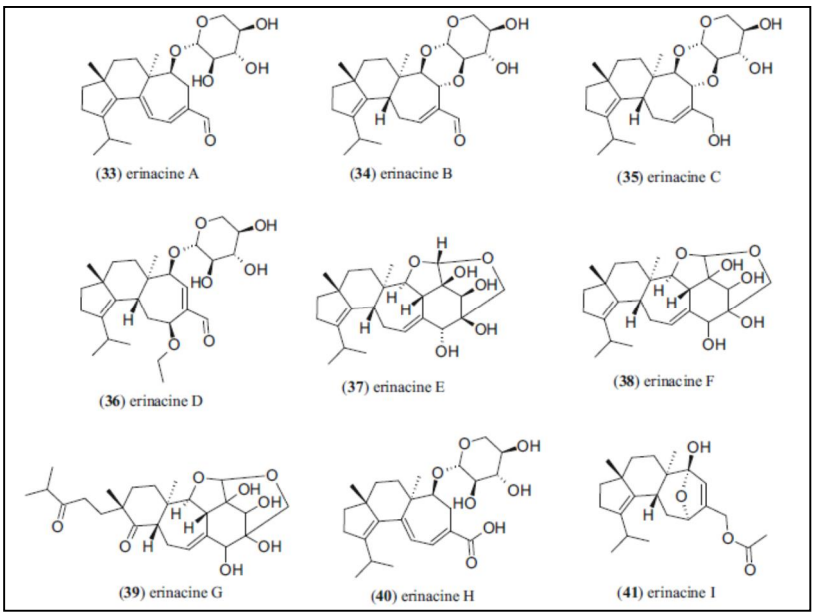

Fig. 4: chemical structures of erinacines (Thongbai *et al*., 2015).

The main features of these compounds are the poor water solubility and the necessity to use organic solvents for their extraction. The figure 5 shows some organic solvents listed according to their polarity and the related molecules extracted.

In particular, for the experiments conducted during the present PhD project, the procedures reported by Gerbec *et al*. (2015) was slightly modify using a concentration of 70% ethanol, and maintaining the material at 50 °C for 24 h.

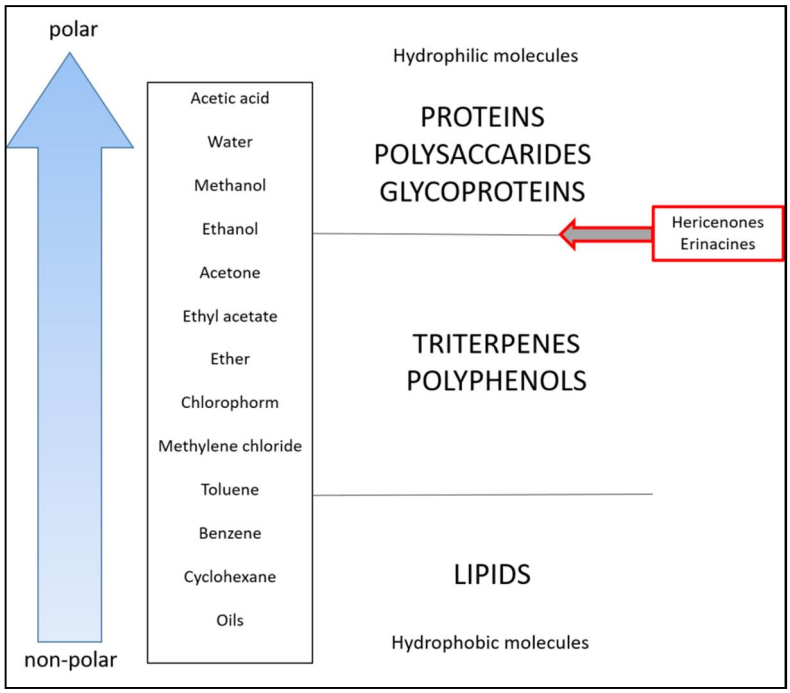

Fig. 5 (drawing by V. Cesaroni): polarity of different solvents and hydrophilic and hydrophobic molecules extracted.

The main bioactive compounds isolated from both sporophore and mycelium of *H. erinaceus* are reported in table 1.

Table 1: bioactive compounds isolated from *H. erinaceus*.

Bioactive compounds	***H. erinaceus* sample**	**Activity**	**Treatment**	**References**
Polysaccharides	sporophore	immunomodulatory, anticancer	cancers	Wang *et al*., 2001
		anti-fatigue	sport nutrition	Li *et al*., 2015
		skin antioxidant enzymes	anti-skin-aging	Xu *et al*., 2010
		lipid-lowering	obesity	Shang *et al*., 2013
Hericenones A-B	sporophore	cytotoxic	cancers	Kawagishi *et al*., 1990
		anti-platelet aggregation	vascular diseases, stroke, trombosis	Mori *et al*., 2010
Hericenones C,D,E,F,G,H	sporophore	neuroprotective, neuroregenerative	Alzheimer's and Parkinson's diseases, dementia, depression	Kawagishi *et a*l., 1991; Kawagishi *et al*., 1992
Hericenone L	sporophore	cytotoxic	cancers	Ma *et al*., 2012
Erinacines A,B,C,D,E,F,G,H, I,S	mycelium	nerve growth factor (NGF)-synthesis, reducing amyloid-β deposition by increasing insulin-degrading enzyme (IDE) expression	Alzheimer's and Parkinson's diseases, dementia, depression, neuropathic pain	Kawagishi *et al*., 1994; Kawagishi *et al*., 1996; Saito *et al*., 1998; Lee *et al*., 2000; Chen *et al*., 2016
Hericirine	sporophore	reduction of pro-inflammatory mediators and cytokines	inflammatory diseases	Wang *et al*., 2014
Erinacerines C-L	sporophore	α-Glucosidase inhibitory activity	Pompe disease, diabetes	Wang *et al*., 2015
Erinacene D	sporophore	NF-kB inhibitory activity	inflammatory disorders, cancers	Li *et al*., 2014
Erinapyrones A,B,C	subemerged cultures, sporophore	cytotoxic, antimicrobial	cancers	Kawagishi *et al*., 1992; Arnone *et al*., 1994; Mizuno *et al*., 1999
Hericenales A,B,C	subemerged cultures	antihyperglucemic. antihypersterolemic	diabetes	Vertesy *et al*., 1999
Hericenes A, B, C, D	mycelium	antimicrobial	bacteria	Kobayashi *et al*., 2018

1.2.1 *H. erinaceus* and its effects on Central Nervous System (CNS)

Hericenones and erinacines exert a predominant neuroprotective effect promoting Nerve Growth Factor (NGF) synthesis *in vitro* and *in vivo* (Kawagishi *et al.*, 2008). NGF is a highly conserved protein with an high molecular weight. It plays a crucial role for survival and proliferation and is involved in repair, regeneration and protection of neurons, supporting synapse formation and enhancing memory function (Obara and Nakahata, 2002; Kawagishi *et al.*, 2008). It is assumed that functional deficiency of NGF is related to Alzheimer's disease, a progressive neurodegeneration of the brain, commonly diagnosed in the aging population over 65 years old. Nowadays, degenerative brain diseases such as dementia are one of the major causes of decreased quality of life in humans during aging.

However, NGF cannot pass the blood-brain-barrier (BBB) and its effect by directly injection into the brain is an invasive treatment for patients. On the other hand, erinacines and hericenones were the first natural compounds with a low molecular weight capable to pass the BBB.

Until now, there were identified 15 erinacines and 8 of them display neuroprotective properties, such as enhancement of NGF-release (erinacines A-I), reduction of amyloid-β deposit, and management of neuropathic pain (erinacine E) (Li *et al.*, 2018) (see table 2).

Table 2: biological activities of erinacines isolated from *H. erinaceus* in *in vitro* and *in vivo* tests (Li *et al.*, 2018).

Erinacines	Tests	Concentration	Biological activities
Erinacine A	*In vitro*	1 mM	Induced 250.1 ± 36.2 pg/ml NGF synthesis
	In vivo	30 mg/kg body weight/day	(1) Reduced amyloid burden by 38.1 ± 19.7% (2) Increased IDE levels by 141.1 ± 63.7%
	In vivo	1 mg/kg body weight/day	Inhibited DLD-1 tumor growth by 66%
	In vivo	30 mg/kg body weight/day	(1) Reduced both the size and number of amyloid plaques (2) Increased IDE levels by 303.5% (3) Recovered from impairments in burrowing, nesting, and Morris water maze tasks
Erinacine B	*In vitro*	1 mM	Induced 129.7 ± 6.5 pg/ml NGF synthesis
Erinacine C	*In vitro*	1 mM	Induced 299.1 ± 59.6 pg/ml NGF synthesis
Erinacine D	*In vitro*	1.67 mM	Induced 141.5 ± 18.2 pg/ml NGF synthesis
Erinacine E	*In vitro*	5 mM	Induced 105.0 ± 5.2 pg/ml NGF synthesis
	In vitro	IC_{50}	Binding inhibitor for κ-opioid receptor at 0.8 μM
Erinacine F	*In vitro*	5 mM	Induced 175.0 ± 5.2 pg/ml NGF synthesis
Erinacine H	*In vitro*	70.8 mM	Induced 31.5 ± 1.7 pg/ml NGF synthesis
Erinacine S	*In vivo*	30 mg/kg body weight/day	(1) Reduced amyloid burden by 40.2 ± 15.2% (2) Increased IDE levels by 130.5 ± 68.9%
	In vivo	30 mg/kg body weight/day	(1) Reduced the size of amyloid plaques (2) Increased IDE levels by 269.8% (3) Recovered from impairments in burrowing, nesting, and Morris water maze tasks

Several experimental works, starting from 1994, reported the stimulation of NGF synthesis by a group of erinacines (Kawagishi *et al*., 1994). Ethanolic extract of *H. erinaceus* increases the level of NGF mRNA in 1321N1 human astrocytoma cells and its expression in the hippocampus via activation of the c-jun N-terminal kinase pathway (Mori *et al*., 2008). Furthermore, ethanolic extract of *H. erinaceus* promotes normal development of cultivated cerebellar cells suggesting a regulatory effect on the process of myelin genesis *in vitro* (Kolotushkina *et al*., 2003).

H. erinaceus sporophore extract was studied by Moldavan *et al*. (2007) on rat brain slices *in vitro*, using whole-cell patch clamp recording demonstrating that it exerts neurotropic action and improves the myelination process in the mature myelinating fibers, not affects nerve cell growth, and not evokes a toxic effect or nerve cell damage.

Furthermore, also *an H. erinaceus* aqueous extract promotes functional recovery following crush injury to the peroneal nerve in rats (Wong *et al*., 2011).

Phan *et al.* (2014) showed a potential neurotrophic activity in PC12 cells by the hericenone E mediated partially by TrKA phosphorylation by NGF.

In vivo studies on the effect of *H. erinaceus* assessed the effect on memory performance both in animal models and in humans. In particular in animal models recognition memory is valuated through different behavioural tests. Recognition memory is considered one of the most important features of human and mammalians personality. Recognition memory is defined as a long-term, declarative, episodic memory (fig. 5). Recognition memory is based on two processes: a “knowledge” process that allows the recognition of a novel or a familiar person or object and a “remember” that allows the reconstruction of a spatial and temporal context of the event (fig. 6). By using different test is possible to discriminate between the two steps of the recognition memory.

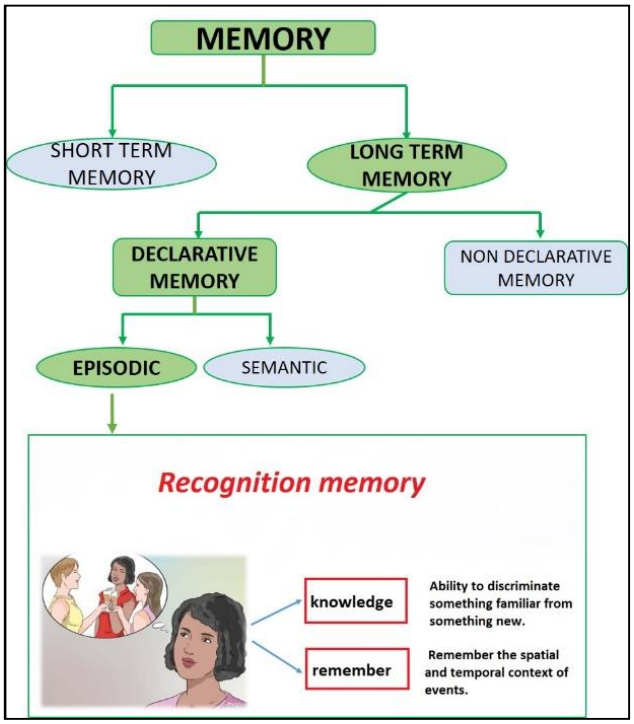

Fig. 5 (drawing by V. Cesaroni): schematic diagram of different types of memory.

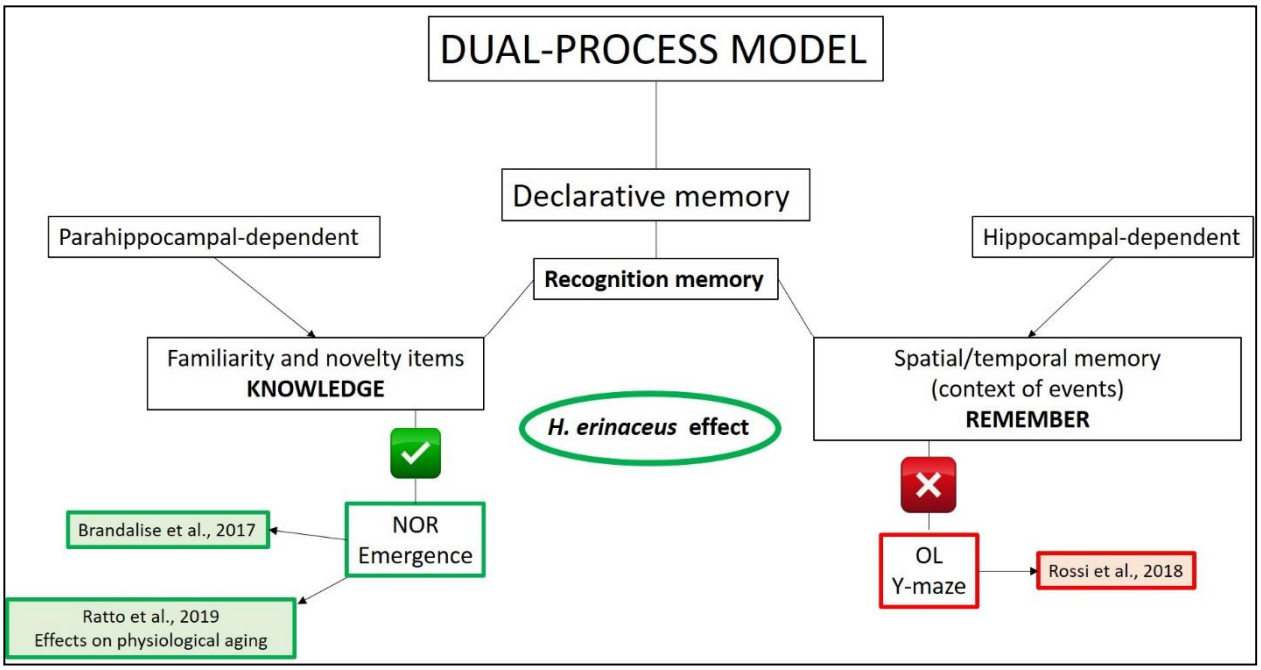

Fig. 6 (drawing by V. Cesaroni): schematic drawing of the effects of *H. erinaceus* on recognition memory and the hippocampal network.

Mori *et al*. (2011) examined the effect of a 23 days diet with lyophilized sporophore of *H. erinaceus* on memory and learning of mice with an Aβ(25-35)-induced cognitive impairment by using *in vivo* tests. Data revealed that *H. erinaceus* reduces impairments of spatial short-term memory and visual recognition memory, suggesting that it may be useful for short-term spatial memory performances.

In 2017, with the paper published by Brandalise *et al.* (2017), we tested the effect of two months oral supplement made by AVD reform (Casal Noceto, Parma) of *H. erinaceus* on middle aged wild-type mice, both *in vitro* and *in vivo*, considering cognitive skills and hippocampal neurotransmission. In particular, by means of patch-clamp whole-cell recordings we demonstrated that spontaneous glutamatergic synaptic currents (sEPSCs) recorded in CA3 neurons increased in both frequency and amplitude, and the mossy fiber-CA3 glutamatergic evoked synaptic currents (EPSCs) increased in amplitude. The mechanism involved in this increase in excitatory neurotransmission between mossy fiber and CA3 neurons is due to an increase in neurotransmission. Furthermore, *H. erinaceus* supplementation induced a significantly improvement in the novelty seeking behaviour and in the novel object recognition, in particular in the "knowledge" component of the recognition memory. After that, we evaluated the effect of an *H. erinaceus* daily diet in wild-type mice in spatial memory (the "remember" component of the recognition memory) by behavioural tests (Rossi *et al*., 2018). Results showed an increasing in general locomotor activity but no effect on spatial memory, leading us to the conclusion that *H. erinaceus* has a selective and discriminating effect between the two components of recognition memory. This data has allowed us to conferm the hypothesis that recognition memory involves two different anatomical structures (fig. 5, 6).

Among all the erinacines molecules erinacine A is described to display both *in vitro* and *in vivo* neuroprtective effects (see table 1). Results obtained in preclinical studies suggest that erinacine A is effective in reducing neurodegenerative disease-induced cell death, by enhancing NGF synthesis *in vitro* and NGF level in locus coeruleus and hippocampus in rats (Shimbo *et al*., 2005), by reducing infarcted volume in cortex and subcortex of transient stroke rats (Lee *et al*., 2014), and by reducing amyloid plaque burden in cerebral cortex in a transgenic mice model (Chen *et al*., 2016).

To date, there are growing preclinical studies that demontrate how *H. erinaceus* mycelium enriched with erinacines is able to reduce the risk of dementia and cognitive impairment in their early and intial stages (fig. 7).

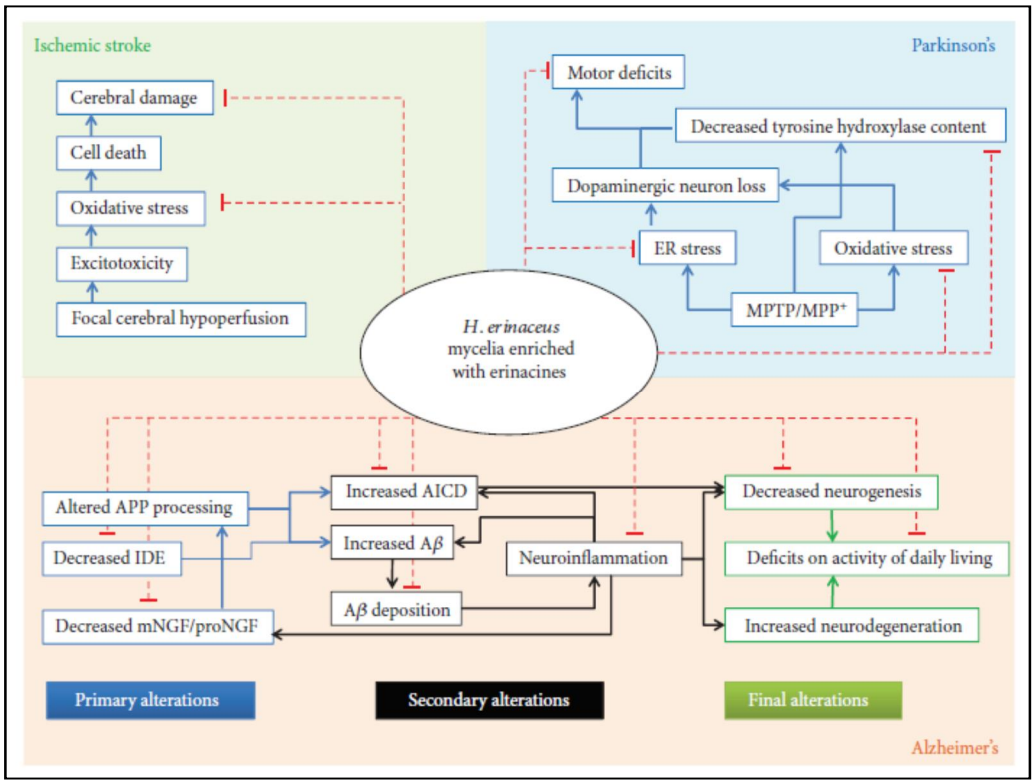

Fig. 7: mechanisms of action of *H. erinaceus* mycelium enriched with erinacines in stroke, Parkinson's and Alzheimer's diseases, (Li *et al*., 2018).

1.2.2 Human clinical trials

H. erinaceus was also tested in human trials. The first human clinical trial have been carried out by Kasahara *et al*. (2001) in a rehabilitation hospital in Japan. Patients suffering from dementia received 5 g of dried sporophore of *H. erinaceus* for 6 months and have been evaluated as functional independence measure (FIM) before and after the treatment. The majority of the patients improved the overall FIM scores and perceptual capacities; furthermore, some bedridden patients were able to get up and eat.

In another clinical test, Ohtomo *et al*. (2011) examined the efficacy of oral supplementation of powdered *H. erinaceus* for 3 months to dementia patients. Data were evaluated by the mini-mental state examination (MMSE) test that is used not only to screen dementia but also to estimate the severity of cognitive impairments following patients in the course of the pathology. Results obtained showed that people treated with *H. erinaceus* have significantly improved the MMSE score.

In a double-blind, parallel-group, placebo-controlled trial Mori et al. (2009) confirmed the beneficial effect of an oral supplementation with *H. erinaceus* tablets for 4 months in patients with mild cognitive impairments (MCI), evaluating through their functional independence scores (FIS).

Effects of oral *H. erinaceus* supplementation on depression, menopause, anxiety and sleep disorders were first investigated by Nagano *et al.* in 2010 in a randomized, double-blind, placebo-controlled trial over 4 weeks. By using Kupperman Menopausal Index (KMI), Center for Epidemiologic Studies Depression Scale (CES-D), the Pittsburgh Sleep Quality Index (PSQI) and the Indefinite Complain Index (ICI), researchers suggested that *H. erinaceus* may reduce depression and anxiety.

A pilot study examined the effect of *H. erinaceus* (Amyloban3399, a product made of amycenone, a standardized extract of *H. erinaceus* containing hericenones and amyloban) in female students, declaring the improvement of sleep quality, mood, attentiveness to one's surroundings, voluntary interactions with others and the level of mental alertness.

In the 2015, it was reported a case of schizophrenia who recovered from schizophrenia with Amyloban3399 (Inanaga, 2015), with a significantly improving of all the average scores on PANSS (positive and negative syndrome scale), including positive, negative, and general psychopathology.

Recently, mood disorder such as depression, anxiety, binge eating and sleep disorders have been investigated by Vigna *et al*. (2019) in an 8 weeks pilot study. The aim of the study was to evaluate the effect of the *H. erinaceus* supplement provided by AVD reform (the same supplement tested in Brandalise *et al.*, 2017; Rossi *et al*., 2018), in patients affected by overweight or obesity and subjected to a low calorie dietary regimen. Obesity and depression are connected by a bidirectional link and it has been reported that about 50% of overweight patients are depressed and *vice versa*.

Two months of *H. erinaceus* supplementation reduced anxiety, depression, and sleep disorders in overweight patients following a hypocaloric diet. The beneficial effect of oral supplementation persist also after 8 weeks wash-out.

Based on experimental studies, both on mice and humans, the *H. erinaceus* is considered safety and it could be used as functional ingredient with medicinal properties for the brain and nerve health. Daily consumption may keep people away from life-threatening disorders. More extensive clinical trials are needed to substantiate results obtained till now.

Besides the wide range of bioactivities reported, there could be other potential attractive properties not yet discovered. Furthermore, *H. erinaceus* is used from millennia in the Traditional Chinese Medicine and our experimental data support the popular knowledge, and connect like a bridge Western and Eastern medicine.

1.2.3 References

- Arnone A, Cardillo R, Nasini G, De Pava OV. Secondary mold metabolites: Part 46. 1994. Hericenes A-C and erinapyrone C, new metabolites produced by the fungus Hericium erinaceus. J. Nat. Prod. 57,602–606.
- Brandalise F, Cesaroni V, Gregori A, Repetti M, Romano C, Orrù G, Botta L, Girometta C, Guglielminetti ML, Savino E. 2017. Dietary Supplementation of Hericium erinaceus Increases Mossy Fiber-CA3 Hippocampal Neurotransmission and Recognition Memory in Wild-Type Mice. Evid. Based Complement. Alternat. Med. 3864340:1-13.
- Chen CC, Tzeng TT, Chen CC, Ni CL, Lee LY, Chen WP, Shiao YJ, Shen CC. 2016. Erinacine S, a Rare Sesterterpene from the Mycelia of Hericium erinaceus. J Nat Prod. 79(2):438-441.
- Friedman M. 2015. Chemistry, Nutrition, and Health-Promoting Properties of Hericium erinaceus (Lion's Mane) Mushroom Fruiting Bodies and Mycelia and Their Bioactive Compounds J Agric Food Chem. 63(32):7108-7123.
- Gerbec B, Tavcar E, Gregori A, Kreft, S, Berovic M. 2015. Solid State Cultivation of Hericium erinaceus Biomass and Erinacine: A Production. J. Bioproces. Biotech., 5,1-5.
- He X, Wang X, Fang J, Chang Y, Ning N, Guo H, Huang L, Huang X, Zhao Z. 2017. Structures, biological activities, and industrial applications of the polysaccharides from Hericium erinaceus (Lion's Mane) mushroom: A review. Int J Biol Macromol. 97:228-237.
- Inanaga K. 2015. Recovery from Schizophrenia with Bioactive Substances in Hericium erinaceum. Int J Sch Cog Psychol. S1:1.
- Jiang S, Wang S, Sun Y, Zhang Q. 2014. Medicinal properties of Hericium erinaceus and its potential to formulate novel mushroom-based pharmaceuticals. Appl Microbiol Biotechnol. 98:7661-7670.
- Kasahara K, Kaneko N, Shimizu K. 2001. Effects of Hericium erinaceum on aged patients with impairments. Gunma Med Suppl. 76:77-78 (in Japanese).
- Kawagishi H, Zhuang C. 2008. Compounds for dementia from Hericium erinaceum. Drugs Fut. 33(2):149.
- Kawagishi H, Ando M, Mizuno T. 1990. Hericenone A and B as cytotoxic principles from the mushroom Hericium erinaceum. Tetrahedron Lett. 31,373–376.

- Kawagishi H, Ando M, Sakamoto H, Yoshida S, Ojima F, Ishiguro Y, Ukai N, Furukawa SC. 1991. Hericenones C, D and E, stimulators of nerve growth factor (NGF)-synthesis, from the mushroom Hericium erinaceum. Tetrahedron Lett. 32,4561–4564.
- Kawagishi H, Ando M, Shinba K, Sakamoto H, Yoshida S, Ojima F, Ishiguro Y, Nobuo U, Shoei F. 1992. Chromans, hericenones F, G and H from the mushroom Hericium erinaceum. Phytochemistry. 32(1):175-178.
- Kawagishi H, Shimada A, Hosokawa S, Mori H, Sakamoto H, Ishiguro Y, Sakemi S, Bordner J, Kojima N, Furukawa S. 1996. Erinacines E, F, and G, stimulators of nerve growth factor (NGF) synthesis from the mycelia of Hericium erinaceum. Tetrahedron Lett. 37,7399–7402.
- Kawagishi H, Shimada A, Shirai R, Okamoto K, Ojima F, Sakamoto H, Ishiguro Y, Furukawa S. 1994. Erinacines A, B and C strong stimulators of nerve growth factor (NGF)-synthesis from the mycelia of Hericium erinaceum. Tetrahedron Lett. 35,1569–1572.
- Kawagishi H, Shimada A, Shirai R, Okamoto K, Ojima F, Sakamoto H, Ishiguro Y, Furukawa S. 1994. Erinacines A, B and C strong stimulators of nerve growth factor (NGF)-synthesis from the mycelia of Hericium erinaceum. Tetrahedron Lett. 35,1569–1572.
- Kawagishi H, Simada A, Shizuki K, Mori H, Sakamoto H, Furukawa S. 1996. Erinacine D, a stimulator of NGF-synthesis from the mycelia of Hericium erinaceum. Heterocycl Commun. 2,51–54.
- Khan A, Tania M, Liu R, Rahman MM. 2013. Hericium erinaceus: an edible mushroom with medicinal values. J Complement Integr Med. 10(1):253-258.
- Kolotushkina EV, Moldavan MG, Voronin KY, Skibo GG. 2003. The influence of Hericium erinaceus extract on myelination process in vitro. Fiziologicheskii zhurnal. 49(1):38-45.
- Kobayashi S, Hamada Y, Yasumoto T, Hashino Y, Masuyama A, Nagai K. 2018. Total syntheses and endoplasmic reticulum stress suppressive activities of hericenes A-C and their derivatives. Tetrahedron Lett. 59:1733–1736.
- Lee EW, Shizuki K, Hosokawa S, Suzuki M, Suganuma H, Inakuma T, Li J, Ohnishi-Kameyama M, Nagata T, Furukawa S, Kawagish H. 2000. Two novel diterpenoids, erinacines H and I from the mycelia of Hericium erinaceum. Biosci. Biotechnol. Biochem. 64:2402-2405.

- Lee KF, Chen JW, Teng CC, Shen CH, Hsieh MC, Lu CC, Lee KC, Lee LY, Chen WP, Chen CC, Huang WS, Kua HC. 2014. Protective effects of Hericium erinaceus mycelium and its isolated erinacine a against ischemia-injury-induced neuronal cell death via the inhibition of iNOS/p38 MAPK and nitrotyrosine. Int J Mol Sci. 15:15073–15089.
- Li G, Yu K, Li F, Xu K, Li J, He S, Cao S, Tan G. 2014. Anticancer potential of Hericium erinaceus extracts against human gastrointestinal cancers. Journal of Ethnopharmacology. 153(2):521-530.
- Li IC, Lee LY, Tzeng TT, Chen WP, Chen YP, Shiao YJ, Chen CC. 2018. Neurohealth properties of Hericium erinaceus mycelia enriched with erinacines. Behavioural Neurology. 2018:1-10.
- Li W, Zhou W, Kim EJ, Shim SH, Kang HK, Kim YH. 2015. Isolation and identification of aromatic compounds in Lion's Mane Mushroom and their anticancer activities. Food Chem. 170,336–342.
- Ma BJ, Ma JC, Ruan Y. Hericenone L, a new aromatic compound from the fruiting bodies of Hericium erinaceums. 2012. Chinese Journal of Natural Medicines. 10(5):363-365.
- Mizuno T, Wasa T, Ito H, Suzuki C, Ukai N. 1992. Antitumor-active polysaccharides isolated from the fruiting body of Hericium erinaceum, an edible and medicinal mushroom called yamabushitake or houtou. Biosci Biotechnol Biochem. 56(2):347-8.
- Mizuno T. 1999. Bioactive Substances in Hericium erinaceus (Bull.: Fr.) Pers. (Yamabushitake), and Its Medicinal Utilization. 1(2):105-119.
- Moldavan MG, Grygansky AP, Kolotushkina OV, Kirchhoff B, Skibo GG, Pedarzani P. 2007. Neurotropic and Trophic Action of Lion's Mane Mushroom Hericium erinaceus (Bull.: Fr.) Pers. (Aphyllophoromycetideae) Extracts on Nerve Cells in Vitro. IntJMedMushr. 9(1):15-28.
- Mori K, Inatomi S, Ouchi K, Azumi Y, Tuchida T. 2009. Improving effects of the mushroom Yamabushitake (Hericium erinaceus) on mild cognitive impairment: a double-blind placebo-controlled clinical trial. Phytother Res. 23(3):367-72.
- Mori K, Kikuchi H, Obara Y, Iwashita M, Azumi Y, Kinugasa S, Inatomi S, Oshima Y, Nakahata N. 2010. Inhibitory effect of hericenone B from Hericium erinaceus on collagen-induced platelet aggregation. Phytomedicine. 17(14):1082-5.

- Mori K, Obara Y, Hirota M, Azumi Y, Kinugasa S, Inatomi S, Nakahata N. 2008. Nerve growth factor-inducing activity of Hericium erinaceus in 1321N1 human astrocytoma cells. Biol Pharm Bull. 31(9):1727-1732.
- Mori K, Obara Y, Moriya T, Inatomi S, Nakahata N. 2011. Effects of Hericium erinaceus on amyloid β(25-35) peptide-induced learning and memory deficits in mice. Biomedical Research. 32(1):67-72.
- Nagano M, Shimizu K, Kondo R, Hayashi C, Sato D, Kitagawa K, Ohnuki K. 2010. Reduction of depression and anxiety by 4 weeks Hericium erinaceus intake. Biomed Res. 31(4):231-7.
- Obara Y, Nakahata N. 2002. The signaling pathway of neurotrophic factor biosynthesis. Drug News Perspect. 15(5):290-8.
- Ohtomo E, Shimizu M, Komatsu Y. 2011. Clinical effects of Yamabushitake on senile dementia of Alzheimer type. Rouneikagu. 47:1061-1066 (in Japanese).
- Phan CW, Lee GS, Hong SL, Wong YT, Brkljača R, Urban S, Malek SNA, Sabaratnam V. 2014. Hericium erinaceus (Bull.: Fr) Pers. cultivated under tropical conditions: isolation of hericenones and demonstration of NGF-mediated neurite outgrowth in PC12 cells via MEK/ERK and PI3K-Akt signaling pathways. Food Funct. 5:3160-9.
- Rai M, Tidke G, Wasser S. 2005. Therapeutic potential of mushrooms. Natural Product Radiance. 4(4).
- Rossi P, Cesaroni V, Brandalise F, Occhinegro A, Ratto D, Perrucci F, Lanaia V, Girometta C, Orrù G, Savino E. 2018. Dietary Supplementation of Lion's Mane Medicinal Mushroom, Hericium erinaceus (Agaricomycetes), and Spatial Memory in Wild-Type Mice. Int. J. Med. Mushrooms. 20,485–494.
- Saito T, Aoki F, Hirai H, Inagaki T, Matsunaga Y, Sakakibara T, Sakemi S, Suzuki Y, Watanabe S, Suga O, Sujaku T, Smogowicz AA, Truesdell SJ, Wong JW, Nagahisa A, Kojima Y, Kojima N. 1998. Erinacine E as a kappa opioid receptor agonist and its new analogs from a basidiomycete, Hericium ramosum. J Antibiot. 51(11):983-90.
- Shang X, Tan Q, Liu R, Yu K, Li P, Zhao GP. 2013. In vitro anti-Helicobacter pylori effects of medicinal mushroom extracts, with special emphasis on the Lion's Mane mushroom, Hericium erinaceus (higher Basidiomycetes). Int J Med Mushrooms 15:165–174.

- Shimbo M, Kawagishi H, Yokogoshi H. 2005. Erinacine A increases catecholamine and nerve growth factor content in the central nervous system of rats. Nutrition Research. 25(6):617-623.
- Sokol S, Golak-Siwulska I, Sobieralski K, Siwulski M, Gorka K. 2016. Biology, cultivation, and medicinal functions of the mushroom Hericium erinaceum. Acta Mycologica. 50(2):1-18.
- Thongbai B, Rapior S, Hyde KD, Wittstein K, Stadler M. 2015. Hericium erinaceus, an amazing medicinal mushroom. Mycological Progress. 14(91):1-23.
- Vertesy L, Kurz M, Schindler P, Stump H. 1999. Novel phthalaldehyde derivatives, process for their preparation, and their use CA 2246928.
- Vigna L, Morelli F, Agnelli GM, Napolitano F, Ratto D, Occhinegro A, Di Iorio C, Savino E, Girometta C, Brandalise F, Rossi P. 2019. Hericium erinaceus Improves Mood and Sleep Disorders in Patients Affected by Overweight or Obesity: Could Circulating Pro-BDNF and BDNF Be Potential Biomarkers? Evid Based Complement Alternat Med. 7861297:1-12.
- Wang JC, Hu SH, Su CH, Lee TM. 2001. Antitumor and immunoenhancing activities of polysaccharide from culture broth of Hericium spp. Kaohsiung J Med Sci. 17(9):461-7.
- Wang M, Gao Y, Xu D, Gao Q. 2015. A polysaccharide from cultured mycelium of Hericium erinaceus and its anti-chronic atrophic gastritis activity. Int J Biol Macromol. 81:656-61.
- Wang M, Gao Y, Xu D, Konishi T, Gao Q. 2014. Hericium erinaceus (Yamabushitake): a unique resource for developing functional foods and medicines. Food Funct. 5(12):3055-3064.
- Wong KH, Naidu M, David P, Abdulla MA, Abdullah N, Kuppusamy UR, Sabaratnam V. 2011. Peripheral Nerve Regeneration Following Crush Injury to Rat Peroneal Nerve by Aqueous Extract of Medicinal Mushroom Hericium erinaceus (Bull.: Fr) Pers. (Aphyllophoromycetideae). Evid Based Complement Alternat Med. 2011:580752.
- Xu H, Wu PR, Shen ZY, Chen XD. 2010. Chemical analysis of Hericium erinaceum polysaccharides and effect of the polysaccharides on derma antioxidant enzymes, MMP-1 and TIMP-1 activities. Int J Biol Macromol. 47(1):33-6.

1.3 Medicinal mushrooms and supplementation

1.3.1 "The mushrooms pyramid"

Chang and Wasser (2012) stated that about more than 100 medicinal functions are thought to be related to medicinal mushrooms (MM).

According to an old Chinese saying, medicine and food have a common origin. This quote can be attributed also to medicinal mushrooms for their nutritional qualities. From more than 3000 years, Chinese population used mushrooms as a food for the human healthy state, as a medicine to treat the ill states and as dietary supplement to strengthen the sub-healthy state between the healthy and the ill states (fig. 1).

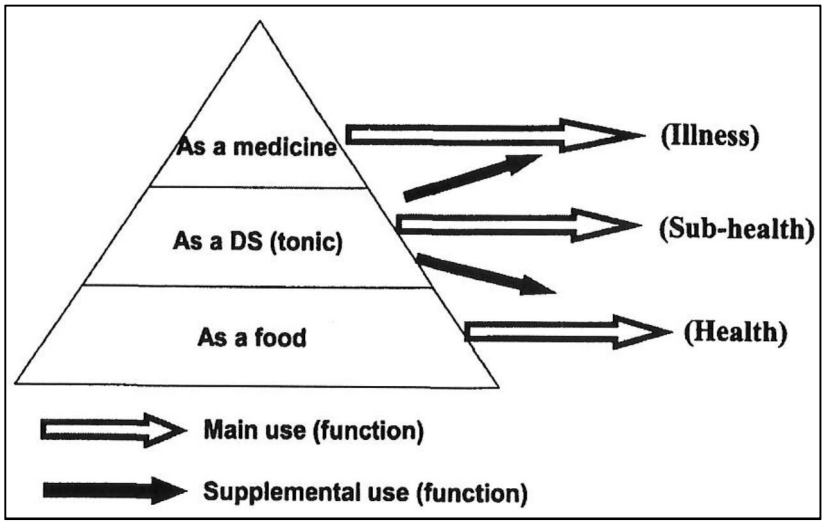

Fig. 1: a pyramid model of the mushrooms usage (Chang and Wasser, 2012).

Nowadays, medicinal mushrooms, such as *H. erinaceus*, produce beneficial effects not only as a drugs but also as a novel class of products: dietary supplements (DS), functional foods, nutraceuticals and mycopharmaceuticals.

Medicinal mushrooms have been used in many different cultures and are considered safe for their long traditional use. Nevertheless, safety on MM preparations is strictly lacking and must be mandatory for any regulation measure and should be verified and proven as thoroughly as possible.

The interest in the treatment of physiological disorders with natural remedies and the recognition of some beneficial properties of mushrooms has led to the birth of "mushrooms nutriceuticals", mycelium or sporophore extract (or dried), consumed in tablets or capsules as a DS with a potential therapeutic effect.

One of the most important criticism in studying dietary supplements from medicinal mushroom preparations regards the products variety available on markets:

- powders of cultivated sporophores;
- hot water or alcoholic extracts of sporophores;
- dried and pulverized preparations of mycelia, primordia and sporophores (single or combined);
- mycelium harvested from submerged liquid cultures;
- dried sporophores in capsules or tablets;
- spores or their extracts.

Chang (2006) suggested a list of guidelines to improve the quality of mushroom products:

- good laboratory practice (GLP) for maintaining and preserving the nature and the source of the stain used;
- good agriculture practice (GAP) for monitoring the growth and environmental conditions;
- good manufacturing practice (GMP) for monitoring the extraction processes;
- good production practice (GPP) for analyzing chemical and microbial composition;
- good clinical practice (GCP) for evaluate the appropriate dosage and formulation of products.

Many problems regard labeling of MM products because the majority are marketed as mushrooms although they contain different fungal ingredients (mycelium or sporophore) or a mixture of grains and mushrooms, sometimes not fully specified.

To date, approximately 80% of MM products are composed of cultivated sporophores and their composition results considerably diverse, inconstant and unpredictable because the growth substrate and ingredients. One primary step in mushrooms cultivation could be optimizing the culture medium composition and the growth condition to obtain a high yield of biomass and known amounts of specific metabolites.

Up to now, there are already many MM species without a correct scientific name. So, future studies could be helpful to check the correct identification, correlating classical taxonomical methods and DNA barcoding.

1.3.2 Criticisms about dietary supplements production

Unresolved issues including standardization, regulation and safety of medicinal mushrooms dietary supplements are still in the early stages of the debate, as reported in various papers (Chang and Wasser, 2012; Wasser, 2014; Zied *et al.*, 2017) (fig. 2).

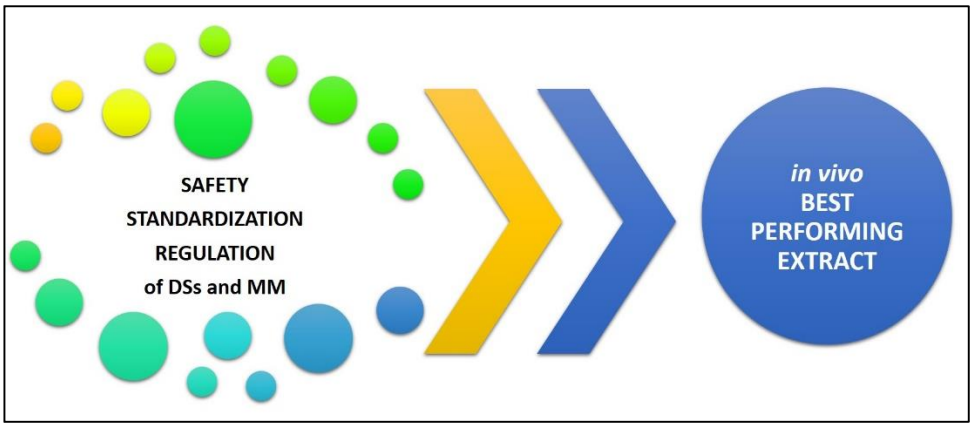

Fig. 2 (drawing by V. Cesaroni): criticisms about dietary supplements production that could led to the resolution of a standardized extract.

Until now, there are not internationally recognized standards and protocols for the production and testing MM products. This leads to a difference in the composition and in the related medicinal effect expected among the various products available on the markets. In fact, it is not known whether bioactive effects are attributable to the activity of a single component or if the effect is the consequence of a synergistic mechanism between the various bioactive molecules of the fungus.

At the present time, the majority of the medicinal properties of MM products have been evaluated through *in vitro* and animal-based experiments, with a lack to the application to the human context.

Improvements in the creation of standards for the production and protocols for the control of quality are essential to retain the consumer, protect public health, and to maintain a high level of quality and safety set by regulatory authorities.

One of the advantages that would be useful to exploit should be to combine the knowledge of Western and Eastern medicine. They adopted different regulation systems among countries for medicinal plants and fungi to evaluate the quality control and the best standards for the consumers of commercial products. One of the major differences between the two regulation regard the necessity of clinical studies before the introduction to the markets of MM dietary supplements.

1.3.3 References

- Chang ST, Wasser SP. 2012. The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. Int J Med Mushrooms. 14(2):95-134.
- Wasser SP. 2014. Medicinal mushroom science: Current perspectives, advances, evidences, and challenges. Biomed J. 37(6):345-56.
- Chang ST. 2006. The World Mushroom Industry: Trends and Technological Development. Int J Med Mushrooms. 8(4):297-314.
- Zied DC, Pardo-Giménez A. 2017. Edible and medicinal mushrooms: technology and applications. Wiley, West Sussex.

2. AIM OF THE PHD PROJECT

The final goal of my PhD project was to reconstruct the experimental chain, starting from samplings of *Hericium* sporophores in Italy to functional behavioural tests in mice, to obtain the best performing extract on recognition memory.

Furthermore, to achieve the aim of my PhD project, I set myself some key points in the experimental development and procedures:

- preserve the biodiversity of this Mediterranean resource through *ex situ* conservation;
- collect Italian samples to evaluate the intraspecific biological variability, comparing with those collected in other countries of the world;
- maintain a translational approach in functional tests in order to obtain data in mice that could be transferred to humans, “from bench to bedside”;
- recognize, quantify and screen some neuroactive metabolites present in Italian samples by using analytical chemistry methods.

Thanks to an interdisciplinary approach and solid collaborations between various laboratories, I was able to follow all the stages of the supply chain to obtain the best performing MM extract (fig. 1).

Fig. 1 (drawing by V. Cesaroni): gears of different activities done during my PhD project that led to the creation of high quality products, containing the best performing medicinal mushroom extract used in behavioural tests.

3. RESULTS AND DISCUSSION

The necessary steps to achieve the goals of my PhD project on *H. erinaceus* Italian samples, never investigated before, were:

1. to collect wild sporophores in order to isolate the strains;
2. to deposit the ITS sequences in GeneBank;
3. to detect and to quantify a few metabolites affecting the central nervous system (CNS) in order to select the potentially best performing strain/s;
4. to test the *H. erinaceus* selected materials (mycelium, cultivated and wild-type sporophores) *in vivo* experiments on memory performances in mice.

Results obtained in point 1 and 2 were published in Cesaroni *et al*. (2019). Sporophores of *H. alpestre*, *H. coralloides* and *H. erinaceus* were collected in Italy and the corresponding mycelia were isolated in pure culture. Internal transcribed spacer (ITS) sequences from the Italian strains were phylogenetically compared along with 64 other sequences available from GenBank, the CBS Strain Database and the European Nucleotide Archive (ENA) for the same *Hericium* species. Bayesian phylogenetic analysis produced a phylogram that permitted a good discrimination between *Hericium* species, providing an updated phylogeny within the genus *Hericium* and a better understanding of affinity among the species analyzed. The phylogenetic comparison further confirmed the importance of a joint morphological-molecular approach to avoid misidentification and to guarantee the quality of strains for further chemical and medicinal characterization.

Once obtained the molecular identification of the strains, it was possible to proceed with the identification and quantification of the neuroactive metabolites (point 3) present in our samples.

Until now, these analyses regard only *H. coralloides*, as described in the first chapter, *H. erinaceus* strain 1 (Ratto *et al*., 2019) and strain 2 (Corana *et al*., 2019). In this last study, an array of metabolites at different growth stages (mycelium, primordium and cultivated and wild-type sporophores) of H.e.2 was analyzed. In particular, for the first time there were described the metabolites present from the formation of the mycelium to the appearance of the primordium and up to the sporophore development, the hericenes.

The last point of my PhD was obtained thanks to the scientific contribution of two papers, Brandalise *et al*. (2017) and Rossi *et al.* (2018), which investigated the effect of a commercial product provided by AVD Reform, Parma, Italy, containing *H. erinaceus* in a wild type middle aged mouse model with

respect to the double component of the recognition memory ("knowledge" and "remember"). Thanks to these data, it was possible to confirm the dual-component hypothesis of memory recognition, asserting that the two component are placed in two different brain areas, such as hippocampus and parahippocampus.

After discovering these results, it was evaluated if *H. erinaceus* could also improve recognition memory during physiological aging (Ratto *et al*., 2019). At this purpose, it was developed a model of physiological aging in mice, and frailty index (FI) for monitoring cognitive and locomotor decline during aging was tuned. Data revealed that the locomotor and cognitive performances developed in parallel. Locomotor performances anticipate the cognitive decline in recognition memory and decreased to a more extent. These data suggest that, by monitoring locomotor performances, we can intercept cognitive decline in memory. Once obtained a frailty index, the most frailty mice were indivuduated. In frailty mice the effect of two months of oral supplementation of H. *erinaceus* was studied. An ethanolic extract of a blend made by a known amount of erinacine A and hericenones C and D of an Italian strain of *H. erinaceus* (strain H.e.1) was used for the tests. *H. erinaceus* extract reverted the cognitive frailty of about 4 months in mice, corresponding at about 13 years in humans. It should be noted that a translational approach was used with the purpose to transfer the results obtained in preclinical model to clinical trials on humans. For this reason, the blend with a known amount of bioactive metabolites to mimic the dose administered to humans and behavioural test that are comparable to that carried out for human in clinical.

The mechanism involved in this effect was assessed by immunohystochemical experiments on hippocampus and cerebellum sections, testing both the proliferating cell nuclear antigen (PCNA) and doublecortin (DCX), as specific markers of active proliferation and neurogenesis.

Results showed an increased expression of both these markers in hippocampus and cerebellum of adult mice, indicating an effect of *H. erinaceus* on cell proliferation and neurogenesis.

The articles published during my PhD are listed below:

- Cesaroni *et al*., 2019, Int. J. Med. Mushrooms. 24;3511:1-14;
- Corana *et al*., 2019, Molecules, 2019,24:3511;
- Brandalise *et al*., 2017, Evid. Based Complement. Alternat. Med. 3864340:1-13;
- Rossi *et al*., 2018, Int. J. Med. Mushrooms. 20,485–494;
- Ratto *et al*., 2019, Nutrients, 2019(11):1-20.

Phylogenetic Comparison between Italian and Worldwide *Hericium* Species (Agaricomycetes)

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ABSTRACT: A broad literature concerns the genus *Hericium*, mainly regarding the medicinal properties of *H. erinaceus*. Congeneric species of *H. erinaceus* have been poorly investigated. We collected basidiomata of *H. alpestre*, *H. coralloides* and *H. erinaceus* in Italy and isolated the corresponding mycelia in pure culture. Analysis of the respective internal transcribed spacer regions confirmed the morphological identification of the strains. Internal transcribed spacer sequences from the Italian strains were phylogenetically compared along with 64 other sequences available from Gen-Bank, the CBS Strain Database, and the European Nucleotide Archive (ENA) for the same *Hericium* species. Geographic origin and host plant species were cross-checked using the above data banks. Bayesian phylogenetic analysis produced a phylogram that permitted good discrimination among *Hericium* species. It provides an updated phylogeny within the genus *Hericium* and a better understanding of affinity among the species analyzed. The main *Hericium* clade includes the following: the *H. erinaceus* group and the *H. alpestre*/*H. coralloides* group, where the two species cluster separately. This study also allowed us to differentiate the *H. erinaceus* group on a biogeographical basis. The phylogenetic comparison further confirms the importance of a joint morphological-molecular approach to avoid misidentification and to guarantee the quality of strains for further chemical and medicinal characterization.

KEY WORDS: *Hericium*, internal transcribed spacer, medicinal mushrooms, morphological identification, nuclear ribosomal DNA, phylogeny

ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; **ITS**, internal transcribed spacers; **NGF**, nerve growth factor

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TO WHOM IT MAY CONCERN

Our referees have now considered manuscript titled ñPhylogenetic Comparison between Italian and Worldwide *Hericium* Species (Agaricomycetes), *Cesaroni Valentina, Brusoni Maura, Cusaro Carlo Maria, Girometta Carolina, Perini Claudia, Picco Anna Maria, Rossi Paola, Salerni Elena, & Savino Elena* and have recommended publication in the International Journal of Medicinal Mushrooms. We are pleased to accept your manuscript in its current form which will now be forwarded to the publisher for copy editing and typesetting.

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Article

Array of Metabolites in Italian *Hericium erinaceus* Mycelium, Primordium, and Sporophore

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Abstract: *Hericium erinaceus* is a medicinal mushroom that contains many molecules promising a plethora of therapeutic properties. In this study, the strain H.e.2 (MicUNIPV, University of Pavia, Italy) was isolated from a sporophore collected in Tuscany (Italy). Mycelium, primordium, and wild type and cultivated sporophores were analyzed by HPLC-UV-ESI/MS. Erinacine A in the mycelium and hericenones C and D in the sporophores were quantified by comparison with their standard molecules. For the first time, *H. erinaceus* primordium was also investigated for the presence of these molecules. Comparing with the literature data, hericenes, molecules structurally similar to hericenones, were present in all our samples. The highest contents of hericenones C and D were detected in cultivated sporophores, compared to the wild type. The comparison of these data with those of another Italian *H. erinaceus* strain (H.e.1 MicUNIPV) was discussed. The results led us to select *H. erinaceus* strains more suitable for mycelium production or sporophore cultivation to obtain extracts with a higher content of bioactive compounds. This work provides a further step towards standardizing the procedures in the development of dietary supplements made from mushrooms.

Keywords: medicinal mushroom; *Hericium erinaceus*; bioactive compounds; mycelium; sporophore; primordium; erinacines; hericenones; hericenes

1. Introduction

Hericium erinaceus (Bull.) Pers. is a fungus belonging to Basidiomycota, Agaricomycetes, Russulales, and Hericiaceae [1]. Among all mushrooms, *H. erinaceus*, an edible and medicinal mushroom in traditional Chinese medicine, has been widely reported to have healthy effects on: the central nervous system [2,3]; different cancerous cell lines, such as HepG2 (hepatoma), MCF7 (breast cancer), HL-60 (human acute promyelocytic leukemia), and SGC-7901 (human gastric cancer cells) [4–6]; depression [7]; diabetes [8]; lipedema [9]. It also exhibited a reversion of frailty cognitive decline during aging [10]. Up to now, about 70 different secondary metabolites have been isolated from either sporophore or mycelium, or both. The investigation of chemical constituents promising for their

properties is constantly being updated in the search for the discovery of a new drug source [11]. Both the high-weight metabolites (e.g., polysaccharides) and low-weight metabolites (e.g., polyketides, phenols, and terpenoids) include bioactive molecules, although each substance category provides in turn an extremely various bouquet of molecules, where only a fraction shows evidence for bioactivity [12–15]. Hericenones are low-weight aromatic compounds first isolated by Kawagishi et al. (1990) [14] from the sporophore of *H. erinaceus.* Up to now, eight different compounds have been recognized as hericenones (A–H) [11,16]. Hericenones C, D, and E have stimulating activity on the synthesis of nerve growth factor (NGF) [17]. Hericenone F has been reported to be responsible for an anti-inflammatory effect by reducing nitrogen monoxide (NO) release [18]. Analyses of dried sporophores have detected different volatile compounds, some of them represented by hexadecanoic acid, linoleic acid, phenylacetaldehyde, and benzaldehyde. The erinacines in *H. erinaceus* are a group of cyathane-type diterpenoids, including 20 members of 24 diterpenoids described by Tang et al. [19]. To date, 15 erinacines (A, B, C, D, E, F, G, H, I, P, Q, J, K, R, S) isolated from *H. erinaceus* mycelium have been identified and eight out of 15 show neuroprotective properties, such as enhancing NGF release (erinacines A–I), reducing amyloid-β deposition, increasing insulin-degrading enzyme (IDE) expression (erinacines A and S), and managing neuropathic pain (erinacine E), while the others have different pharmacological activities [12,20–26].

At present, *H. erinaceus* is widely used as a dietary supplement. Nevertheless, the lack of standardization strongly affects the quality and effective bioactivity of the final product. As above described, only a few molecules have been reported to stimulate NGF release, namely erinacines A–I from mycelium (the most studied being erinacine A) and hericenones C–D from sporophore. To obtain this specific target on neuroprotection, the standardization process of dietary supplements therefore relies on the selective detection and quantification of such molecules. A major problem at this concern is the availability of pure analytical standards, due to the difficulty in the isolation and achievement of suitable amounts.

The aim of this study is to analyze and compare different stages of *H. erinaceus* (mycelium, primordium, and sporophore) sampled in Tuscany (Italy), in order to detect the presence and to quantify the concentration of the target bioactive metabolites erinacine A and hericenones C and D. The results could be useful for suggesting optimization strategies for future dietary supplements.

2. Results

2.1. H. erinaceus Samples for Chemical Analyses

The *H. erinaceus* wild type (WT) sporophore analyzed in the present study was collected on a living holm oak (*Quercus ilex* L.) in the hilly area around Siena (Italy) in 2018. The sporophore was identified based on the macro- and micromorphological characteristics of the species [27]. The main features are reported in Table 1.

Table 1. Characteristics of *Hericium erinaceus* wild type sporophore.

	H. erinaceus
fresh weight (g)	620
dried weight (g)	153
diameter (cm)	about 20
remarks on the sporophore	the collected specimen was mature, without any alteration by atmospheric or animal agents

The strain obtained from the WT was confirmed to belong to *H. erinaceus* [28] and is maintained in the Fungal Research Culture Collection of Pavia University (MicUNIPV) as H.e.2.

The cultivation of *H. erinaceus* at the Botanical Garden of the University of Pavia (Italy) led to the collection of 44 sporophores, total weight 1344.2 g. The diameter of the collected samples was 6–15 cm.

The primordium can be considered the transition stage before sporophore. It is formed by dense mycelial cords developing with negative geotropism (Figure 1). Primordia were harvested after 60 days, at the maximum of their development, and the fresh material was analyzed.

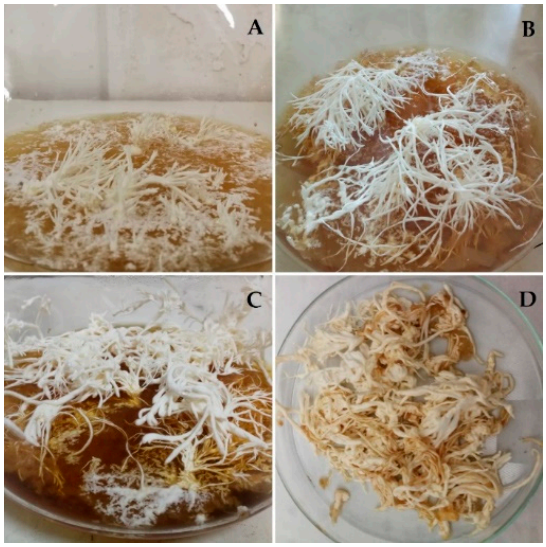


Figure 1. Different stages of growth of the primordium. 30 (**A**), 45 (**B**), 60 (**C**) days of growth and fresh collected material (**D**).

2.2. Chemical Analyses

We processed 1 g of lyophilized mycelium, dried WT and cultivated sporophores, and fresh primordium for chemical analyses.

In order to identify and analyze the bioactive metabolites present in our samples, we compared them with the standard molecules of erinacine A and hericenones C and D by HPLC-UV-ESI/MS. The molecular formula, chemical structures, and molecular weight of these molecules are reported in Table 2 [29].

Table 2. Molecular formula, chemical structures, and molecular weights of erinacine A and hericenones C and D.

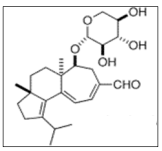
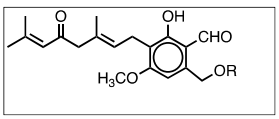
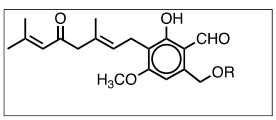
	Erinacine A	**Hericenone C**	**Hericenone D**
molecular formula	$C_{25}H_{36}O_6$	$C_{35}H_{54}O_6$	$C_{37}H_{58}O_6$
molecular weight (MW) (g/mol)	432	570	598
chemical structure		 R = palmitoyl	 R = stearoyl

Figure 2 shows the mass spectrum (MS) chromatographic traces of H.e.2 mycelium and the standard molecule of erinacine A. The standard erinacine A was detected using HPLC-UV-ESI/MS at the retention time (RT) of 10.44 min. By comparing the RT and molecular ion or mass spectra, the presence of this molecule in the H.e.2 mycelium was detected too. Besides, the chromatographic trace of H.e.2 mycelium also showed a peak at RT 12.58 min that belongs to a molecule not yet identified with a MW of 430 Da.

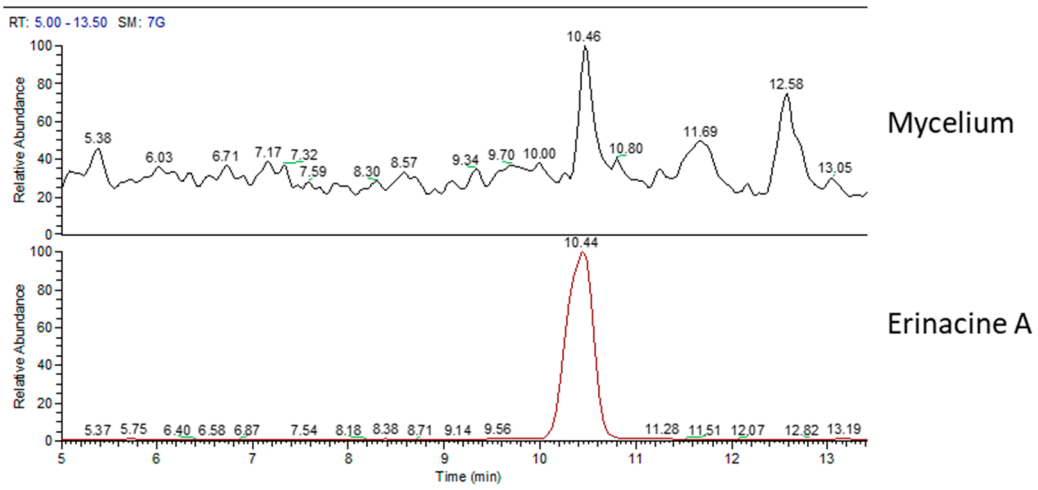


Figure 2. Mass spectrum (MS) traces of mycelium (top) and erinacine A molecule standard (bottom).

Figure 3 shows the UV chromatographic traces of H.e.2 mycelium and the standard molecules of hericenones C and D. In mycelium, hericenones C and D were not present. Moreover, the peaks at RT 46.46 min, 47.41 min, 47.63 min, and 48.83 min were supposed to be, respectively, hericene D, hericene A, hericene B, and hericene C, based on the data reported by Arnone et al. (1994) and Kobayashi et al. (2018) [30,31]. The molecular formula, chemical structures, and molecular weights of the hericenes are reported in Table 3 [29].

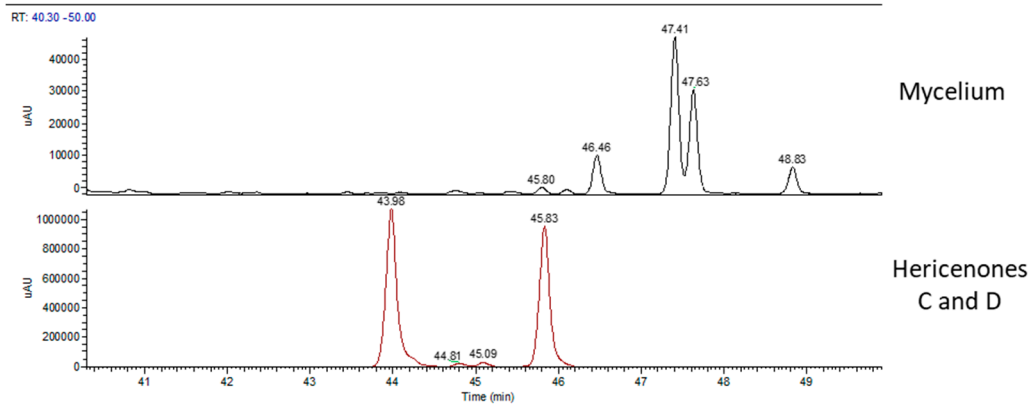
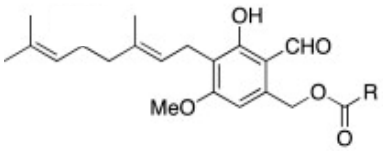


Figure 3. UV traces of mycelium (top) and the standard molecules of hericenones C and D (bottom).

Table 3. Molecular formula, chemical structures, and molecular weights of hericenes.

	Hericene A	**Hericene B**	**Hericene C**	**Hericene D**
molecular formula	$C_{35}H_{56}O_5$	$C_{37}H_{58}O_5$	$C_{37}H_{60}O_5$	$C_{37}H_{56}O_5$
molecular weight (MW) (g/mol)	556	582	584	580
chemical structure				
	R = palmitoyl	R = oleoyl	R = stearoyl	R = lineoyl

Our hypothesis was also confirmed by ion fragments in MS/MS spectra. Figure 4 reports the MS/MS-ESI spectra of hericenes: from top to bottom, hericenes D, A, B, and C are shown. The ion *m*/*z* 301 was present in all the spectra and derives from the loss of the side chain R. Spectra of hericenes D and B also show ions derived from fragmentation close to double bonds of R.

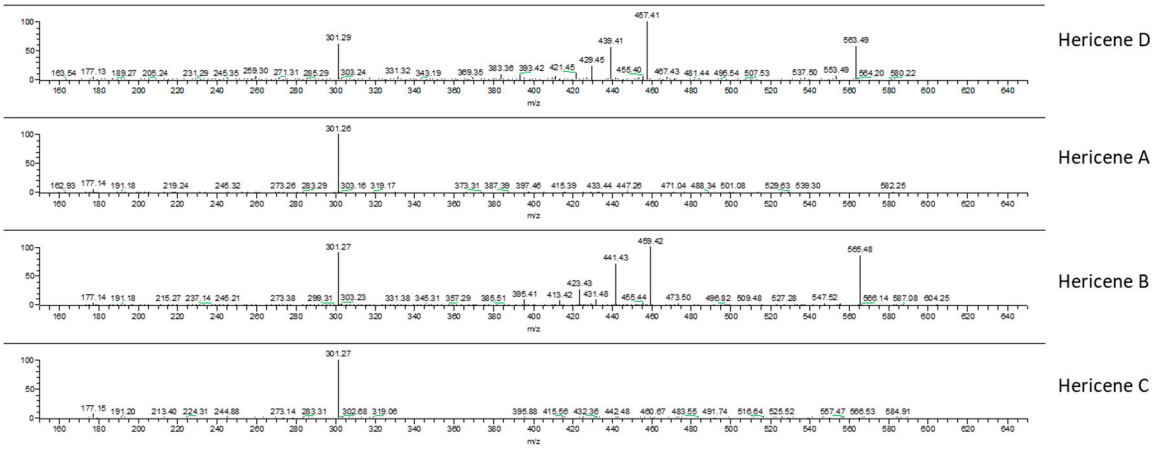


Figure 4. MS/MS-ESI spectra of hericenes D, A, B, and C (from top to bottom).

WT and cultivated sporophores were analyzed. The data were compared with the standard molecules hericenones C and D.

Figure 5 shows the UV chromatographic traces of the WT sporophore collected in Italy, the cultivated and the two standard molecules of hericenones: C detected at RT of 43.98 min and D detected at RT of 45.83 min. Hericenone C was detected in the WT at RT of 43.92 min and at RT of 43.94 in the cultivated sporophore. Hericenone D was detected at RT of 45.74 min in both samples. There were also other peaks close to these hericenones that could be attributed to hericenone E (RT of 42.72 min), hericenone I (RT of 44.18 min), and hericenone H (RT of 47.37 min) on the basis both of previous publications [16] and the similarity of their fragmentation pattern to that of hericenones C and D standard. In the same chromatogram (Figure 5), there were peaks that could be attributed to hericenes: from lower to higher RT hericene D (RT 46.28 min), hericene A (RT 47.37 or 47.38 min), hericene B (RT 47.52 or 47.53 min), and hericene C (RT 48.83 min).

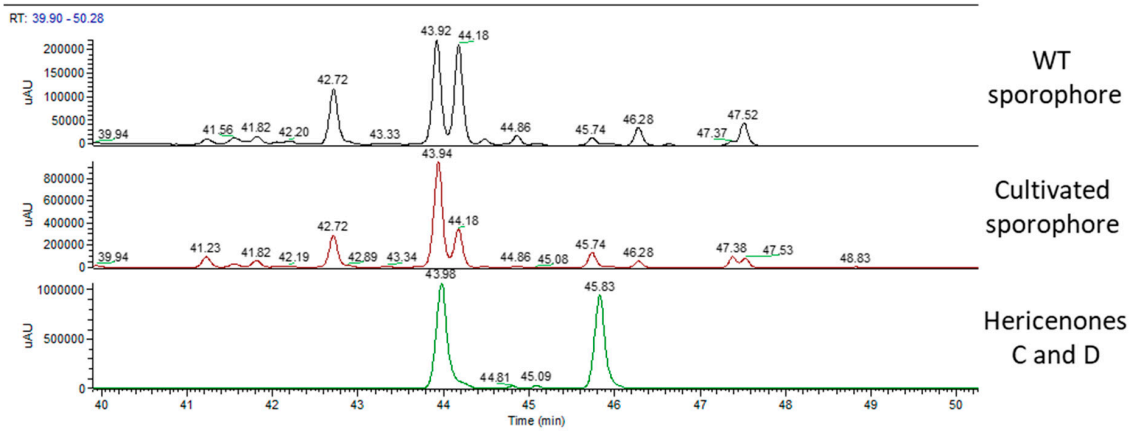


Figure 5. UV traces of wild type sporophore (top), cultivated sporophore (middle), and hericenones C (RT 43.98 min) and D (RT 45.83 min) standards (bottom).

Figure 6 displays MS chromatographic traces of the two sporophores and the standard molecule of erinacine A. The erinacine A molecule was not present in both sporophores. Besides, the

chromatographic traces also showed other peaks at RT of 11.29 and 12.52 min for the WT sporophores and at 11.44 and 12.50 min for the cultivated sporophores that belong to molecules not yet identified.

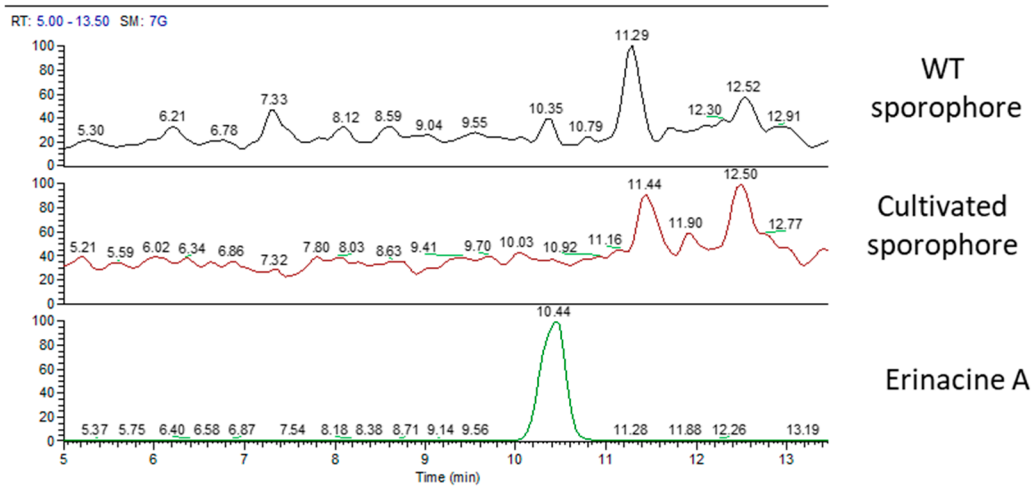


Figure 6. MS traces of wild type sporophore (top), cultivated sporophore (middle), and erinacine A standard molecules (bottom).

Chemical analyses of primordium showed neither erinacines (Figure 7) nor hericenones (Figure 8). MS trace of primordium (Figure 7) also showed a peak at RT of 11.37 min that has not yet been determined. Instead, the UV trace of primordium showed peaks at the same RT described for hericenes D, A, B, and C (Figure 8), as mentioned for the mycelium, and the WT and cultivated sporophores.

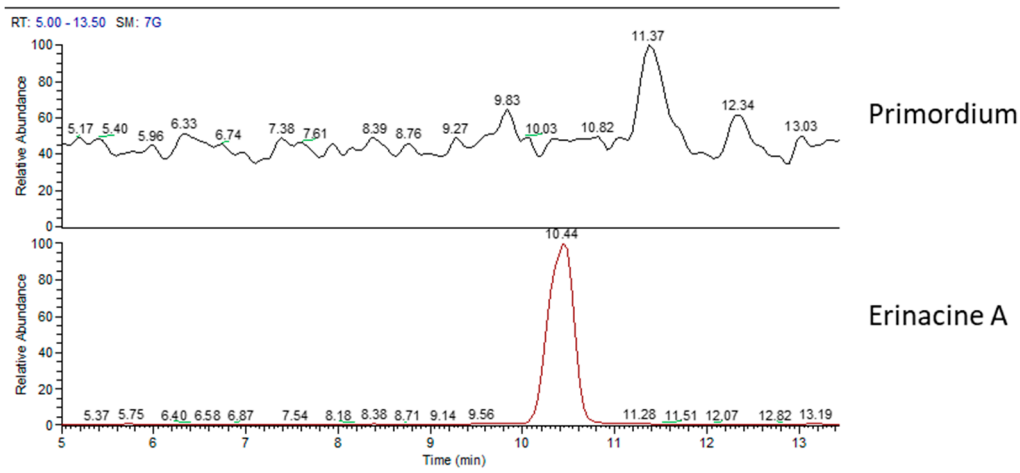


Figure 7. MS (mass spectrum) traces of primordium (top) and erinacine A molecule standard (bottom).

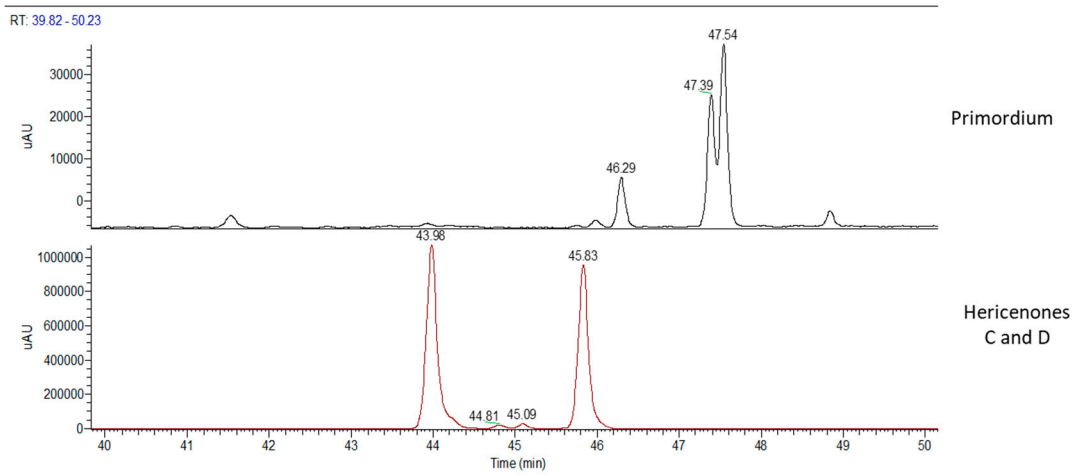


Figure 8. UV traces of primordium (top) and hericenones C and D standards (bottom).

Table 4 summarizes the array of metabolites present in different stages of *H. erinaceus*.

Table 4. Presence of different molecules (erinacine A, hericenones C and D, and hericenes) in the lyophilized mycelium, fresh primordium, and dried sporophores.

	erinacine A	hericenone C	hericenone D	hericenes
mycelium	✓	-	-	✓
primordium	-	-	-	✓
WT sporophore	-	✓	✓	✓
cultivated sporophore	-	✓	✓	✓

The content of erinacine A in H.e.2 mycelium and of hericenone C and D in sporophores were measured by the calibration curves [10] (Table 5).

Table 5. Content of erinacine A and hericenones C and D in H.e.2 lyophilized mycelium, fresh primordium and dried sporophores.

	Erinacine A (μg/g)	Hericenone C (μg/g)	Hericenone D (μg/g)
mycelium	105	-	-
primordium	-	-	-
WT sporophore	-	760	100
cultivated sporophore	-	1560	188

Figure 9 summarizes the UV chromatographic traces of different samples where it is possible to identify peaks that are attributed to hericenes. From lower to higher RT, hericene D (RT at 46.47 or 42.29 or 46.28), hericene A (RT at 47.41 or 47.39 or 47.37 or 47.38), hericene B (RT at 47.63 or 47.54 or 47.52 or 47.53), and hericene C (RT at 48.83 or 48.84) were identified. As previously reported, hericenes were present in all the samples in different amounts.

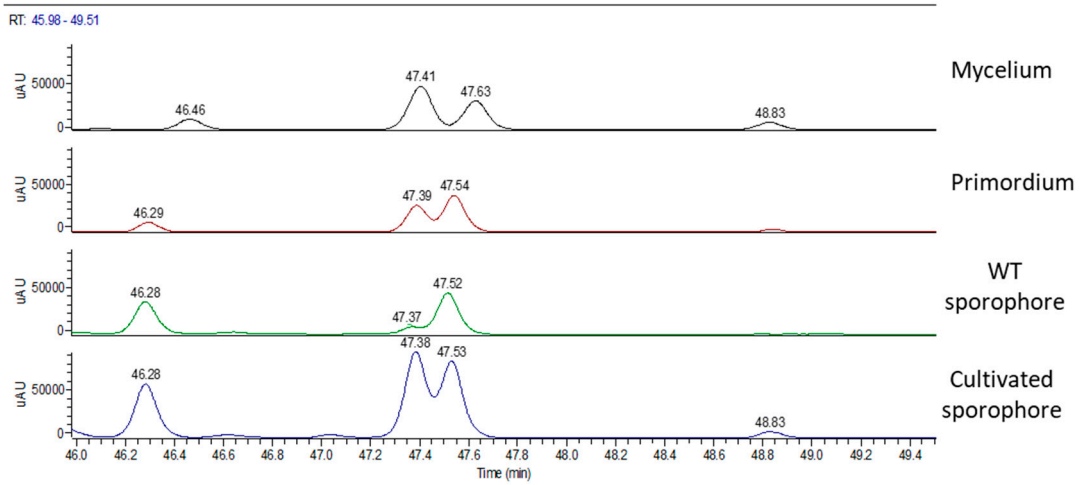


Figure 9. UV chromatographic traces of hericenes in (from top to bottom) mycelium, primordium, and wild type and cultivated sporophores.

Table 6 reports peak area values of hericenes A, B, C, and D in different samples.

Table 6. Content of hericenes in mycelium, primordium, and wild type (WT) and cultivated sporophores.

	Total Area 10^3	Hericene A Area 10^3	Hericene B Area 10^3	Hericene C Area 10^3	Hericene D Area 10^3
mycelium	684	327	232	51	74
primordium	557	201	262	21	73
WT sporophore	627	70	305	/	252
cultivated sporophore	1685	645	588	42	410

3. Discussion

At present, medicinal mushrooms such as *H. erinaceus* are exploited as dietary foods or supplements, producing beneficial effects by daily use in a balanced and varied diet. There are different products available on the market, increasing in number year by year. Despite this, there are still unresolved issues, including standardization and safety for the production of fungal supplement. Standardization is still in its early stage because of the lack of protocols and international guidelines [32].

This study is placed within an interdisciplinary research project to draw up the steps for the production of high quality dietary supplements to improve cognitive functions. The project has been following all the stages of the supply chain: strains selection, production, extraction, chemical analysis, and finally a pre-clinical test on animal models. More specifically, this study is the first step planned to analyze the array of some metabolites present in different growth stages of the *H. erinaceus* collected in Italy.

Thanks to the comparison with standard molecules, we were able to identify and quantify erinacine A in mycelium, hericenones C and D, and in wild type (WT) and cultivated sporophores.

In MicUNIPV, the Fungal Research Culture Collection at the University of Pavia (Italy), two strains of *H. erinaceus* collected in Italy are present to date: H.e.1 and H.e.2 [10,28]. The content of erinacine A in H.e.2 (105 μg/g) is slightly less compared to H.e.1 mycelium (150 μg/g) [10]. These amounts of erinacine A are comparable to that reported by Krzyczkowski et al. (2010) in improved submerged cultivation [33].

The same comparison between the WT sporophores showed that H.e.2 contains more hericenones C and D (760 μg/g and 100 μg/g, respectively) compared to H.e.1 (500 μg/g and <20 μg/g, respectively) [10]. These values are comparable with those of some strains reported by Lee et al. (2016) [18].

Therefore, given these data, the H.e.2 strain must be used for sporophore cultivation, whereas the H.e.1 for mycelium production.

By comparing the WT and cultivated sporophores of H.e.2, hericenones C and D in the cultivated sporophores are about two folds higher (1560 µg/g vs. 760 µg/g and 188 µg/g vs. 100 µg/g, respectively). Generally, wild sporophores exhibit biological variability, depending on the different growth environment and on seasonality. Conversely, the cultivated conditions are more stable. In particular, for the mycelial colonization, for the appearance of primordia, and the development of sporophores, the medium components (nitrogen, carbon, and mineral sources) and environmental factors (pH, temperature, and relative humidity) are fundamental in order to optimize the growth and to influence the bioactive metabolites production.

The primordium is an intermediate stage of the fungus between the mycelium and the sporophore development, characterized by the formation of spider-like aerial spines that grow above the culture medium. Generally, there are still few studies concerning primordium and none for *H. erinaceus* [34,35]. In our primordium, only hericenes were present, without any hericenone and erinacine. Hericenes are also found in mycelium and sporophores, both WT and cultivated, in agreement to what was reported by Arnone et al. (1994) [30] and Kobayashi et al. (2018) [31]. Preliminarily, in order to obtain a relative measure among the different samples, we compared the peak areas of the single hericenes detected, which were supposed to be A, B, C, and D, by HPLC-UV-ESI/MS. In particular, it is notable that in H.e.2 mycelium and primordium, hericenes A and B are more present compared to hericenes C and D. In cultivated sporophores, all hericenes are present, with a wider peak area if compared to the other samples. In WT sporophores, hericene C is absent and hericene A is at a lower level, whereas hericenes B and D peak areas have considerable values. Hericene B is present in fairly constant quantities in all samples, whereas hericene C is present in smaller quantities.

It should be noted that the cultivated sporophore has higher content than all the hericenes compared to the WT one. Similarly, the contents of hericenones C and D are higher in the cultivated sporophores than in the WT.

We could hypothesize a chemical correlation between hericenes and hericenones. Hericene A has a side chain with palmitoyl acid similar to hericenone C. Hericene C is similar to hericenone D with a side chain with stearic acid. Hericene D is similar to hericenone H and contains a side chain with a linoleoyl acid. Thus, in these paired molecules the side chain is maintained but hericenones differ from hericenes for their oxidation state. We can speculate that hericenone C derives from the oxidation of hericene A and hericenone D from the oxidation of hericene C. Other hericenones, such as I and E, could derive from the oxidation of hericenes B and D, respectively.

Up to now, ethanolic extracts obtained from H.e.1 mycelium and sporophores, with the standardized amounts of erinacine A and hericenones C and D, have been used to evaluate the effects of oral supplementation on cognitive decline in a mice model, during physiological aging [10]. Because of the different amounts of the neuroactive metabolites present in the different strains, now it is possible to prepare the best extract blend for in vivo tests. The present study contributes as it re-addresses the selection of raw material.

Further investigation will be carried out by setting different cultivation conditions to maximize the yield of bioactive metabolites.

4. Materials and Methods

4.1. Study Area and Sampling

Samplings were conducted in the hilly area around Siena (Tuscany, Italy), where both Mediterranean and temperate environments are present. The plant communities are dominated by holm oak (*Quercus ilex*), strawberry tree (*Arbutus unedo*), heather (*Erica arborea*), Mediterranean buckthorn (*Rhamnus alaternus*), juniper (*Juniperus communis*), and other deciduous species such as the

downy oak (*Q. pubescens*). *Q. ilex* is an important feature in the landscape, being usually prevalent and resistant to anthropic stress.

The wild type (WT) sporophore was collected from an old living specimen of *Q. ilex* and kept at 4 °C until experimental use.

4.2. H. erinaceus Samples for Chemical Analyses

The *H. erinaceus* samples processed for chemical analyses were: the WT sporophore (the sample was dried and maintained in a freezer at −20 °C for at least one month in order to avoid any further degradation); the strain isolated from it; the sporophores cultivated at the Botanical Garden of the University of Pavia (Italy) using the above mentioned isolated strain; the primordium that was the first aerial part consisting of mycelial cords.

4.3. H. erinaceus Strain Isolation

The isolation of mycelium in a pure culture from the WT was performed in accordance with the usual procedures [36–38]. Small pieces (up to 10 mm^3) were aseptically cut off from the center of the WT and inoculated into Petri dishes containing 2% malt extract agar (MEA, Biokar Diagnostics). Chloramphenicol at 50 ppm was added in this first step. Incubation was performed at 24 °C in complete darkness. The isolated strain is maintained in the Fungal Research Culture Collection of Pavia University (MicUNIPV).

4.4. H. erinaceus Sporophores Cultivation

The cultivation of *H. erinaceus* sporophores was performed in the mushroom greenhouse of the Botanical Garden at the University of Pavia (Italy). As the substrate, a mix of 70% oak sawdust, 20% rice bran, and 10% wheat straw, combined with 1% sucrose and 1% calcium carbonate, was used [36,39–41]. The substrate was mixed and hydrated, and then 300 g were placed in polypropylene bags with filters to allow gas exchange. Each bag was sterilized twice at 120 °C for 60 min.

In parallel, the spawn with *H. erinaceus* was prepared: the mycelium grew in sterilized polypropylene bags containing 300 g of hydrated barley. They were taken at 24 °C with 90% relative humidity (RH) in the dark for two weeks, until complete colonization.

We aseptically put and mixed 5% of spawn into each substrate bag. The cultivation room was kept at 24 °C and bathed to maintain high relative humidity (95%-100%).

Soon after the substrate was completely colonized by the mycelium, the bags were moved to a room where it was possible to carry out the light-dark cycle, maintaining the temperature at 18 °C–24 °C, the RH of 90%–95%, and good aeration condition to induce primordia formation.

In correspondence to the appearance of primordia, holes were made in the bags to allow the development of sporophores [36,39–41]. Once collected, the sporophores were weighed, measured, dried, and maintained frozen.

4.5. Extraction Procedures

The procedure of alcoholic extraction described by Lee et al. (2016) and Gerbec et al. (2015) [18,42] was followed with slight modification: 1 g of lyophilized mycelium/dried WT/cultivated sporophores/fresh primordium was blended with 10 mL of ethanol 70% and left in the thermostat at 50 °C for 24 h. At the end, the material was transferred for centrifugation (4000 rpm for 3 min) and the supernatant was stored at −20 °C for HPLC analysis.

4.6. HPLC-UV-ESI/MS Method

HPLC-UV-ESI/MS analyses were carried out on a LCQ FLEET system (Thermo Fisher Scientific, San Jose, CA, USA), equipped with a PAD-UV detector working at 254 nm. The chromatographic separation was performed using an F5 HPLC column 150 × 3.0 mm, 2.7 µm particle size

(Ascentis®Express, Merck KGaA, Darmstadt, Germany) maintained at 40 °C, with a flow rate of 0.3 mL/min and an injection volume of 20 µL. The mobile phase consisted of water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) (Table 7). The following gradient method was utilized: 0–9 min (30%–50% B), 9–27 min (50%–60% B), 27–54 min (60%–100% B), 54–69 min (100%–30% B), and 69–75 min (30% B).

Table 7. The mobile phase and the gradient method.

Time	Solvent A	Solvent B
0	70	30
9	50	50
27	40	60
54	00	100
69	70	30
75	70	30

An Electro Spray Ionization (ESI) interface was used as an ion source, under positive ion conditions (ESI+). The Ion Spray voltage and Capillary voltage were set at 5 kV and 10 V in positive ion mode. The capillary temperature was 400 °C. Acquisition was performed both in Full Scan mode (mass range 200–2000 Da) and Dependent Scan mode. The data station utilized the Xcalibur MS Software Version 2.1.

Stock solutions of erinacine A and hericenones C and D (1 mg/mL) were prepared in 70% ethanol. Standard solutions with the final concentration range of 1–25 µg/mL for erinacine A and 20–100 µg/mL for hericenones C and D were obtained by the proper dilution of stock solutions.

Calibration curves were constructed by injecting the standard mixture solutions at five concentrations (1, 5, 10, 15, 25 µg/mL) for erinacine A and at four concentrations (20, 50, 75, 100 µg/mL) for hericenone C and D. Linear least-square regression analysis for the calibration curves showed correlation coefficients of 0.9968, 09945, and 0.9951, respectively, for erinacine A, hericenones C, and hericenones D with respect to the peak area, demonstrating a good linear relationship in the different ranges tested. Each concentration was analyzed in triplicate [10].

5. Conclusions

In this study, an array of metabolites at different growth stages of the fungus *H. erinaceus* collected in Italy was analyzed. In particular, for the first time we described the array of metabolites present in primordium stage, i.e., the hericenes. These molecules are also present from the formation of the mycelium to the appearance of the primordium and up to the sporophore development. Experiments in the future will focus on testing the functional role of these molecules in vitro and in vivo.

In conclusion, in our opinion this methodological approach is a necessary step for developing dietary supplements with a higher and standardized content of bioactive metabolites.

Author Contributions: Conceptualization: P.R., E.S., F.C., V.C., H.K.; Methodology: V.C., B.M., F.C., R.M.B., C.E.G., D.R.; Samplings: C.P., V.C., C.E.G.; Supervision: P.R., E.S., F.C., A.M.P.; Writing–original draft: V.C., P.R., E.S.; Writing–review and editing: P.R., H.K., C.E.G. All authors have made a substantial contribution to the revision of the work and approved it for publication.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mycobank. Available online: www.mycobank.org (accessed on 24 September 2019).
2. Rossi, P.; Cesaroni, V.; Brandalise, F.; Occhinegro, A.; Ratto, D.; Perrucci, F.; Lanaia, V.; Girometta, C.; Orrù, G.; Savino, E. Dietary Supplementation of Lion's Mane Medicinal Mushroom, *Hericium erinaceus* (Agaricomycetes), and Spatial Memory in Wild-Type Mice. *Int. J. Med. Mushrooms* **2018**, *20*, 485–494. [CrossRef] [PubMed]
3. Brandalise, F.; Cesaroni, V.; Gregori, A.; Repetti, M.; Romano, C.; Orrù, G.; Botta, L.; Girometta, C.; Guglielminetti, M.L.; Savino, E. Dietary Supplementation of *Hericium erinaceus* Increases Mossy Fiber-CA3 Hippocampal Neurotransmission and Recognition Memory in Wild-Type Mice. Evid. Based Complement. *Alternat. Med.* **2017**, 3864340. [CrossRef]
4. Li, Y.; Zhang, G.; Ng, T.B.; Wang, H. A novel lectin with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from dried fruiting bodies of the monkey head mushroom *Hericium erinaceum*. *BioMed Res. Int.* **2010**, *2010*, 716515. [CrossRef] [PubMed]
5. Li, W.; Zhou, W.; Kim, E.J.; Shim, S.H.; Kang, H.K.; Kim, Y.H. Isolation and identification of aromatic compounds in Lion's Mane Mushroom and their anticancer activities. *Food Chem.* **2015**, *170*, 336–342. [CrossRef] [PubMed]
6. Zan, X.; Cui, F.; Li, Y.; Yang, Y.; Wu, D.; Sun, W.; Ping, L. *Hericium erinaceus* polysaccharide-protein HEG-5 inhibits SGC-7901 cell growth via cell cycle arrest and apoptosis. *Int. J. Biol. Macromol.* **2015**, *76*, 242–253. [CrossRef] [PubMed]
7. Vigna, L.; Morelli, F.; Agnelli, G.M.; Napolitano, F.; Ratto, D.; Occhinegro, A.; Di Iorio, C.; Savino, E.; Girometta, C.; Brandalise, F.; et al. *Hericium erinaceus* Improves Mood and Sleep Disorders in Patients Affected by Overweight or Obesity: Could Circulating Pro-BDNF and BDNF Be Potential Biomarkers? *Evid Based Complement Alternat Med.* **2019**, *2019*, 1–12. [CrossRef] [PubMed]
8. De Silva, D.D.; Rapior, S.; Hyde, K.D.; Bahkali, A.H. Medicinal mushrooms in prevention and control of diabetes mellitus—A review. *Fungal Divers.* **2012**, *56*, 1–29. [CrossRef]
9. Yang, B.K.; Park, J.B.; Song, C.H. Hypolipidemic Effect of an Exo-biopolymer Produced from a Submerged Mycelial Culture of *Hericium erinaceus*. *Biosci. Biotech. Bioch.* **2003**, *67*, 1292–1298. [CrossRef] [PubMed]
10. Ratto, D.; Corana, F.; Mannucci, B.; Priori, E.C.; Cobelli, F.; Roda, E.; Ferrari, B.; Occhinegro, A.; Di Iorio, C.; De Luca, F.; et al. *Hericium erinaceus* improves recognition memory and induces hippocampal and cerebellar neurogenesis in frail mice during aging. *Nutrients* **2019**, *11*, 715. [CrossRef]
11. Friedman, M. Chemistry, nutrition, and health-promoting properties of *Hericium erinaceus* (Lion's Mane) mushroom fruiting bodies and mycelia and their bioactive compounds. *J. Agric. Food Chem.* **2015**, *63*, 7108–7123. [CrossRef]
12. Kawagishi, H.; Shimada, A.; Shirai, R.; Okamoto, K.; Ojima, F.; Sakamoto, H.; Ishiguro, Y.; Furukawa, S. Erinacines A, B and C strong stimulators of nerve growth factor (NGF)-synthesis from the mycelia of *Hericium erinaceum*. *Tetrahedron Lett.* **1994**, *35*, 1569–1572. [CrossRef]
13. Ma, B.J.; Yu, H.Y.; Shen, J.W.; Ruan, Y.; Zhao, X.; Zhou, H.; Wu, T.T. Cytotoxic aromatic compunds from *Hericium erinaceum*. *J. Antibiotics* **2010**, *63*, 713–715. [CrossRef] [PubMed]
14. Kawagishi, H.; Ando, M.; Mizuno, T. Hericenone A and B as cytotoxic principles from the mushroom *Hericium erinaceum*. *Tetrahedron Lett.* **1990**, *31*, 373–376. [CrossRef]
15. Keong, C.Y.; Rashid, B.A.A.; Ing, Y.S.; Ismail, Z. Quantification and identification of polysaccharide contents in *Hericium erinaceus*. *Nutr. Food Sci.* **2007**, *37*, 260–271. [CrossRef]
16. Ma, B.J.; Shen, J.W.; Yu, H.Y.; Ruan, Y.; Wu, T.T.; Zhao, X. Hericenones and erinacines: Stimulators of nerve growth factor (NGF) biosynthesis in *Hericium erinaceus*. *Mycology* **2010**, *1*, 2–92. [CrossRef]
17. Kawagishi, H.; Ando, M.; Sakamoto, H.; Yoshida, S.; Ojima, F.; Ishiguro, Y.; Ukai, N.; Furukawa, S.C. Hericenones C, D and E, stimulators of nerve growth factor (NGF)-synthesis, from the mushroom *Hericium erinaceum*. *Tetrahedron Lett.* **1991**, *32*, 4561–4564. [CrossRef]
18. Lee, D.G.; Kang, H.W.; Park, C.G.; A, Y.S.; Shin, Y. Isolation and identification of pyotochemicals and biological activities of *Hericium erinaceus* and their contents in *Hericium* strains using HPLC/UV analysis. *J. Ethnopharmacol.* **2016**, *184*, 219–225. [CrossRef] [PubMed]
19. Tang, H.Y.; Yin, X.; Zhang, C.C.; Jia, Q.; Gao, J.M. Structure diversity, synthesis, and biological activity of cyathane diterpenoids in higher fungi. *Curr. Med. Chem.* **2015**, *22*, 2375–2391. [CrossRef]

20. Kawagishi, H.; Shimada, A.; Hosokawa, S.; Mori, H.; Sakamoto, H.; Ishiguro, Y.; Sakemi, S.; Bordner, J.; Kojima, N.; Furukawa, S. Erinacines E, F, and G, stimulators of nerve growth factor (NGF) synthesis from the mycelia of *Hericium erinaceum*. *Tetrahedron Lett.* **1996**, *37*, 7399–7402. [CrossRef]
21. Kawagishi, H.; Simada, A.; Shizuki, K.; Mori, H.; Sakamoto, H.; Furukawa, S. Erinacine D, a stimulator of NGF-synthesis from the mycelia of *Hericium erinaceum*. *Heterocycl Commun.* **1996**, *2*, 51–54. [CrossRef]
22. Lee, E.W.; Shizuki, K.; Hosokawa, S.; Suzuki, M.; Suganuma, H.; Inakuma, T.; Kawagishi, H. Two novel diterpenoids, erinacines H and I from the mycelia of *Hericium erinaceum*. *Biosci. Biotech. Biochem.* **2000**, *64*, 2402–2405. [CrossRef] [PubMed]
23. Kenmoku, H.; Sassa, T.; Kato, N. Isolation of erinacine P, a new parental metabolite of cyathane-xylosides from Hericium erinaceus and its biomimetic conversion into erinacines A and B. *Tetrahedron Lett.* **2000**, *41*, 4389–4393. [CrossRef]
24. Kenmoku, H.; Shimai, T.; Toyomasu, T.; Kato, N.; Sassa, T. Erinacine Q, a new erinacine from Hericium erinaceus, and its biosynthetic route to erinacine C in the basidiomycete. *Biosci. Biotech. Biochem.* **2002**, *66*, 571–575. [CrossRef] [PubMed]
25. Kawagishi, H.; Masui, A.; Tokuyamab, S.; Nakamurac, T. Erinacines J and K from the mycelia of *Hericium erinaceum*. *Tetrahedron Lett.* **2006**, *62*, 8463–8466. [CrossRef]
26. Ma, B.J.; Zhou, Y.; Li, L.Z.; Li, H.M.; Gao, Z.M.; Ruan, Y. A new cyathanexyloside from the mycelia of *Hericium erinaceum*. *Z Naturforsch* **2008**, *63b*, 1241–1242. [CrossRef]
27. Bernicchia, A.; Gorjon, S.P. *Hericium erinaceus*. In *Corticiaceae s.l. Fungi europaei*; Candusso: Alassio, Italy, 2010; Volume 12, pp. 318–319. ISBN 978-88-901057-9-8.
28. Cesaroni, V.; Brusoni, M.; Cusaro, C.M.; Girometta, C.; Perini, C.; Picco, A.M.; Rossi, P.; Salerni, E.; Savino, E. Phylogenetic Comparison between Italian and Worldwide *Hericium* Species (Agaricomycetes). *Int. J. Med. Mushrooms* **2019**, accepted.
29. PubChem. Available online: http://pubchem.ncbi.nlm.nih.gov/ (accessed on 26 September 2019).
30. Arnone, A.; Cardillo, R.; Nasini, G.; De Pava, O.V. Secondary mold metabolites: Part 46. Hericenes A-C and erinapyrone C, new metabolites produced by the fungus *Hericium erinaceus*. *J. Nat. Prod.* **1994**, *57*, 602–606. [CrossRef]
31. Kobayashi, S.; Hamada, Y.; Yasumoto, T.; Hashino, Y.; Masuyama, A.; Nagai, K. Total syntheses and endoplasmic reticulum stress suppressive activities of hericenes A-C and their derivatives. *Tetrahedron Lett.* **2018**, *59*, 1733–1736. [CrossRef]
32. Wasser, S.P. Medicinal Mushroom Science: Current Perspectives, Advances, Evidences, and Challenges. *Biomed. J.* **2014**, *37*, 345–356. [CrossRef]
33. Krzyczkowski, W.; Malinowska, E.; Herold, F. Erinacine A biosynthesis in submerged cultivation of *Hericium erinaceum*: Quantification and improved cultivation. *Eng. Life Sci.* **2010**, *10*, 446–457. [CrossRef]
34. Zhang, G.; Sun, Z.; Ren, A.; Shi, L.; Shi, D.; Li, X.; Zhao, M. The mitogen-activated protein kinase GlSlt2 regulates fungal growth, fruiting body development, cell wall integrity, oxidative stress and ganoderic acid biosynthesis in *Ganoderma lucidum*. *Fungal Genet. Biol.* **2017**, *104*, 6–15. [CrossRef] [PubMed]
35. Xie, C.; Gong, W.; Zhu, Z.; Yan, L.; Hu, Z.; Peng, Y. Comparative transcriptomics of *Pleurotus eryngii* reveals blue-light regulation of carbohydrate-active enzymes (CAZymes) expression at primordium differentiated into fruiting body stage. *Genomics* **2018**, *110*, 201–209. [CrossRef] [PubMed]
36. Stamets, P. The lion's mane mushroom. In *Growing Gourmet and Medicinal Mushrooms*, 3rd ed.; Ten Speed Press: Berkeley, Toronto, 2000; pp. 387–394. ISBN 13 978-1-58008-175-7.
37. Stalpers, J.A. Identification of wood-inhabiting Aphyllophorales in pure culture. *Stud. Mycol.* **1978**, *16*, 114.
38. Sturini, M.; Girometta, C.; Maraschi, F.; Savino, E.; Profumo, A. A preliminary investigation on Metal Bioaccumulation by *Perenniporia fraxinea*. *Bull. Environ. Contam. Toxicol.* **2017**, *98*, 508–512. [CrossRef] [PubMed]
39. Ko, H.G.; Park, H.G.; Park, S.H.; Choi, C.W.; Kim, S.H.; Park, W.M. Comparative study of mycelial growth and basidiomata formation in seven different species of the edible mushroom genus *Hericium*. *Bioresource Technol.* **2005**, *96*, 1439–1444. [CrossRef] [PubMed]
40. Hassan, F.R.H. Cultivation of the Monkey Head Mushroom (*Hericium erinaceus*) in Egypt. *J. App. Sci. Res.* **2007**, *3*, 1229–1233.

41. Savino, E.; Girometta, C.; Baiguera, R.M.; Cesaroni, V.; Guglielminetti, M.L.; Rodolfi, M.; Rossi, P.; Picco, A.M. Different approaches for *Hericium erinaceus* spawn in the perspective of gluten free products. *ISMS* **2016**, *19*, 106.
42. Gerbec, B.; Tavčar, E.; Gregori, A.; Kreft, S.; Berovic, M. Solid State Cultivation of *Hericium erinaceus* Biomass and Erinacine: A Production. *J. Bioproces. Biotech.* **2015**, *5*, 1–5. [CrossRef]

Sample Availability: Samples of the compounds are available from one of the authors (H. K.) under an MTA.

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Research Article

Dietary Supplementation of *Hericium erinaceus* Increases Mossy Fiber-CA3 Hippocampal Neurotransmission and Recognition Memory in Wild-Type Mice

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Hericium erinaceus (Bull.) Pers. is a medicinal mushroom capable of inducing a large number of modulatory effects on human physiology ranging from the strengthening of the immune system to the improvement of cognitive functions. In mice, dietary supplementation with *H. erinaceus* prevents the impairment of spatial short-term and visual recognition memory in an Alzheimer model. Intriguingly other neurobiological effects have recently been reported like the effect on neurite outgrowth and differentiation in PC12 cells. Until now no investigations have been conducted to assess the impact of this dietary supplementation on brain function in healthy subjects. Therefore, we have faced the problem by considering the effect on cognitive skills and on hippocampal neurotransmission in wild-type mice. In wild-type mice the oral supplementation with *H. erinaceus* induces, in behaviour test, a significant improvement in the recognition memory and, in hippocampal slices, an increase in spontaneous and evoked excitatory synaptic current in mossy fiber-CA3 synapse. In conclusion, we have produced a series of findings in support of the concept that *H. erinaceus* induces a boost effect onto neuronal functions also in nonpathological conditions.

1. Introduction

Hericium erinaceus (Bull.) Pers. (also known as Yamabushitake, Lion's Mane, or Satyr's beard) is a mushroom that grows on both living and dead broadleaf trees. It has been used for centuries as food source and herbal medicine in several Asian countries. However, over the last 10 years, the potential nootropic capabilities of *H. erinaceus* in neurodegenerative diseases have attracted considerable attention [1].

In mice, dietary supplementation with *H. erinaceus* prevents the impairment of spatial short-term and visual recognition memory induced by amyloid β(25–35) peptide [2]. Recently, Hazekawa et al. [3] described the neuroprotective effects of *H. erinaceus* dietary supplementation in mice subjected to middle cerebral artery occlusion. Furthermore, scores on the cognitive function scale improved after oral administration of *H. erinaceus* in patients suffering from mild cognitive impairment [4]. Finally, in humans, reduction of depression and of anxiety following 4 weeks of *H. erinaceus* intake was described in 30 females by means of a questionnaire investigation for psychometric measures [5].

Most of the effects induced by *H. erinaceus* have been correlated with an increase in the NGF production. NGF is a small secreted protein that acts as a neurotrophin that plays

pivotal roles in neuronal survival in the adult mammalian brain and modulates forms of structural and functional plasticity, like the neurogenesis and memory (for a review see [6]).

What is the link between NGF and *H. erinaceus* dietary supplementation? A study conducted by Mori et al. 2008 [7] has shown that the extract of the fruiting body of *H. erinaceus* presents a 5-fold increase in the level of expression of NGF mRNAs in isolated human astrocytoma cells with respect to the control. Similar effect was also described by others [8, 9]. Interestingly, NGF effect was detected in the hippocampus but not in the cortex, thus suggesting an essential role of NGF in hippocampal learning and memory [10]. Furthermore, the presence of a persistent neurogenesis has recently been discovered in a specific region of the hippocampus, the dentate gyrus; it involves the main neuronal type present there, the granule cells (for a review see [10]).

Despite an extensive investigation of the preventing action of *H. erinaceus* in cognitive pathological conditions, to date, no studies have investigated the effects of dietary supplementation with *H. erinaceus* in healthy mice. To approach this point a group of wild-type mice were fed with a dextrin dietary supplementation and another one with *H. erinaceus* dietary supplementation and we investigated the effect on behaviour and on the neuronal network involved in memory skills. We therefore performed in vivo and in vitro experiments to assess novelty exploration and recognition memory by a battery of spontaneous behavioural tests and the effect on hippocampal mossy fiber to CA3 pyramidal cells synapses in wild-type mice after two months of dietary supplementation with *H. erinaceus*.

The *H. erinaceus* supplemented mice were able to perform better in a novel object recognition test. They were also more adventurous in exploring a novel environment. Electrophysiology recording in the mossy fiber-CA3 region suggested that there was a higher amount of neurotransmitter release from mossy fiber to CA3. This conclusion was supported by higher frequency and higher amplitude of spontaneous excitatory activities and lower number of stimulation failures and decreased pair-pulse ratio of evoked activities in Hr-fed mice.

2. Materials and Methods

2.1. Subjects. Blind experiments were carried out in wild-type mice (strain C57BL-6J). To avoid any potential differences related to the oestrous cycle in females, only males were selected. One-month-old wild-type male mice were divided into two groups: control mice received 2 months of a diet containing 5% dextrin dietary supplementation (dx mice), and *Hericum erinaceus* treated mice (Hr mice) received two months of a diet containing 5% "Micotherapy Hericium" supplement (corresponding to 0.025 g/g body weight). Water was provided *ad libitum* for both groups.

After 2 months of dietary supplementation in vivo experiments were performed in dx and Hr mice. At the end of behavioural test session, the same animals were maintained with the same diet and about 1 week later were used for patch-clamp experiments.

Table 1: Nutrient composition of dietary supplements (supplied by A. V. D. Reform, Noceto, Parma, Italy).

Components	Per capsule (mg)
Hericium mycelium	400
Hericium fruiting bodies extract	100
Titled in polysaccharides	38.6

Table 2: Nutritional composition of *H. erinaceus* extract.

Analyte	Result	Unit
Calorie	2.23	Kcal/g
Crude proteins	8.25	%wt
Crude fat	0.17	%wt
Crude fiber	5.92	%wt
Polysaccharides/total glucan	>45	%wt
Sodium	0.0146	%wt

Table 3: Nutritional composition of *H. erinaceus* mycelium.

Analyte	Result	Unit
Calorie	1.98	Kcal/g
Crude proteins	10.22	%wt
Crude fat	1.02	%wt
Crude fiber	39.2	%wt
Polysaccharides/total glucan	>37	%wt
Sodium	0.0031	%wt

2.2. Fungal Supplementation. The supplement "Micotherapy Hericium" was provided by A. V. D Reform s.r.l. (Noceto, Parma, Italy). The supplement contains mycelium and fruiting body extract of *Hericium erinaceus* in a ratio 4/1 (Table 1). *H. erinaceus* (Her. Erin. strain) culture was obtained from the fungal culture bank of MycoMedica d.o.o., Slovenia, and was cultivated in the dark on PDA (Potato Dextrose Agar; Difco, USA) at 24°C. After 20 days, cultures were transferred onto lignocellulosic substrates and further incubated for 60 days at 24°C. After incubation, fruiting bodies and fungal biomass were harvested.

Fruiting bodies extractions were performed for three hours using water and ethanol as solvents in a 1 : 15 extraction ratio (w/v). The remaining extracted liquid as well as mycelium was dried under vacuum at 70°C and −0.9 bar and further milled by using a UPZ mill (Hosokawa Alpine Aktiengesellschaft, Augsburg, Germany) to obtain particles mostly smaller than 100 μm.

The polysaccharide content of *H. erinaceus* fruiting bodies extract and of *H. erinaceus* mycelium, contained in "Micotherapy Hericium" supplement, was determined using β-Glucan Assay Kit (Megazyme, Ireland) and expressed as total (α plus β) glucan content (Tables 2 and 3).

All experiments were carried out according to the guidelines laid down by the institution's animal welfare committee, the Ethics Committee of Pavia University.

2.3. Apparatus and Procedures for Behavioural Test. Motor activity was quantified by means of a SMART video tracking

system (2 Biological Instruments, Besozzo, Varese, Italy) and a Sony CCD colour video camera (PAL).

2.3.1. Emergence Test. To assess approach and exploratory behaviour in rodents, we performed the emergence test, which is a variant of the open-field test that was designed to reduce anxiety by providing a safe enclosure within the open field. This test has been used to test anxiety-like behaviour in mice [11–13]. The free exploration test entails housing animals in a compartment prior to giving the animal a free choice between a novel compartment and a familiar one [14]. In our experimental conditions, the mouse is situated in a familiar environment (a cage 33 cm long, 15 cm wide, and 13 cm high) with a hole in one side (5 cm long and 4 cm wide) through which it can emerge in a larger arena (90 cm long and 60 cm wide) with a laminated floor but without walls. While the primary measure of anxiety-like behaviour is taken to be the latency to emerge into the novel arena, auxiliary markers of anxiety-like behaviour may include the number of emergences and the time spent out of the cage.

2.3.2. Novel Object Recognition Task. The novel object recognition task (NOR) is used to test novelty exploration behaviour and recognition memory in rodents [15, 16]. The task consists of three phases: habituation, familiarization, and the test. In the habituation phase, for the first two days each mouse is given 10 minutes to freely explore the open-field arena in the absence of objects, after which it is removed from the arena and placed in the holding cage. On the third day, during the familiarization phase, each mouse is placed into the open-field arena and left free to explore two identical objects for 5 minutes. After the retention phase has elapsed (15 minutes), the mouse is put back into the open box where it is exposed both to a familiar object, identical to the one previously encountered in the familiarization phase, and to a novel object with a different size and shape. Approaches are defined as nose entries within 2 cm far from the object.

In both behavioural tests, the apparatus was wiped clean with water and dried after every trial.

2.4. Behavioural Test Analysis. In the emergence test, the software collected the following:

(1) The number of times a mouse completely emerged from the cage with all four limbs.

(2) The amount of time a mouse spent exploring the large environment outside the cage.

(3) The latency before the first exit from the cage.

In the NOR task, the software collected the following:

(1) The number of approaches.

(2) Total duration of approaches.

(3) Average duration of an approach.

(4) Latency of the first approach.

(5) Total latency between approaches.

(6) Average latency between approaches.

Table 4: Electrical properties of neurons in Dx mice ($n = 16$) and Hr mice ($n = 16$). R_{in}, input resistance; C_m, membrane capacitance; and R_s, series resistance. Differences are not statistically significant.

	Dx mice	Hr mice
R_{in} (Mohm)	122 ± 22	115 ± 11
C_m (pF)	294 ± 17	211.8 ± 15.2
R_s (Mohm)	6.7 ± 1.7	5.7 ± 0.5

2.5. Hippocampal Slices Preparation. 250 μm thick hippocampal sagittal slices were prepared from mice treated with either placebo or *H. erinaceus* by oral administration for two months. Briefly, mice were anesthetized by isofluorane inhalation (Aldrich, Milwaukee, WI, USA) before they were decapitated (the experimental procedure was approved by the Ethical Committee of the University of Pavia; Regulation of the Italian Ministry of Sanity, number 68/97-A). Artificial cerebrospinal fluid (ACSF) solution for slice cutting and recovery contained (in mM) the following: NaCl 120, KCl 2, $MgSO_4$ 1.2, $NaHCO_3$ 26, KH_2PO_4 1.2, $CaCl_2$ 2, and glucose 11. This solution was equilibrated with 95% O_2 and 5% CO_2, pH 7.4. Slices were maintained at room temperature before being transferred to the recording chamber (1.5 ml) mounted on the stage of an upright microscope (OLYMPUS BX51WI, Japan). The preparation was then superfused at a rate of 2 ml/min with Krebs' solution and maintained at 30°C by a feedback Peltier device (TC-324B, Warner Instr. Corp., Hamden, CT, USA).

2.6. Electrophysiological Data Collection and Analysis. Patch-clamp whole-cell recordings were performed in CA3 pyramidal neurons. CA3-CA3 and mossy fiber-CA3 synapses were studied; spontaneous and evoked postsynaptic current were recorded in placebo and treated mice. No more than two neurons have been recorded from the same mice.

Whole-cell patch-clamp recordings were made from the soma of visually identified CA3 pyramidal neurons and membrane currents were recorded by using an Axopatch 200B amplifier. Data were sampled with a Digidata-1440 interface. The resting membrane potential was between −60 and −70 mV. Table 4 shows electrical properties of recorded CA3 neurons.

The detection of the spontaneous excitatory currents was accomplished by using an automated routine based on the derivatives of the recording waveform with an algorithm similar to that previously published with a detection threshold of 10 pA compared to a noise level of about 2 pA [17]. Miniature and EPSCs recordings were performed at the reversal potential for GABAergic IPSCs (−70 mV).

Mossy fiber axons were electrically stimulated with a bipolar electrode (glass pipette with tip diameter of ~4 μm filled with media solution, glued to a fine tungsten rod) placed in *stratum lucidum* (30–50 μm from the edge of the pyramidal cell layer) at a lateral distance of 75–200 μm from the recording pipette. The minimal stimulation intensity and duration were adjusted to observe failures of synaptic transmission (stimulus intensity and duration ranged between 140 and 160 microampere and 20 and 30 μsec, Figure 4(b)).

In some experiments, a stimulating electrode was placed in *stratum radiatum* (150–200 μm from *stratum pyramidale*) to activate CA3-CA3 synapses. Test pulses were delivered every 10 seconds; a hyperpolarizing current pulse (20 pA, 300 ms) was injected into the cell between test pulses to monitor input resistance and series resistance throughout each experiment. Accepted deviations from these parameters over the time-windows used for statistical analysis were less than 10%. Patch pipettes were pulled from borosilicate capillaries (Hingelberg, Malsfeld, Germany) and had 5–8 MΩ resistance before a seal was formed. The filling solution contained (in mM) the following: potassium gluconate 126, NaCl 4, $MgSO_4$ 1, $CaCl_2$ 0.02, BAPTA 0.1, glucose 15, ATP 3, HEPES 5, and GTP 0.1 and 1 mM picrotoxin (pH was adjusted to 7.2 with KOH). After obtaining a whole-cell configuration we waited 5 minutes before starting the recordings, in order to inhibit all IPSCs by picrotoxin.

At the end of the experiments we perfused in the bath 2 μM DCG-IV, a receptor agonist of Group II metabotropic glutamate that blocks glutamate release from mossy fibers terminals.

Experimental traces were analyzed by using P-Clamp (Axon Instruments, Foster City, CA, USA) and Origin (Microcal Software, Northampton, MA, USA) software.

2.7. Statistics. After using Bartlett's test [17] for Homogeneity of Variances, two-way ANOVA repeated measures were used, where parameters reported in Figures 2(b) and 2(c) (number of approaches or total duration of approaches or average duration of approaches or latency of first approaches) were the dependent variable, the within subjects factor was "familiar object" or "novel object," and the between subjects factor was "dextrin" or "*Hericium*" treatment. For emergence test a one-way ANOVA test was used.

Descriptive statistics, expressed as data, are reported as means ± standard error of the mean (SEM), and statistical comparisons in electrophysiological experiments analysis were made by using Student's t-test. Before applying the Student t-test, a QQ plot was generated and the Shapiro-Wilk test was performed for each pool of data to confirm a normal distribution.

In figures symbols indicate $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

More details about each statistical analysis are given in Section 3 and figures captions.

3. Results

3.1. Behavioural Tests

3.1.1. Hr Supplementation Increases Novelty Exploration Behaviour of a New Environment. We first investigated the effect of oral supplementation with *H. erinaceus* on novelty exploration behaviour in healthy mice by using the emergence test (Figure 1(a)). We tested 18 dx mice and 22 Hr mice. The results show that the Hr mice had a higher frequency of complete exits (9.5 ± 0.8 versus 4.1 ± 0.8; T test $P < 0.001$, Figure 1(b)), spent more time out of the cage exploring the new environment (129.6 sec ± 14 versus 59.2 sec ± 15.8; T test $P < 0.01$, Figure 1(c)), and had a lower latency before the first exit (13.1 ± 2.6 sec versus 31.7 ± 10.3 sec; T test $P < 0.05$, Figure 1(d)). Overall, the number of complete exits (2.3-fold increase), the exploring time (2.2 times longer), and the decreased latency to the first exit (2.4 times shorter) all of them indicate an increase in novelty-seeking behaviour after the oral supplementation with *H. erinaceus* for two months.

3.1.2. Hr Supplementation Increases Recognition Memory and Exploration of a Novel Object. Previous studies showed that *H. erinaceus* oral supplementation in mice with learning and memory deficits induced by intracerebroventricular administration of A β(25–35) amyloid β(25–35) peptide prevented cognitive deficits in a memory recognition task but not in a spatial working task [3]. Based on these findings, we decided to test the effect of *H. erinaceus* on recognition memory in wild-type mice by using the NOR task (Figure 2).

In the NOR task, in dextrin mouse ($n = 12$) the animals' natural inquisitiveness regarding the presence of a new object in the arena is expressed by a longer latency of the first approach to the novel object (42.7 ± 9 sec versus 17.6 ± 5.7 sec; $F_{3,46} = 6.47$, $P < 0.001$, Figure 2(e)). The number (13.9 ± 1.7 versus 14 ± 0.8, Figure 2(b)) and the total duration of approaches (45.4 ± 7.9 sec versus 33 ± 5.6 sec, Figure 2(c)) and consequently the average duration of an approach to the novel object did not reach the significance (3.2 ± 0.5 sec versus 2.3 ± 0.4 sec, Figure 2(d)).

When we compared the behaviour of dx and Hr mice ($n = 19$) toward the familiar object (Figures 2(b), 2(c), 2(d), and 2(e)), the only parameter that was statistically significantly lower in the latter was the latency for the first approach (Figure 2(e), 17.6 ± 5.7 sec versus 6.7 ± 2.4 sec, resp., $P < 0.05$), indicating that Hr and dx mice approach a familiar object in the arena in fairly similar ways.

Interestingly, the comparison of the behaviour of dx and Hr mice toward the novel object revealed that the number of approaches (13.9 ± 1.7 versus 18.9 ± 1.5; $F_{3,46} = 3.24$, $P < 0.05$, Figure 2(b)) and the total duration of approaches (45.4 ± 7.9 sec versus 65.4 ± 5.7 sec; $F_{3,46} = 8.86$, $P < 0.05$, Figure 2(c)) were significantly different, with the Hr mice showing 36% and 44% increases, respectively, in exploratory behaviour toward the novel object. Furthermore, the latency of the first approach (42.7 ± 9 sec versus 19.5 ± 5.9 sec; $F_{3,46} = 6.47$, $P < 0.05$, Figure 2(e)) and the average latency between approaches (8.6 ± 1 sec versus 5.9 ± 0.4, T test, $P < 0.05$, Figure 2(g)) were significantly lower (54% and 31.4% decrease) in Hr mice. However, neither the average duration of an approach (3.2 ± 0.5 sec versus 3.5 ± 0.3 sec, Figure 2(d)) nor the total latency (221.6 ± 12 sec versus 203 ± 7 sec, Figure 2(f)) reached significance. Briefly, Hr mice showed increased numbers and total duration of approaches when exploring the novel object in the arena and are less distrustful when approaching a novel object for the first time.

As expected, three out of four parameters that we measured in the behaviour of Hr mice with the familiar and novel objects were significantly different, with the total duration of approaches (31.5 ± 3.6 sec versus 65.4 ± 5.7 sec; $F_{3,46} = 8.86$, $P < 0.001$, Figure 2(c)), the average duration of an

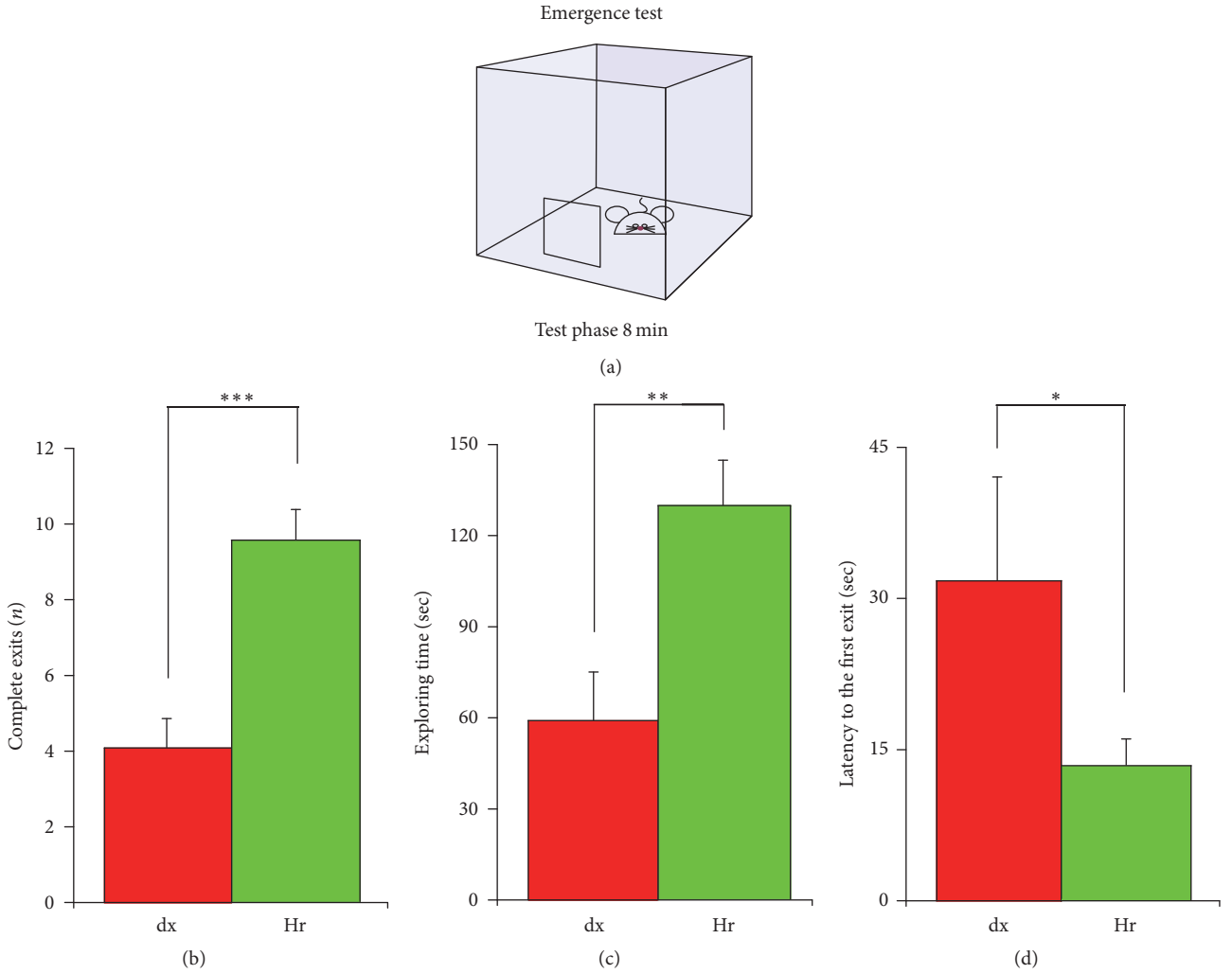

FIGURE 1: *H. erinaceus* increases novelty exploration behaviour. Emergence test in dx (n = 12) and Hr-dietary supplemented mice (n = 22) during an 8 min session. (a) Schematic of the experimental set-up and procedure used. Histograms show (b) the number of complete exits; (c) duration of exploring time; and (d) the latency of the first exit.

approach (1.9 ± 0.2 sec versus 3.5 ± 0.3 sec; $F_{3,46}$ = 8.86, $P < 0.001$, Figure 2(d)), and the latency of the first approach (6.6 ± 2.4 sec versus 19.5 ± 5.9 sec; $F_{3,46}$ = 6.47, $P < 0.05$, Figure 2(e)) all being significantly higher. We conclude that, after *H. erinaceus* dietary supplementation, mice spent more time exploring the novel object and increased the latency of the first approach compared with the familiar object.

To evaluate the discrimination between novel and familiar objects in dx and Hr mice, we calculated the mean novelty discrimination index (NI) by using the following formula: NI = $(n-f)/(n+f)$ [18], where n is the average time with the novel object, and f is the average time with the familiar object. This index ranges from −1 to 1, where −1 means complete preference for the familiar object, 0 means no preference, and 1 means complete preference for the novel object. The NI index was 0.15 for the dx mice and 0.35 for the Hr mice.

In conclusion, Hr mice displayed very different behaviour compared with dx mice specifically when exploring novel objects, not familiar objects. The data are concordant and indicate that Hr mice show increased recognition memory performance.

3.2. Electrophysiological Data. Pyramidal cells in hippocampal area CA3 receive both excitatory and inhibitory inputs. We recorded spontaneous and evoked synaptic currents in single CA3 pyramidal cells, in which GABAA receptor mediated responses were reduced with 1 mM intracellular picrotoxin (Figure 3(a)) and cells were voltage-clamped at −70 mV. In this experimental condition only excitatory currents are detected. CA3 pyramidal cells received excitatory synaptic inputs mainly from two sources: collateral axons from other CA3 pyramidal cells synapse on the medial apical and basal dendrites (CA3-CA3 synapse), and mossy fiber axons of dentate granule cells on the proximal, basal, and apical dendrite (mf-CA3 synapse).

3.2.1. Hr Supplementation Increases the Frequency and Amplitude of Spontaneous Synaptic Excitatory Currents of Mossy Fiber-CA3 Synapse. As a direct measurement of the synaptic

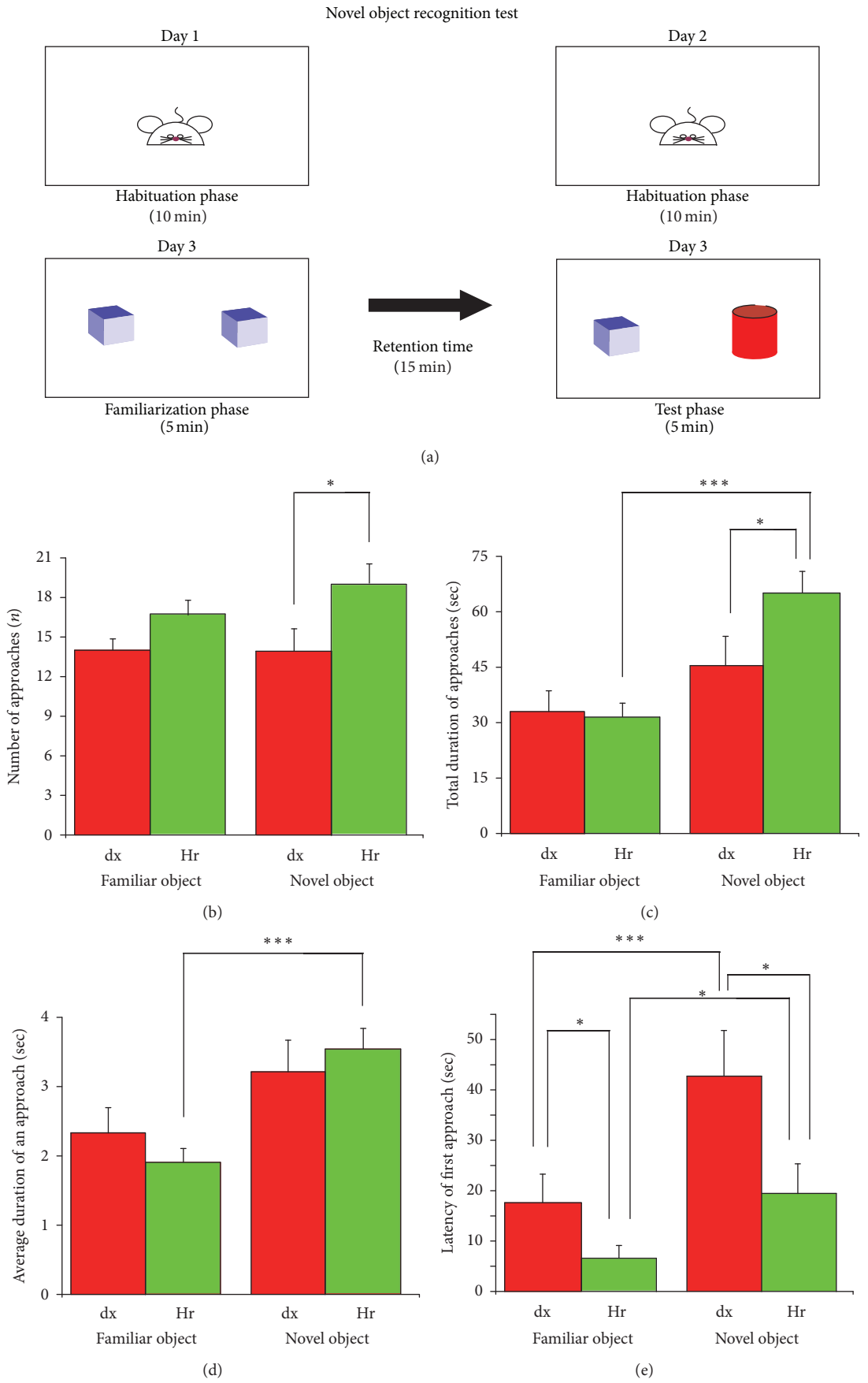

Figure 2: Continued.

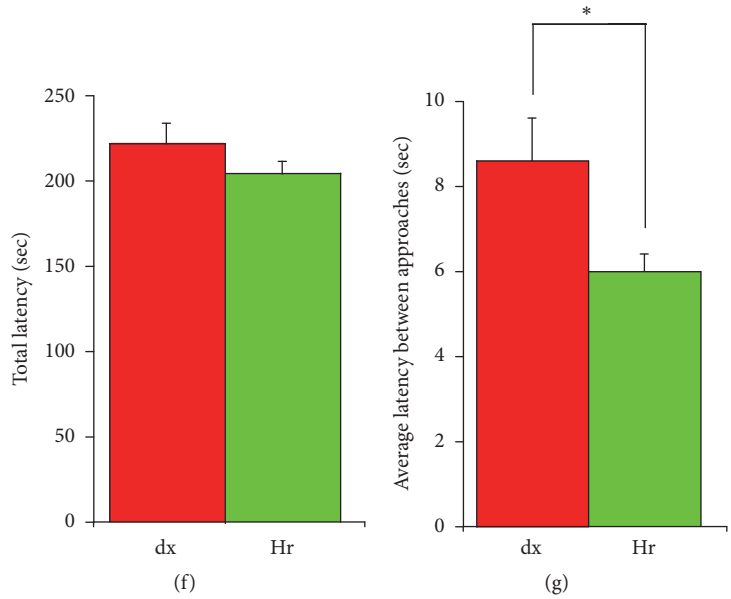

Figure 2: *H. erinaceus* increases recognition memory. Novel object recognition test (NOR) in dx ($n = 10$) and Hr-dietary supplemented mice ($n = 15$) during a 10 min session. (a) Schematic of the experimental set-up and procedure used. Histograms show (b) the number of approaches to the familiar and novel objects; (c) the total duration of approaches; (d) the average duration of an approach; (e) the latency to the first approach; (f) total latency; and (g) average latency between approaches.

activity received by the CA3 neuron, we recorded spontaneous excitatory postsynaptic currents (sEPSCs) in single hippocampal CA3 pyramidal neurons voltage-clamped at −70 mV in mice after two months of oral supply with dextrin or *H. erinaceus* (Figure 3(a)).

By a multimodal fitting of the frequency histogram of spontaneous excitatory currents we can distinguish between collateral-CA3 and mossy fiber-CA3 events [19].

Figures 3(b) and 3(c) show representative traces (left) and frequency histogram (right) of spontaneous postsynaptic currents recorded in dx ($n = 6$ cells, Figure 3(b)) and Hr mice ($n = 5$ cells, Figure 3(c)). The frequency histograms obtained by representative neurons can be fitted by a trimodal Gaussian distribution with data binned into 10 pA bins (Figures 3(b) and 3(c), right). In our experimental condition the first Gaussian peak could be due to the multiquantal release of the recurrent transmission CA3-CA3. The frequency of the events contributing to the first peak is not statistically different between dx and Hr mice (0.32 ± 0.07 Hz for dx versus 0.28 ± 0.09 Hz for Hr).

Giant spontaneous EPSCs at the hippocampal mossy fiber to CA3 pyramidal cell are monoquantal [19–22] and in our experimental condition should correspond to the second Gaussian peak, at about 120 pA. The frequency of the spontaneous events contributing to the second peak is statistically higher in Hr mice compared to dx mice (0.13 ± 0.03 Hz for Hr versus 0.05 ± 0.01 Hz in dx; T test $P < 0.01$, Figure 3(c) compared to Figure 3(b), right). The third peak of the histogram at about 200–250 pA (Figure 3(c) compared to Figure 3(b), right) is only just sketched in dx mice, while it is clearly visible in Hr mice. This peak could be due to the mossy fiber-CA3 synapses originating from the younger granule cells that, as reported in literature, display an increase of the input resistance giving a dramatic increase in granule cells excitability ([23] see discussion). The overall spontaneous activity is significantly higher in Hr as compared to dx mice (Figure 3(d), 1.32 ± 0.19 Hz in dx versus 1.79 ± 0.26 Hz in Hr; T test $P < 0.001$).

In conclusion, spontaneous activity at hippocampal mossy fiber to CA3 pyramidal cell is significantly higher in Hr as compared to dx mice.

3.2.2. Hr Supplementation Increases the Amplitude and Decreases the Failures Rate in Evoked Excitatory mf-CA3 Currents. By stimulating mossy fiber in the dentate gyrus (see Materials and Methods), we recorded evoked mf-CA3 synaptic excitatory currents (EPSCs). Minimal mossy fiber stimulation was applied at low frequency (0.1 Hz). Two key inclusion criteria were stated for differentiating EPSCs evoked by mossy fibers and CA3 recurrent fibers: the latency of evoked responses is longer when stimulating mossy fibers ($n = 16$ cells, 6.4 ± 0.8 ms; T test $P < 0.001$) versus CA3 recurrent fibers ($n = 16$ cells, 2.1 ± 0.3 ms; T test $P < 0.001$) [24] and the inhibition by DCG-IV, an agonist of the metabotropic glutamate receptor 2. At the end of the experiments we blocked the mf-CA3 neurotransmission by local perfusion of DCG-IV (2 μM, [25], see Figure 3(c) plot on the right).

EPSCs evoked by minimal mossy fiber stimulation were studied in the failures rate and in the amplitude (see representative traces in Figure 4(a), upper traces in dx mice

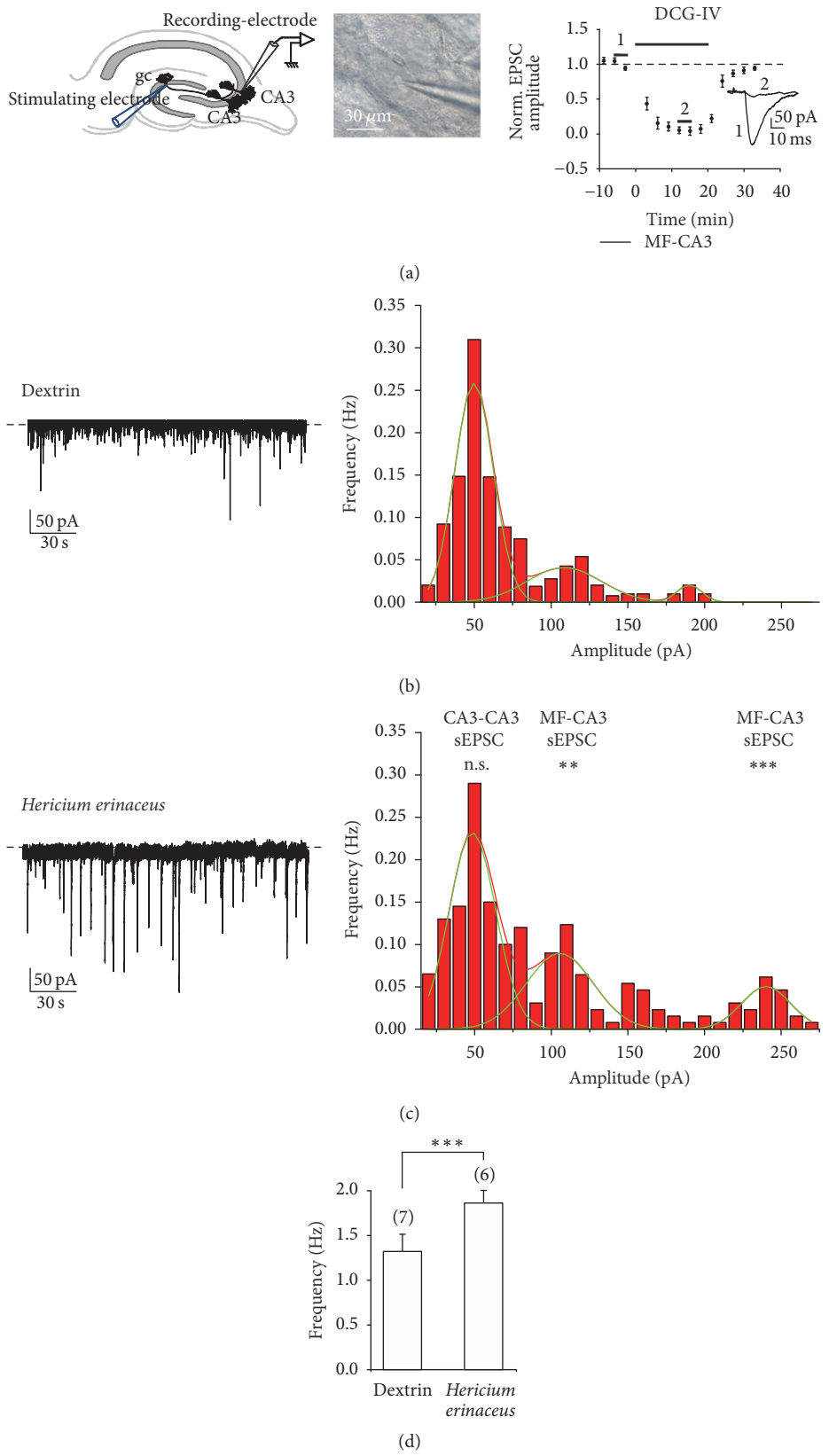

Figure 3: Spontaneous synaptic excitatory currents (sEPSCs) recorded from CA3 pyramidal cells. (a) Left, schematic drawing of experimental set-up. gc: granule cell, CA3 pyramidal cell. Middle, patch-pipette in a CA3 neuron. Right, bath-application of 2 μM DCG-IV, a receptor agonist of Group II metabotropic glutamate that blocks glutamate release from mossy fibers terminals, selectively decreases mossy fiber response (see inset). ((b) and (c)) Excitatory currents (sEPSCs) and (d) frequency histogram of spontaneous activity recorded from CA3 pyramidal cells in dextrin treated mice (Dextrin, $n = 6$) and in *Hericium* supplemented mice (*Hericium erinaceus*, $n = 5$).

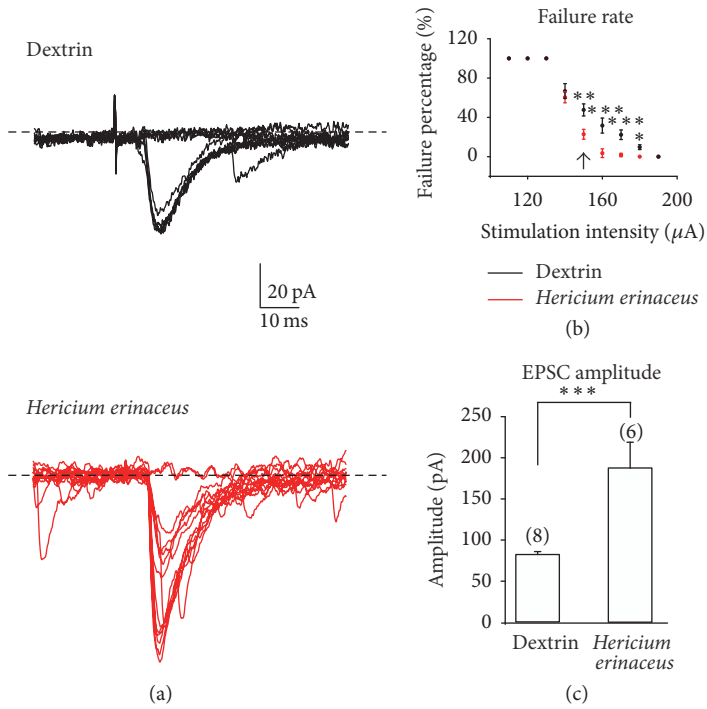

FIGURE 4: Mossy fiber-CA3 synapse evoked synaptic excitatory current (EPSCs) recorded after minimal stimulation at low frequency (0.1 Hz). (a) Experimental traces of evoked synaptic excitatory currents recorded in CA3 pyramidal cells for dextrin treated mice (top, Dextrin $n = 8$) and *H. erinaceus* oral supplemented mice (bottom, *Hericium erinaceus* $n = 6$). (b) Experimental design: mossy fibers were stimulated at different intensities (15 stimulations for each point) and the percentage of failure was calculated as the number of sweeps where no EPSC was evoked over the total number of stimulations per intensity point. The arrow inside the plot indicates the intensity point at which the representative traces in (a) are taken. (c) EPSCs amplitude histogram in dextrin treated mice (Dextrin) and in *H. erinaceus* oral supplemented mice (*Hericium erinaceus*).

and lower traces in Hr mice). We measured the failures percentage at minimal mossy fiber stimulation (see Materials and Methods). In dx mice ($n = 8$ cells) the failure percentage is significantly higher than in Hr mice ($n = 8$ cells, Figure 4(b), $P < 0.001$). The EPSC mean peak amplitude is significantly lower in dx mice compared to Hr mice (85.9 ± 17.9 pA versus 187.9 ± 68 pA, Figure 4(a); T test $P < 0.001$). *H. erinaceus* oral supply causes a significant decrease in percentage of failures and an increase in EPSCs amplitude.

3.2.3. Hr Supplementation Increases Neurotransmitter Release in mf-CA3 Synapse. Paired-pulse facilitation (PPF) is a relatively simple experimental protocol to estimate the dynamic of presynaptic release during evoked neurotransmission [26]. PPF of the excitatory synaptic transmission at mossy fiber-CA3 synapse in the hippocampus was studied. While stimulating mossy fibers at low frequency (0.1 Hz), EPSCs were elicited in pairs with an interpulse interval of 50 ms between the two stimuli in both dx and Hr mice (Figure 5(a), representative traces in dextrin and *H. erinaceus*).

Mossy fiber-CA3 synapse always results in paired-pulse facilitation as described [27] and as confirmed in our dx mice (Figure 5(a), upper traces). In dx mice the ratio between the 2nd and 1st EPSP in a pair was 1.9 ± 0.3 ($n = 8$ cells, Figure 5(b)), indicating an increase in the release probability in the second of the two stimuli applied. In Hr mice, the ratio between the 2nd and 1st EPSP was 1.2 ± 0.2 (Figure 5(b), $n = 6$ cells) showing a significant reduction (T test $P < 0.001$).

If the neurotransmission is increased the paired-pulse ratio (PPR, i.e., the ratio between the 2nd and 1st EPSP in a pair) should decrease in the 2nd peak for the lower availability of neurotransmitter vesicle [28] and/or for the postsynaptic receptor desensitization [29].

A decrease in paired-pulse ratio, a decrease in failure rate, and an increase in EPSCs amplitude are concordant data indicating an increase in neurotransmission between mossy fiber and CA3 hippocampal neuron probably due to an increase in neurotransmitter release from mossy fiber axon.

4. Discussion

Our in vivo data concordantly demonstrate that wild-type mice supplemented with *H. erinaceus* increased their exploration of novel stimuli. It has been recognized that several parameters of a task need to be recorded to support the validity and interpretation of the data of a behavioural experiment [30]. In our emergence test, the decreased latency of the first exit, the increase in the frequency of complete exits, and the longer duration of exploring time offer convergent evidences of increased novelty-seeking behaviour.

Recognition memory, a form of declarative memory, can generally be defined as the ability to discriminate the novelty

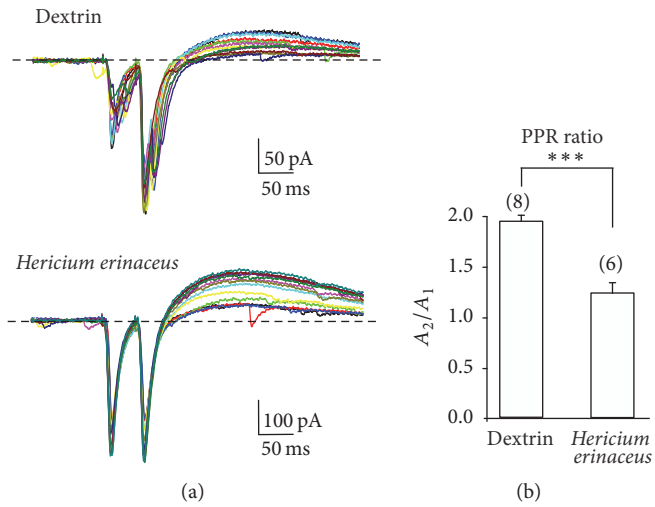

Figure 5: Paired-pulse stimulation: mossy fiber-CA3 synapse was stimulated by two pulses (interpulse interval 50 ms). (a) Experimental traces recorded in paired-pulse protocol for dextrin treated mouse (Dextrin, $n = 8$) and *H. erinaceus* supplemented mouse (*Hericium erinaceus*, $n = 6$). (b) Histograms of peak amplitude of the ratio between the second peak (A_2) and the first peak (A_1) for dextrin and Hr mice.

or familiarity of previous experiences by identifying when something (e.g., an object or an environment) has already been encountered. The results of the NOR task indicate that the Hr mice spent more time approaching the novel object than the familiar object; the decreased latency of the first approach and the increase in the frequency of approaches, combined with the longer duration of approaches, further support a state of increased novelty exploration behaviour. Conversely, the NOR test revealed no differences between Hr and dx mice in the exploration of the familiar object, indicating that the increase in exploratory activity is specifically oriented to the novel object.

The ability to cope with novelty is essential in all mammal species [31]. Novelty-seeking has been identified as one of the six major human personality dimensions, whereas neophobia describes hesitancy to engage with novel objects and places and can be considered a risk factor for anxiety disorders [32, 33]. A low level of exploratory activity towards novelty is interpreted as a sign of anxiety-like behaviour, whereas a high level reflects less anxiety [34]. Furthermore, reduced novelty-seeking and, in turn, increased neophobia can be considered core symptoms of depression; these behaviours are closely related to rigid evaluative patterns and reduced coping flexibility that also characterize the depressive state [35–38]. A recently published paper described the reduction of depression and anxiety by 4 weeks' intake of *H. erinaceus* dietary supplementation in 30 female subjects [5].

As we did, several studies have used the novelty approach as a measure of anxiety and the parameters measured were the latency, the frequency, and the duration of approaches [39, 40].

When rodents explore novel objects the pathway from the perirhinal cortex to lateral entorhinal cortex and then to the dentate gyrus and CA3 is engaged, whereas the pathway from the perirhinal cortex to lateral entorhinal cortex and then to CA1 is involved when familiar object s are explored [41].

Therefore, we focused our attention in this pathway, in particular on the final projection from the dentate gyrus (the mossy fiber tract) to the CA3 area (the pyramidal CA3 neurons).

The differential effect in Hr for novel versus familiar objects is reflected in the network as an increased neurotransmission at the mossy fiber-CA3 activity on both spontaneous (amplitude and frequency) and evoked glutamatergic events (amplitude).

The electrophysiological data obtained by recording in CA3 pyramidal neurons show some substantial differences in a number of features on both spontaneous excitatory postsynaptic currents (sEPSCs) and evoked excitatory postsynaptic currents (EPSCs) in mf-CA3 synapse in mice treated with *H. erinaceus* compared to those treated with dextrin.

In Hr mice, sEPSCs recorded from mf-CA3 neurons are increased in frequency and amplitude while a nonstatistical change in CA3-CA3 spontaneous currents was observed. In vivo and in vitro it was demonstrated that only a 2–5% fraction of granule cells in the hippocampal dentate gyrus neurons are spontaneously active and a monoquantal release was described [42–44].

Again, in Hr mice evoked mf-CA3 EPSCs are higher in amplitude and the frequency of the failures is lower. Furthermore, in paired-pulse protocol, we revealed a decrease in paired-pulse facilitation in EPSCs recorded in Hr mice. All these data together are in agreement with an increase in neurotransmitter release by the mossy fiber axons.

Adult hippocampal neurogenesis is the most interesting of the neurogenic zones in the adult brain, because it is involved in higher cognitive function, most notably memory processes, and certain affective behaviour [45]. In particular, in the dentate gyrus, adult and persistent hippocampal neurogenesis generates new excitatory granule cells in the dentate gyrus and contributes significantly to plasticity across the life span.

The hypothesis of on increased neurogenesis is in agreement with our data. As extensively reported by both in vitro and in vivo investigations [24] the newborn granule cells (GCs) have peculiar electrophysiological properties. Among them the most relevant one consists of a higher input resistance [46, 47] and of a lower rheobase [48]. Both of these parameters lower the threshold for the generation of the action potential in the newborn compared to the mature GCs, so increasing the neuron excitability. Consequently, a lower threshold for the generation of the action potential increases the likelihood that an action potential is triggered from the newborn GCs with two major consequences: an increase in the frequency of spontaneous events and a decrease in the frequency of the failures in evoked currents recorded in the postsynaptic CA3 pyramidal cells.

Moreover, while the mature GCs spontaneous firing activity is normally characterized by a single spike, the new born GCs deliver a burst of action potentials at high frequency [48]. Consequently, due to the temporal summation, a higher number of action potentials will provide a larger amount of the neurotransmitter released for every event and this will be detected as an increase in the amplitude of the excitatory spontaneous and evoked postsynaptic currents. Briefly, even though more experiments must be performed in future, the findings described in our investigation support the hypothesis of an increased neurogenesis in the hippocampal dentate gyrus as a consequence of *H. erinaceus* dietary supplementation in wild-type animals.

Hippocampal neurogenesis would impact the animal behaviour. A neurogenetic hypothesis of depression was originally formulated upon the demonstration that neurogenesis is regulated negatively by stressful experiences and positively by treatment with antidepressant. Since then much work has established that newborn neurons in the dentate gyrus are required for mediating some of the beneficial effects of antidepressant treatment [49–51].

What might be the molecular mechanism at the base of all those effects mediated by *H. erinaceus*?

Interestingly it was demonstrated that chronic intracerebroventricular infusion of nerve growth factor improves recognition memory in the rat [52] through an increase in the expression of NGF and of its receptor, TrkA, and of the synaptic vesicle protein, synapsin. This effect was paralleled by an increase in cell proliferation in the dentate gyrus of NGF treated rats. These data indicate that NGF chronic infusion can stimulate an improvement in learning and memory that is associated with specific cellular changes in the hippocampus, including synaptogenesis and cell proliferation.

Overall, our data show that *H. erinaceus* supplementation for two months has influential effects on wild-type mice, increasing glutamatergic synaptic drive novelty exploration behaviour and recognition memory in hippocampus.

Our study on the effects in wild-type mice of *H. erinaceus* oral supplementation on brain in both in vivo and in vitro experiments yielded several key findings that we hope will pave the way for new studies in healthy humans and bridge the gap between the millenary Eastern medicine and our Western medicine [53–55].

Competing Interests

The authors have declared that no competing interests exist.

Acknowledgments

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References

[1] S. T. Chang and S. P. Wasser, "The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health," *International Journal of Medicinal Mushrooms*, vol. 14, no. 2, pp. 95–134, 2012.

[2] K. Mori, Y. Obara, T. Moriya, S. Inatomi, and N. Nakahata, "Effects of *Hericium erinaceus* on amyloid β(25–35) peptide-induced learning and memory deficits in mice," *Biomedical Research*, vol. 32, no. 1, pp. 67–72, 2011.

[3] M. Hazekawa, A. Kataoka, K. Hayakawa et al., "Neuroprotective effect of repeated treatment with *Hericium erinaceum* in mice subjected to middle cerebral artery occlusion," *Journal of Health Science*, vol. 56, no. 3, pp. 296–303, 2010.

[4] K. Mori, S. Inatomi, K. Ouchi, Y. Azumi, and T. Tuchida, "Improving effects of the mushroom Yamabushitake (*Hericium erinaceus*) on mild cognitive impairment: a double-blind placebo-controlled clinical trial," *Phytotherapy Research*, vol. 23, no. 3, pp. 367–372, 2009.

[5] M. Nagano, K. Shimizu, R. Kondo et al., "Reduction of depression and anxiety by 4 weeks *Hericium erinaceus* intake," *Biomedical Research*, vol. 31, no. 4, pp. 231–237, 2010.

[6] J. M. Conner, K. M. Franks, A. K. Titterness et al., "NGF is essential for hippocampal plasticity and learning," *The Journal of Neuroscience*, vol. 29, no. 35, pp. 10883–10889, 2009.

[7] K. Mori, Y. Obara, M. Hirota et al., "Nerve growth factor-inducing activity of Hericium erinaceus in 1321N1 human astrocytoma cells," *Biological and Pharmaceutical Bulletin*, vol. 31, no. 9, pp. 1727–1732, 2008.

[8] Y. S. Park, H. S. Lee, M. H. Won, J. H. Lee, S. Y. Lee, and H. Y. Lee, "Effect of an exo-polysaccharide from the culture broth of Hericium erinaceus on enhancement of growth and differentiation of rat adrenal nerve cells," *Cytotechnology*, vol. 39, no. 3, pp. 155–162, 2002.

[9] C.-W. Phan, G.-S. Lee, S.-L. Hong et al., "*Hericium erinaceus* (Bull.: Fr) Pers. cultivated under tropical conditions: isolation of hericenones and demonstration of NGF-mediated neurite outgrowth in PC12 cells via MEK/ERK and PI3K-Akt signaling pathways," *Food & Function*, vol. 5, no. 12, pp. 3160–3169, 2014.

[10] G. Kempermann, "Adult neurogenesis: an evolutionary perspective," *Cold Spring Harbor Perspectives in Biology*, vol. 8, no. 2, Article ID a018986, pp. 1–9, 2016.

[11] G. W. Smith, J.-M. Aubry, F. Dellu et al., "Corticotropin releasing factor receptor 1–deficient mice display decreased

anxiety, impaired stress response, and aberrant neuroendocrine development," *Neuron*, vol. 20, no. 6, pp. 1093–1102, 1998.

[12] S. C. Dulawa, D. K. Grandy, L. J. Low, M. P. Paulus, and M. A. Geyer, "Dopamine D4 receptor-knock-out mice exhibit reduced exploration of novel stimuli," *The Journal of Neuroscience*, vol. 19, no. 21, pp. 9550–9556, 1999.

[13] A. Holmes, J. W. Kinney, C. C. Wrenn et al., "Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze," *Neuropsychopharmacology*, vol. 28, no. 6, pp. 1031–1044, 2003.

[14] G. Griebel, C. Belzung, R. Misslin, and E. Vogel, "The free-exploratory paradigm: an effective method for measuring neophobic behaviour in mice and testing potential neophobia-reducing drugs," *Behavioural Pharmacology*, vol. 4, no. 6, pp. 637–644, 1993.

[15] A. Ennaceur and J. Delacour, "A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data," *Behavioural Brain Research*, vol. 31, no. 1, pp. 47–59, 1988.

[16] A. Ennaceur, "One-trial object recognition in rats and mice: methodological and theoretical issues," *Behavioural Brain Research*, vol. 215, no. 2, pp. 244–254, 2010.

[17] G. W. Snedecor and W. G. Cochran, *Statistical Methods*, Iowa State University Press, Ames, Iowa, USA, 9th edition, 1989.

[18] J. M. Silvers, S. B. Harrod, C. F. Mactutus, and R. M. Booze, "Automation of the novel object recognition task for use in adolescent rats," *Journal of Neuroscience Methods*, vol. 166, no. 1, pp. 99–103, 2007.

[19] N. N. Urban, D. A. Henze, and G. Barrionuevo, "Revisiting the role of the hippocampal mossy fiber synapse," *Hippocampus*, vol. 11, no. 4, pp. 408–417, 2001.

[20] D. A. Henze, D. B. T. McMahon, K. M. Harris, and G. Barrionuevo, "Giant miniature EPSCs at the hippocampal mossy fiber to CA3 pyramidal cell synapse are monoquantal," *Journal of Neurophysiology*, vol. 87, no. 1, pp. 15–29, 2002.

[21] P. Jonas, G. Major, and B. Sakmann, "Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus," *The Journal of Physiology*, vol. 472, no. 1, pp. 615–663, 1993.

[22] D. A. Henze, J. P. Card, G. Barrionuevo, and Y. Ben-Ari, "Large amplitude miniature excitatory postsynaptic currents in hippocampal CA3 pyramidal neurons are of mossy fiber origin," *Journal of Neurophysiology*, vol. 77, no. 3, pp. 1075–1086, 1997.

[23] J. Lopez-Rojas and M. R. Kreutz, "Mature granule cells of the dentate gyrus-passive bystanders or principal performers in hippocampal function?" *Neuroscience and Biobehavioral Reviews*, vol. 64, pp. 167–174, 2016.

[24] M. Morl, M. H. Abegg, B. H. Gähwiler, and U. Gerber, "A frequency-dependent switch from inhibition to excitation in a hippocampal unitary circuit," *Nature*, vol. 431, no. 7007, pp. 453–456, 2004.

[25] D. Debanne, N. C. Guérineau, B. H. Gähwiler, and S. M. Thompson, "Physiology and pharmacology of unitary synaptic connections between pairs of cells in areas CA3 and CA1 of rat hippocampal slice cultures," *Journal of Neurophysiology*, vol. 73, no. 3, pp. 1282–1294, 1995.

[26] P. Schulz, E. Cook, and D. Johnston, "Using paired-pulse facilitation to probe the mechanisms for long-term potentiation (LTP)," *Journal of Physiology—Paris*, vol. 89, no. 1, pp. 3–9, 1995.

[27] P. A. Salin, M. Scanziani, R. C. Malenka, and R. A. Nicoll, "Distinct short-term plasticity at two excitatory synapses in the hippocampus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 23, pp. 13304–13309, 1996.

[28] M. A. Xu-Friedman and W. G. Regehr, "Ultrastructural contributions to desensitization at cerebellar mossy fiber to granule cell synapses," *The Journal of Neuroscience*, vol. 23, no. 6, pp. 2182–2192, 2003.

[29] H. Kamiya, H. Shinozaki, and C. Yamamoto, "Activation of metabotropic glutamate receptor type 2/3 suppresses transmission at rat hippocampal mossy fibre synapses," *Journal of Physiology*, vol. 493, no. 2, pp. 447–455, 1996.

[30] A. Ennaceur, S. Michalikova, A. Bradford, and S. Ahmed, "Detailed analysis of the behavior of Lister and Wistar rats in anxiety, object recognition and object location tasks," *Behavioural Brain Research*, vol. 159, no. 2, pp. 247–266, 2005.

[31] C. R. Cloninger, "A systematic method for clinical description and classification of personality variants: a proposal," *Archives of General Psychiatry*, vol. 44, no. 6, pp. 573–588, 1987.

[32] J. Kagan, "The meanings of personality predicates," *American Psychologist*, vol. 43, no. 8, pp. 614–620, 1988.

[33] T. R. Minor, M. A. Trauner, C.-Y. Lee, and N. K. Dess, "Modeling signal features of escape response: effects of cessation conditioning in 'learned helplessness' paradigm," *Journal of Experimental Psychology: Animal Behavior Processes*, vol. 16, no. 2, pp. 123–136, 1990.

[34] K. A. Stedenfeld, S. M. Clinton, I. A. Kerman, H. Akil, S. J. Watson, and A. F. Sved, "Novelty-seeking behavior predicts vulnerability in a rodent model of depression," *Physiology and Behavior*, vol. 103, no. 2, pp. 210–216, 2011.

[35] J. N. Crawley, "Exploratory behavior models of anxiety in mice," *Neuroscience and Biobehavioral Reviews*, vol. 9, no. 1, pp. 37–44, 1985.

[36] F. C. Celikel, S. Kose, B. E. Cumurcu et al., "Cloninger's temperament and character dimensions of personality in patients with major depressive disorder," *Comprehensive Psychiatry*, vol. 50, no. 6, pp. 556–561, 2009.

[37] T. Kato, "Development of the coping flexibility scale: evidence for the coping flexibility hypothesis," *Journal of Counseling Psychology*, vol. 59, no. 2, pp. 262–273, 2012.

[38] K. A. Jacobs,, "The depressive situation," *Frontiers in Psychology*, vol. 4, 2013.

[39] D. Treit, "Animal models for the study of anti-anxiety agents: a review," *Neuroscience and Biobehavioral Reviews*, vol. 9, no. 2, pp. 203–222, 1985.

[40] M. Wesierska, G. Walasek, J. Kilijanek, R. L. Djavadian, and K. Turlejski, "Behavior of the gray short-tailed opossum (*Monodelphis domestica*) in the open field and in response to a new object, in comparison with the rat," *Behavioural Brain Research*, vol. 143, no. 1, pp. 31–40, 2003.

[41] L. Kinnavane, M. M. Albasser, and J. P. Aggleton, "Advances in the behavioural testing and network imaging of rodent recognition memory," *Behavioural Brain Research*, vol. 285, pp. 67–78, 2015.

[42] M. K. Chawla and C. A. Barnes, "Hippocampal granule cells in normal aging: insights from electrophysiological and functional imaging experiments," *Progress in Brain Research*, vol. 163, pp. 661–821, 2007.

[43] C. B. Alme, R. A. Buzzetti, D. F. Marrone et al., "Hippocampal granule cells opt for early retirement," *Hippocampus*, vol. 20, no. 10, pp. 1109–1123, 2010.

[44] G.-A. Pilz, S. Carta, A. Stäuble, A. Ayaz, S. Jessberger, and F. Helmchen, "Functional imaging of dentate granule cells in the

adult mouse hippocampus," *The Journal of Neuroscience*, vol. 36, no. 28, pp. 7407–7414, 2016.

[45] G. Kempermann, H. Song, and F. H. Gage, "Neurogenesis in the adult hippocampus," *Cold Spring Harbor Perspectives in Biology*, vol. 7, no. 9, 2015.

[46] Y.-B. Liu, P. A. Lio, J. F. Pasternak, and B. L. Trommer, "Developmental changes in membrane properties and postsynaptic currents of granule cells in rat dentate gyrus," *Journal of Neurophysiology*, vol. 76, no. 2, pp. 1074–1088, 1996.

[47] C. Schmidt-Hieber, P. Jones, and J. Bischofberger, "Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus," *Nature*, vol. 429, no. 6988, pp. 184–187, 2004.

[48] L. A. Mongiat, M. S. Espósito, G. Lombardi, and A. F. Schinder, "Reliable activation of immature neurons in the adult hippocampus," *PLoS ONE*, vol. 4, no. 4, Article ID e5320, 2009.

[49] B. L. Jacobs, H. Van Praag, and F. H. Gage, "Adult brain neurogenesis and psychiatry: a novel theory of depression," *Molecular Psychiatry*, vol. 5, no. 3, pp. 262–269, 2000.

[50] B. A. Samuels and R. Hen, "Neurogenesis and affective disorders," *European Journal of Neuroscience*, vol. 33, no. 6, pp. 1152–1159, 2011.

[51] N. D. Hanson, M. J. Owens, and C. B. Nemeroff, "Depression, antidepressants, and neurogenesis: a critical reappraisal," *Neuropsychopharmacology*, vol. 36, no. 13, pp. 2589–2602, 2011.

[52] A. M. Birch and Á. M. Kelly, "Chronic intracerebroventricular infusion of nerve growth factor improves recognition memory in the rat," *Neuropharmacology*, vol. 75, pp. 255–261, 2013.

[53] M. A. Khan, M. Tania, R. Liu, and M. M. Rahman, "*Hericium erinaceus*: an edible mushroom with medicinal values," *Journal of Complementary and Integrative Medicine*, vol. 10, no. 1, 2013.

[54] S. P. Wasser, "Medicinal mushroom science: current perspectives, advances, evidences, and challenges," *Biomedical Journal*, vol. 37, no. 6, pp. 345–356, 2014.

[55] P. Rossi, D. Buonocore, E. Altobelli et al., "Improving training condition assessment in endurance cyclists: effects of *ganoderma lucidum* and *ophiocordyceps sinensis* dietary supplementation," *Evidence-based Complementary and Alternative Medicine*, vol. 2014, Article ID 979613, 11 pages, 2014.

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Dietary Supplementation of Lion's Mane Medicinal Mushroom, *Hericium erinaceus* (Agaricomycetes), and Spatial Memory in Wild-Type Mice

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ABSTRACT: *Hericium erinaceus* is an edible and medicinal mushroom with potential neuroprotective effects. The study of *H. erinaceus* has attracted considerable attention during the past 10 years, particularly with regard to its potential utility in the treatment of motor dysfunction, Alzheimer disease, and other forms of dementia. We previously determined that oral supplementation with *H. erinaceus* results in significant improvements in novelty-seeking behavior and novel object recognition in mice. In this study, *H. erinaceus* was added to the diets of wild-type mice for 2 months, and effects on spatial memory were evaluated by means of a Y maze and an object location task. We found that *H. erinaceus* increased general locomotor activity but had no effect on spatial memory. Thus, oral supplementation with *H. erinaceus* yields specific and selective improvements in recognition memory without altering spatial working memory, which supports the hypothesis that recognition memory can be modeled as a dual process. In this model, the perirhinal cortex supports the recognition of individual items as part of a circuit involved in familiarity with an encountered stimulus, whereas the hippocampus supports recollected associations and relationships between stimuli.

KEY WORDS: dual-processes model, *Hericium erinaceus*, medicinal mushrooms, oral supplementation, recognition memory, spatial working memory

ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; **DX**, dextrin; **NGF**, nerve growth factor

I. INTRODUCTION

The lion's mane medicinal mushroom, *Hericium erinaceus* (Bull.) Pers. (Hericiaceae, Agaricomycetes), can grow on living or dead broadleaf trees and is used in several Asian countries as both food and herbal medicine without any reported harmful effects.[1] *H. erinaceus* comprises numerous compounds with intriguing biologic activities; these include promotion of the synthesis of nerve growth factor (NGF)[2] and brain-derived neurotrophic factor (BDNF),[3] cytotoxic functions,[4] antimicrobial effects,[5] and antitumor and antioxidant activities.[6–9] The compounds hericenones C–H[10] and erinacines A–I[11–14] have been isolated from the fruiting bodies and mycelia, respectively, of *H. erinaceus*. Hericenone and erinacine are low molecular weight, relatively lipid-soluble compounds that can cross the blood-brain barrier. These compounds have been shown to stimulate NGF synthesis in cultured astrocytes.[12,14,15] Moreover, oral administration of *H. erinaceus* increases expression of NGF mRNA in the mouse hippocampus.[15]

NGF has been shown to exert crucial functions in the central nervous system. Results of preclinical studies involving pathological animal models demonstrate that NGF acts on cholinergic neurons, induces neuronal differentiation, promotes neuronal survival and regeneration, and ameliorates neurodegeneration

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and cognitive deficits.[16] In mice, oral administration of *H. erinaceus* prevents amyloid β (25–35) peptide–induced impairment of spatial short-term memory and visual recognition memory.[17] Orally delivered *H. erinaceus* was found to confer neuroprotection in mice in which the middle cerebral artery was occluded.[18] Furthermore, scores on a cognitive function scale improved following oral administration of *H. erinaceus* to patients with mild cognitive impairment.[19] Thus, dietary supplementation with *H. erinaceus* has potential therapeutic benefits, particularly in the management of neurodegenerative diseases (for a review, see Phan et al.[20]).

Herein we describe the effects of *H. erinaceus* on spatial memory in mice. The animals were tested behaviorally by means of an object location task and a Y maze. The object location task—a variant of the spontaneous object recognition task—enables investigation of recognition memory for the spatial location of objects. The Y maze is a simple and automatable task that allows rapid assessment of the willingness of rodents to explore new environments.[21–23] Because the behavior is not reinforced with external rewards or punishments, this task is considered spontaneous; that is, the Y maze yields information about spontaneous alternation and spatial working memory. The free-running Y maze was applied in this study; it has the advantages of avoiding excessive handling of the mice, which stresses the animals, and of providing a measure of locomotor activity in terms of the frequency of arm entries.[24] Our findings of a selective effect of *H. erinaceus* supplementation on these tasks suggest that this medicinal mushroom has a specific target in the central nervous system and that recognition memory is supported by 2 functionally distinct processes in wild-type mice.

II. MATERIALS AND METHODS

A. Animals

Wild-type mice (strain C57BL-6J) used in this study had a mean ± standard deviation body weight of 41 ± 1 g and were 40 to 50 days old. Animals were bred at the Animal Facility of the University of Pavia (Pavia, Italy) in a temperature- and humidity-controlled room. To avoid variations related to the estrous cycle in females, only male mice were included. The mice were housed in groups of 4 or 5 and were given water *ad libitum*. Twelve mice received a normal diet containing 5% dextrin (DX) for 60 days (DX group); 19 mice received powdered *H. erinaceus* mixed with a normal powdered diet (~0.025 g/g body weight, adjusted to contain 5% [w/w] of *H. erinaceus*) for 60 days.

B. Dietary Supplementation

A wild sample of *H. erinaceus* fruiting bodies was collected from a dead oak tree (*Quercus* sp.) in Slovakia in 2009; MycoMedica d.o.o. (Kranjska Gora, Slovenia) isolated the strain (HER.ERIN) and confirmed its identification through a nucleotide sequence that has been submitted to GenBank (accession no. SUB3440287). Culture was cultivated on potato dextrose agar (Difco) at 24°C in the dark; then, after 20 days, cultures were transferred onto lignocellulosic substrates and further incubated at 24°C for 60 days. After that, fruiting bodies and fungal biomass were harvested. Fruiting bodies were extracted in water and ethanol (1:15 [w/v] ratio). The fruiting body extract and mycelia were dried under a vacuum (70°C and −0.9 bar). To obtain particles mostly smaller than 100 μm, samples were milled further using a UPZ Fine Impact Mill (Hosokawa Alpine Aktiengesellschaft, Augsburg, Germany). The *H. erinaceus* supplement was prepared by A.V.D. Reform (Micotherapy Hericium; Noceto, Parma, Italy). One capsule of Micotherapy Hericium contains 400 mg mycelia and 100 mg dried fruiting body extract (50 mg polysaccharide; ratio 4:1). The polysaccharide (expressed as total α- and β-glucan content) was

determined using a β-Glucan Assay Kit (Megazyme; Bray Co., Wicklow, Ireland); the nutrient composition of the supplement has been described previously (see Tables 1–3 in the publication by Brandalise et al.[25]). All experiments were carried out according to guidelines set forth by the Ethics Committee of Pavia University.

C. Apparatus and Procedures

For all experiments, researchers were blinded to the group assignment (DX or *H. erinaceus*). Motor activity was quantified by means of the SMART Video Tracking System (2Biological Instruments, Besozzo VA, Italy) and a charge-coupled device color video camera with a phase-altering line (Sony, New York, NY). The object location (spatial) memory test was carried out in an open box; the task does not involve reinforcement and does not require training.

The object location task comprises 3 phases: habituation, familiarization (sample phase), and testing. During the habituation phase, each mouse was allotted 15 minutes/day during the first 2 days to freely explore the open-field arena in the absence of objects. The mouse was subsequently removed from the arena and placed in a holding cage. On the third day, during the familiarization phase, each mouse was placed in an open-field arena that contained 2 identical objects. The mouse was free to explore these objects for 5 minutes. To prevent coercion to explore the objects, the mouse was placed against the center of the wall opposite the objects, with its back to them. After familiarization, the mouse was placed in the holding cage, and a 10-minute retention phase elapsed. The mouse then was returned to the same open box containing the 2 identical objects, but 1 of these objects had been repositioned. This test phase proceeded for 5 minutes, during which approaches by the mouse with its nose were observed. An approach was defined as the mouse's nose moving within 2 cm of the object.

The software collected the following data from the object location task:

1. Number of approaches (the number of times either of the 2 objects was approached)
2. Total duration of approaches (the amount of time the animal spent exploring the objects)
3. Mean duration of an approach (the amount of time spent exploring the objects divided by the number of approaches)
4. Latency of the first approach (the time elapsed between placement of the animal in the arena and its first approach to either object)
5. Total latency between approaches (the amount of time spent between each approach to either object)
6. Mean latency between approaches (the total latency between approaches divided by the number of approaches)

We subsequently applied the spontaneous Y maze task. This test typically is performed to study spatial working memory and general locomotor activity without any external reward or punishment.[24] A *Y*-shaped maze was constructed with 3 symmetrical grey, solid plastic arms (40 cm long, 12 cm high, 8 cm wide) positioned at 120° angles from each other. Before each session, the maze was cleaned with 10% ethanol to eliminate trace odors. Each session began with the mouse placed in the center of the maze. The mouse could freely explore the 3 arms for 8 minutes.[26–28] In this task, an "arm entry" was defined as all 4 of the animal's limbs entering an arm of the maze. A "triad" was defined as a set of 3 arm entries in which each consecutive entry was into a different arm of the maze. That is, if the arms of the Y maze are designated A, B, and C, entry would proceed as ABC, ACB, BAC, BCA, CAB, or CBA. A failure was defined as repeated entry into the same arm (ABA, ACA, AAB, AAC, BAB, BCB, BBC, BBC, CBC, CAC, CCB, or CCA).[17] The number of arm entries and the number of triads were measured. The percentage alternation

was computed by dividing the number of triads by the number of possible alternations (number of entries − 2) and then multiplying by 100.

The software collected the following data from the Y maze task:

1. Frequency of arm entries (all 4 limbs entering an arm of the maze)
2. Number of triads (sets of 3 arm entries in which each consecutive entry was to a different arm of the maze [ABC, ACB, BAC, BCA, CAB, or CBA])
3. Percentage alternation.

D. Statistical Analysis

Descriptive statistics were expressed as the mean ± standard error of the mean. Statistical significance was ascertained with the Student *t* test after evaluating data on the assumption of normality by means of the Shapiro-Wilk test. Statistical significance was defined as $P < 0.05$.

III. RESULTS

Figure 1A shows a schematic drawing of the experimental protocol for the object location task. In the DX group (control; n = 12 mice), measures when the 2 objects were positioned in a familiar location compared with a novel location indicated no significant differences in the number of approaches (13.7 ± 2 vs. 15.5 ± 2.3; Fig. 1B), the total duration of approaches (28.9 ± 4.3 vs. 30.9 ± 5.4 seconds; Fig. 1C), the mean duration of an approach (2.7 ± 0.6 vs. 2.6 ± 0.6 seconds; Fig. 1D), or the latency of the first approach (7.5 ± 1.9 vs. 10 ± 2.6 seconds; Fig. 1E). In mice fed *H. erinaceus* (n = 19), comparisons of measures related to object placement (familiar vs. novel) indicated no significant differences in the number of approaches (10 ± 0.9 vs. 11.6 ± 1.2; Fig. 1B), the total duration of approaches (18.9 ± 2.5 vs. 19.6 ± 2.5 seconds; Fig. 1C), the mean duration of an approach (2.2 ± 0.4 vs. 1.8 ± 0.2 seconds; Fig. 1D), or the latency of the first approach (8.8 ± 2.1 vs. 12.5 ± 2.1 seconds; Fig. 1E). Thus, when a familiar object was repositioned, mice fed *H. erinaceus* behaved like mice in the DX group with regard to the metrics evaluated. In a comparison of the DX and *H. erinaceus* groups, the total latency (240.1 ± 8.8 vs. 261.5 ± 4.7 seconds; Fig. 1F) and the mean latency between approaches (11.3 ± 2.1 vs. 13.6 ± 1 seconds; Fig. 1G) were similar (Fig. 1). Dietary supplementation with *H. erinaceus* in wild-type mice does not affect exploration of familiar and repositioned objects; hence, *H. erinaceus* supplementation has no effect on spatial memory tasks.

In the Y maze test of spontaneous alternation (Fig. 2A), we measured the number of total entries into arms of the maze, the number of alternation triads, and the alternation triad percentage in mice given DX (control; n = 12) or *H. erinaceus* (n = 19). As shown in Fig. 2, mice fed *H. erinaceus* performed a significantly greater number of spontaneous entries and alternation triads. The number of entries into the maze arms was 50.1 ± 5.5 for the DX group and 63.7 ± 3.7 for the *H. erinaceus* group—a 27.4% increase with *H. erinaceus* treatment ($P < 0.04$; Fig. 2B). The number of alternation triads was 27.3 ± 3.5 for the DX group and 34.1 ± 1.8 for the *H. erinaceus* group—a 24.9% increase with *H. erinaceus* treatment ($P < 0.05$; Fig. 2C). By dividing the number of triads by the number of possible alternations (number of entries − 2), we determined the percentage alternation (Fig. 2D). This value was similar for the 2 groups: 55.4% ± 3.5% for the DX-treated mice and 56.2% ± 1.9% for the *H. erinaceus*–fed mice. Taken together, our data reveal that although spatial working memory did not improve—as indicated by the percentage of alternations—we noted an increase in spontaneous crosses in maze arms in the *H. erinaceus* group compared with the controls.

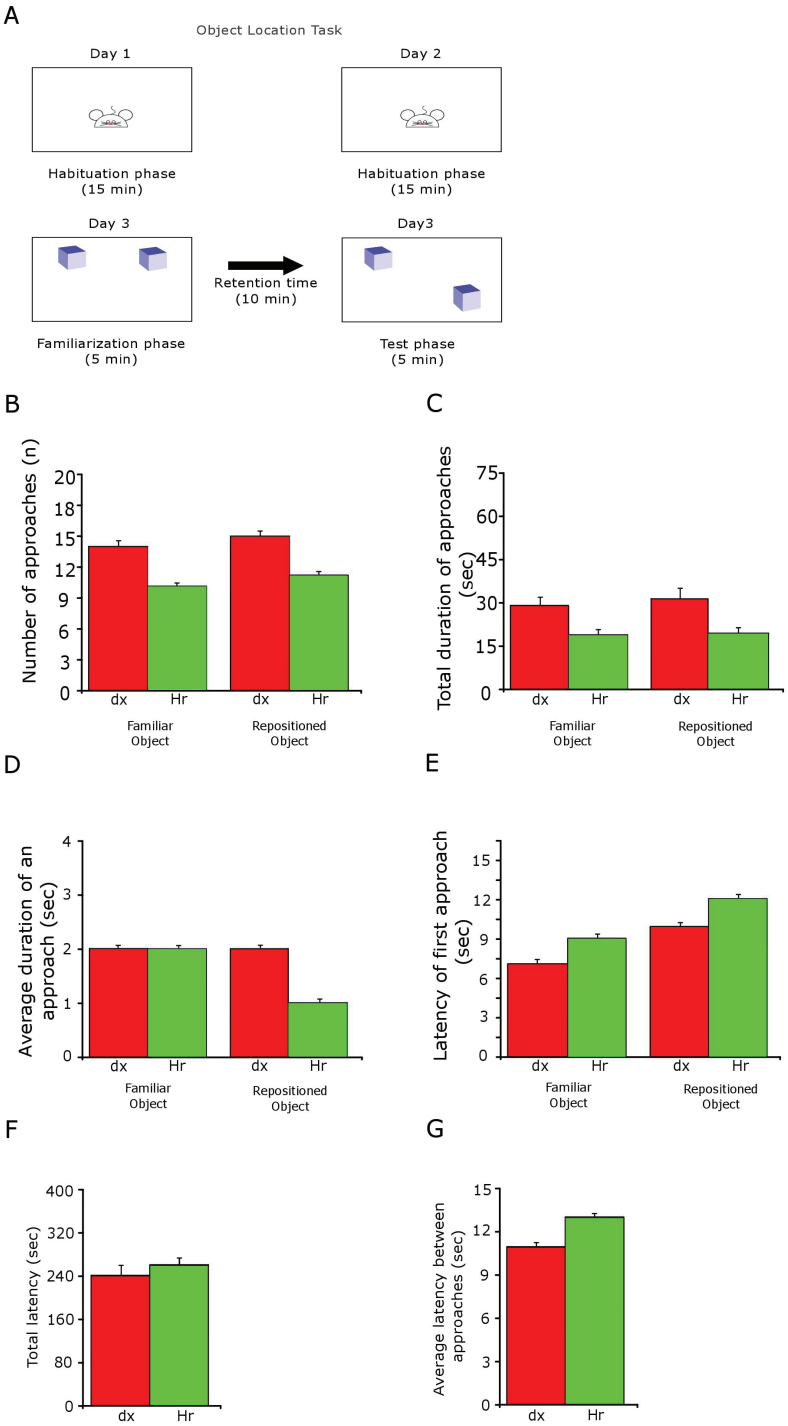

FIG. 1: Results of the object location task (a 5-minute session) for the dextrin group (n = 12) and the *Hericium erinaceus* supplementation group (n = 19). (A) Schematic drawing of the experimental setting and procedure. (B–F) The histograms depict the number of times the mouse approached familiar and novel objects (B), the total duration of approaches (C), the mean duration of an approach (D), the latency of the first approach (E), the total latency (F), and the mean latency between approaches (G).

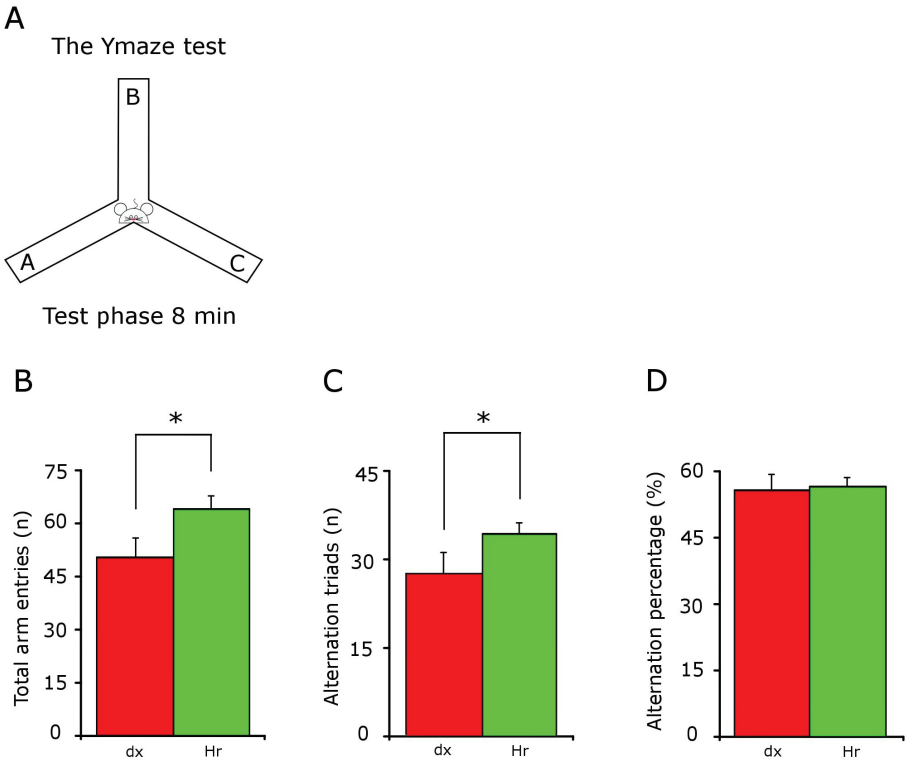

FIG. 2: Results of the Y maze test (an 8-minute session) for the dextrin group (n = 12) and the *Hericium erinaceus* supplementation group (n = 19). (A) Schematic drawing of the experimental setting and procedure. (B–D) The histograms depict the total number of arm entries (B), the number of alternation triads (C), and the alternation percentage (D). Results are presented as the mean ± standard error of the mean. *$P < 0.05$.

IV. DISCUSSION

H. erinaceus has attracted considerable attention owing to its putative neuroprotective role in neurodegenerative diseases such as dementia and motor dysfunction. Hericenone and erinacine are compounds isolated from the fruiting bodies and mycelia, respectively, of *H. erinaceus*. These compounds are able to traverse the blood-brain barrier and stimulate NGF synthesis in cultured astrocytes[12,15] and in the hippocampus of mice after oral administration.[15] We previously determined that oral supplementation with *H. erinaceus* induces significant improvements in novelty-seeking behavior and recognition memory in wild-type mice compared with controls.[25] Moreover, *H. erinaceus* increases spontaneous and evoked glutamatergic synaptic currents in CA3 hippocampal pyramidal cells of these mice.[25]

Recognition memory is a form of declarative memory whereby memories can be consciously recalled. Recognition memory can be defined as the process of identifying when something (e.g., an object, a person, or an environment) has been encountered previously. Researchers have long been interested in the mechanisms underlying recognition memory. Current consensus agrees that 2 distinct cognitive processes, recollection and familiarity, contribute to performance on recognition memory tests.[29] However, it remains debatable whether recollection and familiarity reflect anatomically and functionally distinct, dissociable memory processes (i.e., the dual-process model) or whether these processes constitute a single expression of memory traces of different strengths in the context of a unitary declarative memory system (i.e., the unitary strength model).[29–31] Answering this question is key to understanding recognition memory function.

Some researchers maintain that 2 functionally distinct processes support recognition memory and that these are mediated by different structures in the medial temporal lobe: the parahippocampal region and, in particular, the perirhinal and entorhinal cortices. These structures are thought to contribute to a circuit involved in familiarity with and recognition of individual items that yields a feeling of "knowing."[32,33] In this theoretical framework, the hippocampus supports recollected associations and relations among stimuli to produce a feeling of "remembering" (for a review, see Ameen-Ali et al.[30]). Results of studies involving human patients with amnesia and hippocampal damage have provided useful insight in this debate; some investigators observed selective impairment of recollection with spared familiarity processing,[29] thereby offering support to the dual-process model.[34,35]

Findings from animal studies in which lesions were induced and tests of spontaneous novel object recognition and object location were performed have enhanced our understanding of the anatomic basis for recognition memory. The perirhinal cortex is critical to successful performance on tests of novel object recognition,[36–39] whereas lesions in the hippocampus or fornix have no detrimental effect on novel object recognition.[38–40] Conversely, rats with lesions of the dorsal hippocampus exhibit poor functioning in tests of object location, but lesions of the perirhinal cortex do not interfere with the ability to locate objects.[37]

To explore the role of *H. erinaceus* in recognition memory, we evaluated the effect of 2 months of oral administration of this medicinal mushroom in wild-type mice. We applied 2 spontaneous behavioral tests: the object location test and the Y maze test. Object location tasks involve multiple items and contextual associations. The Y maze reflects spatial memory functioning and the capacity to enter an arm of the maze that had not been entered immediately previously.

We determined that *H. erinaceus* did not affect object location function. Results of all parameters (i.e., number, total duration, and mean duration of approaches, and latency of the first approach) were similar for the DX and *H. erinaceus* groups, suggesting that oral *H. erinaceus* has no effect on spatial working memory in wild-type mice. Findings from the Y maze test indicated that the percentage alternation, a measure of performance in choosing different maze arms at each inspection, was unchanged with *H. erinaceus* supplementation, whereas general locomotor activity—ascertained as the total number of arm entries—was increased with *H. erinaceus* treatment. Hence, we determined that *H. erinaceus* imposes no seemingly hippocampus-dependent effects on object location and Y maze tasks. However, parahippocampus-dependent, recognition memory–based functioning, assessed in terms of performance on novel object recognition and emergence tasks, was enhanced by *H. erinaceus* supplementation. These findings support the hypothesis that *H. erinaceus* has a selective role in increasing parahippocampus-dependent cognitive performance (Fig. 3A). Anatomical/functional evidence is strongly recommended to strengthen our hypothesis, and we are planning experiments to study at the cellular level possible differences in the neural network.

We previously described the effect of oral supplementation of *H. erinaceus* in hippocampal slices from wild-type mice.[25] Through the use of patch-clamp experiments, we observed an increase in mossy fiber–CA3 neurotransmission on both spontaneous and evoked glutamatergic synaptic currents; this possibly was caused by an increase in the release of neurotransmitter from granule cells. We speculate that this finding might entail an increase in the upstream network of the entorhinal cortex that later is transmitted to the hippocampus via the perforant pathway (Fig. 3B). Our *in vivo* results could be strengthened by means of patch-clamp experiments that compare parahippocampal slices from the *H. erinaceus*–supplemented group with slices from the DX group. In particular, neurotransmission in the perirhinal and entorhinal cortices could be recorded to verify potential differences in neurotransmission activity in the upstream hippocampal network.

The effects of *H. erinaceus* supplementation in wild-type animals strongly suggest that recognition memory is a dual process that involves distinct behavioral mechanisms and/or different anatomic structures.

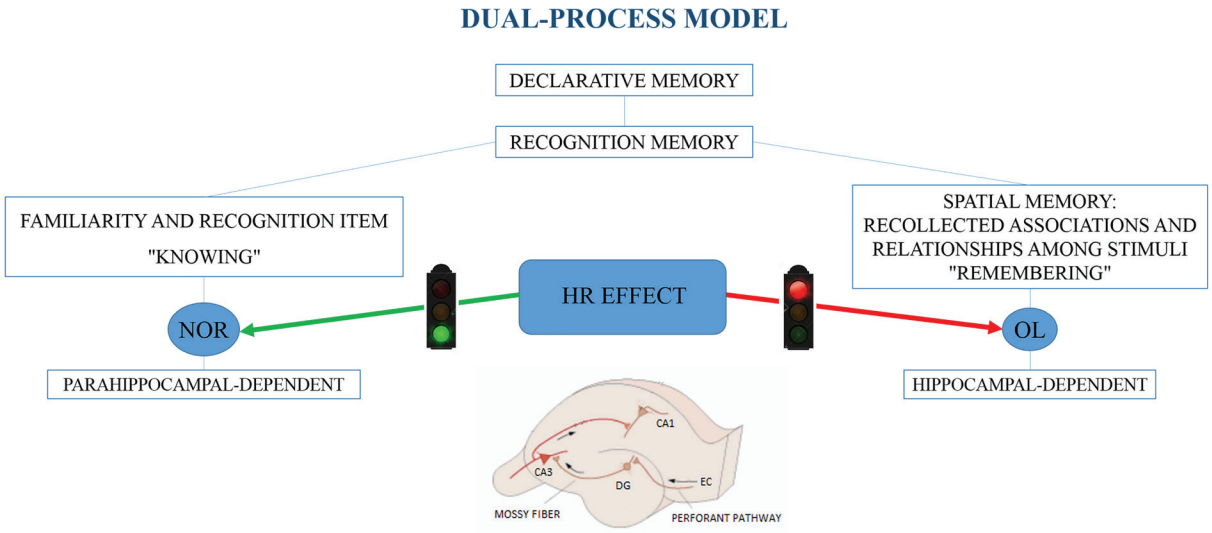

FIG. 3: Schematic drawing of the effects of *Hericium erinaceus* on recognition memory and the hippocampal network. According to the dual-process model, recognition memory can be divided in 2 processes: familiarity and recognition items, and recollected associations and relations among stimuli. The first process is described as a feeling of "knowing"; this process is dependent on the functioning of the parahippocampus and can be evaluated by means of the novel object recognition (NOR) test. The second component of the dual-process model is described as a feeling of "remembering." This process can be examined with the object location (OL) test and is dependent on the hippocampus. Oral supplementation of wild-type mice with *Hericium erinaceus* (HR) had no effect on hippocampus-dependent spatial memory (i.e., object location) but improved parahippocampus-dependent cognitive performance (i.e., novel object recognition).[18] The entorhinal cortex (EC) in the parahippocampal gyrus is connected by the perforant pathway to the dentate gyrus (DG), which contains granule cells. These cells give rise to mossy fibers, which contact CA3 pyramidal neurons. Oral administration of HR yields enhanced mossy fiber–CA3 hippocampal neurotransmission.

Furthermore, *H. erinaceus* supplementation exercises a selective effect, determining recollection memory with spared familiarity processing.

V. CONCLUSIONS

Hippocampal functioning is not directly involved in the improvement of cognitive performance observed in *H. erinaceus*–fed mice. Our findings lend support to the hypothesis that recognition memory involves dual processes. Specifically, the perirhinal cortical region supports recognition of individual items in a circuit associated with familiarity toward an encountered stimulus. Concomitantly, the hippocampus supports recollected associations and relations between stimuli (for a review, see Ameen-Ali et al.[30]). Dietary supplementation with *H. erinaceus* specifically targets parahippocampal structures, increasing glutamatergic synaptic drive in the hippocampus. This effect is reflected through increased novelty-seeking animal behavior and in the "knowing" component of recognition memory.

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REFERENCES

1. Chang ST, Wasser SP. The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. Int J Med Mushrooms. 2012;14(2):95–134.
2. Lai PL, Naidu M, Sabaratnam V, Wong KH, David RP, Kuppusamy UR, Abdullah N, Malek SN. Neurotrophic properties of the lion's mane medicinal mushroom, Hericium erinaceus (higher Basidiomycetes) from Malaysia. Int J Med Mushrooms. 2013;15(6):539–54.
3. Samberkar S, Gandhi S, Naidu M, Wong KH, Raman J, Sabaratnam V. Lion's mane, Hericium erinaceus and tiger milk, Lignosus rhinocerotis (higher Basidiomycetes) medicinal mushrooms stimulate neurite outgrowth in dissociated cells of brain, spinal cord, and retina: an in vitro study. Int J Med Mushrooms. 2015;17(11):1047–54.
4. Kawagishi H, Ando M, Mizuno T. Hericenone A and B as cytotoxic principles from the mushroom Hericium erinaceum. Tetrahedr Lett. 1990;31(3):373–6.
5. Shang X, Tan Q, Liu R, Yu K, Li P, Zhao GP. In vitro anti-Helicobacter pylori effects of medicinal mushroom extracts, with special emphasis on the lion's mane mushroom, Hericium erinaceus (higher Basidiomycetes). Int J Med Mushrooms. 2013;15(2):165–74.
6. Wasser SP. Medicinal mushrooms in human clinical studies. Part I. Anticancer, oncoimmunological, and immunomodulatory activities: a review. Int J Med Mushrooms. 2017;19(4):279–317.
7. Rossi P, Buonocore D, Altobelli E, Brandalise F, Cesaroni V, Iozzi D, Savino E, Marzatico F. Improving training condition assessment in endurance cyclists: effects of Ganoderma lucidum and Ophiocordyceps sinensis dietary supplementation. Evid Based Complement Alternat Med. 2014;2014:979613.
8. Cohen N, Cohen J, Asatiani MD, Varshney VK, Yu HT, Yang YC, Li YH, Mau JL, Wasser SP. Chemical composition and nutritional and medicinal value of fruit bodies and submerged cultured mycelia of culinary-medicinal higher Basidiomycetes mushrooms. Int J Med Mushrooms. 2014;16(3):273–91.
9. Gargano ML, van Griensven LJLD, Isikhuemhen OS, Lindequist U, Venturella G, Wasser SP, Zervakis GI. Medicinal mushrooms: valuable biological resources of high exploitation potential. Plant Biosystem. 2017;151(3):548–65.
10. Kawagishi H, Ando M, Sakamoto H, Yoshida S, Ojima F, Ishiguro Y, Ukai N, Furukawa S. Hericenones C, D and E, stimulators of nerve growth factor (NGF)-synthesis, from the mushroom Hericium erinaceum. Tetrahedr Lett. 1991;32(35):4561–4.
11. Kawagishi H, Shimada A, Shirai R, Okamoto K, Ojima F, Sakamoto H, Ishiguro Y, Furukawa S. Erinacines A, B and C, strong stimulators of nerve growth factor (NGF)-synthesis, from the mycelia of Hericium erinaceum. Tetrahedr Lett. 1994;35(10):1569–72.
12. Kawagishi H, Shimada A, Hosokawa S, Mori H, Sakamoto H, Ishiguro Y, Sakemi S, Bordner J, Kojima N, Furukawa S. Erinacines E, F, and G, stimulators of nerve growth factor (NGF)-synthesis, from the mycelia of Hericium erinaceum. Tetrahedr Lett. 1996;37(41):7399–402.
13. Lee EW, Shizuki K, Hosokawa S, Suzuki M, Suganuma H, Inakuma T, Li J, Ohnishi-Kameyama M, Nagata T, Furukawa S, Kawagishi H. Two novel diterpenoids, erinacines H and I from the mycelia of Hericium erinaceum. Biosci Biotechnol Biochem. 2000;64(11):2402–5.
14. Kawagishi, H, Shimada A, Shizuki K, Mori H, Okamoto K, Sakamoto H, Furukawa S. Erinacine D, a stimulator of NGF-synthesis, from the mycelia of Hericium erinaceum. Heterocycl Comm. 1996;2(1):51–4.
15. Mori K, Obara Y, Hirota M, Azumi Y, Kinugasa S, Inatomi S, Nakahata N. Nerve growth factor-inducing activity of Hericium erinaceus in 1321N1 human astrocytoma cells. Biol Pharm Bull. 2008;31(9):1727–32.
16. Obara Y, Nakahata N. The signaling pathway of neurotrophic factor biosynthesis. Drug News Perspect. 2002;15(5):290–8.
17. Mori K, Obara Y, Moriya T, Inatomi S, Nakahata N. Effects of Hericium erinaceus on amyloid β(25-35) peptide-induced learning and memory deficits in mice. Biomed Res. 2011;32(1):67–72.
18. Hazekawa M, Kataoka A, Hayakawa K, Uchimasu T, Furuta R, Irie K, Akitake Y, Yoshida M, Fujioka T, Egashira N, Oishi R, Mishima K, Uchida T, Iwasaki K, Fujiwara M. Neuroprotective effect of repeated treatment with Hericium erinaceum in mice subjected to middle cerebral artery occlusion. J Health Sci. 2010;56(3):296–303.
19. Mori K, Inatomi S, Ouchi K, Azumi Y, Tuchida T. Improving effects of the mushroom Yamabushitake (Hericium erinaceus) on mild cognitive impairment: a double-blind placebo-controlled clinical trial. Phytother Res. 2009;23:367–72.
20. Phan CW, David P, Naidu M, Wong KH, Sabaratnam V. Therapeutic potential of culinary-medicinal mushrooms for the management of neurodegenerative diseases: diversity, metabolite, and mechanism. Crit Rev Biotechnol. 2015;35(3):355–68.
21. Gerlai R. A new continuous alternation task in T-maze detects hippocampal dysfunction in mice. A strain comparison and lesion study. Behav Brain Res. 1998;95(1):91–101.
22. Gerlai R. Behavioral tests of hippocampal function: simple paradigms complex problems. Behav Brain Res. 2001;125(1–2):269–77.
23. Stewart S, Cacucci F, Lever C. Which memory task for my mouse? A systematic review of spatial memory performance in the Tg2576 Alzheimer's mouse model. J Alzheimers Dis. 2011;26(1):105–26.

24. Hughes RN. The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. Neurosci Biobehav Rev. 2004;28(5):497–505.
25. Brandalise F, Cesaroni V, Gregori A, Repetti M, Romano C, Orrù G, Botta L, Girometta C, Guglielminetti ML, Savino E, Rossi P. Dietary supplementation of Hericium erinaceus increases mossy fiber-CA3 hippocampal neurotransmission and recognition memory in wild-type mice. Evid Based Complement Alternat Med. 2017;2017:3864340.
26. Huang SM, Mouri A, Kokubo H, Nakajima R, Suemoto T, Higuchi M, Staufenbiel M, Noda Y, Yamaguchi H, Nabeshima T, Saido TC, Iwata N. Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. J Biol Chem. 2006;281(26):17941–51.
27. Faizi M, Bader PL, Tun C, Encarnacion A, Kleschevnikov A, Belichenko P, Saw N, Priestley M, Tsien RW, Mobley WC, Shamloo M. Comprehensive behavioral phenotyping of Ts65Dn mouse model of Down syndrome: activation of β1-adrenergic receptor by xamoterol as a potential cognitive enhancer. Neurobiol Dis. 2011;43(2):397–413.
28. Maurice T, Hiramatsu M, Itoh J, Kameyama T, Hasegawa T, Nabeshima T. Behavioral evidence for a modulating role of sigma ligands in memory processes. I. Attenuation of dizocilpine (MK-801)-induced amnesia. Brain Res. 1994;647(1):44–56.
29. Turriziani P, Serra L, Fadda L, Caltagirone C, Carlesimo GA. Recollection and familiarity in hippocampal amnesia. Hippocampus. 2008;18(5):469–80.
30. Ameen-Ali KE, Easton A, Eacott MJ. Moving beyond standard procedures to assess spontaneous recognition memory. Neurosci Biobehav Rev. 2015;53:37–51.
31. Ranganath C, Ritchey M. Two cortical systems for memory-guided behaviour. Nat Rev Neurosci. 2012;13(10):713–26.
32. Tulving E. Memory and consciousness. Can Psychol. 1985;26(1):1–12.
33. Brown MW, Aggleton JP. Recognition memory: what are the roles of the perirhinal cortex and hippocampus? Nat Rev Neurosci. 2001;2(1):51–61.
34. Squire LR, Wixted JT. The cognitive neuroscience of human memory since H.M. Annu Rev Neurosci. 2011;34:259–88.
35. Vann SD, Albasser MM. Hippocampus and neocortex: recognition and spatial memory. Curr Opin Neurobiol. 2011;21(3):440–5.
36. Barker GR, Bird F, Alexander V, Warburton EC. Recognition memory for objects, place, and temporal order: a disconnection analysis of the role of the medial prefrontal cortex and perirhinal cortex. J Neurosci. 2007;27(11):2948–57.
37. Barker GR, Warburton EC. Evaluating the neural basis of temporal order memory for visual stimuli in the rat. Eur J Neurosci. 2011;33(4):705–16.
38. Norman G, Eacott MJ. Impaired object recognition with increasing levels of feature ambiguity in rats with perirhinal cortex lesions. Behav Brain Res. 2004;148(1–2):79–91.
39. Winters BD, Forwood SE, Cowell RA, Saksida LM, Bussey TJ. Double dissociation between the effects of peri-postrhinal cortex and hippocampal lesions on tests of object recognition and spatial memory: heterogeneity of function within the temporal lobe. J Neurosci. 2004;24(26):5901–8.
40. Mumby DG, Gaskin S, Glenn MJ, Schramek TE, Lehmann H. Hippocampal damage and exploratory preferences in rats: memory for objects, places, and contexts. Learn Mem. 2002;9(2):49–57.

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Hericium erinaceus Improves Recognition Memory and Induces Hippocampal and Cerebellar Neurogenesis in Frail Mice during Aging

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Abstract: Frailty is a geriatric syndrome associated with both locomotor and cognitive decline, implicated in both poor quality of life and negative health outcomes. One central question surrounding frailty is whether phenotypic frailty is associated with the cognitive impairment during aging. Using spontaneous behavioral tests and by studying the dynamic change during aging, we demonstrated that the two form of vulnerability, locomotor and recognition memory decline, develop in parallel and therefore, integration of the motoric and cognitive evaluations are imperative. We developed an integrated frailty index based on both phenotypic and recognition memory performances. *Hericium erinaceus* (*H. erinaceus*) is a medicinal mushroom that improves recognition memory in mice. By using HPLC-UV-ESI/MS analyses we obtained standardized amounts of erinacine A and hericenones C and D in *H. erinaceus* extracts, that were tested in our animal model of physiological aging. Two-month oral supplementation with *H. erinaceus* reversed the age-decline of recognition memory. Proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) immunohistochemistry in the hippocampus and cerebellum in treated mice supported a positive effect of an *H. erinaceus* on neurogenesis in frail mice.

Keywords: aging; phenotypic frailty; cognitive decline; *Hericium erinaceus*; erinacines; hericenones; medicinal mushrooms supplementation; neurogenesis

1. Introduction

Recent reports on the European population suggest that by 2060, 30% of Europeans will be over the age of 65. Frailty is a geriatric syndrome associated with poor quality of life and negative health outcomes, such as acute illness, falls, hospitalization, disability, dependency, and mortality, adjusted for comorbidities [1,2,3], in the absence of recognized disabilities or organ-specific diseases. Health declines in frailty are accelerated and accompanied by the failure of homeostatic mechanisms [4,5].

Fried defined phenotypic frailty as an aging-associated phenotype expressing at least three of the following symptoms: weakness, weight loss, slow walking speed, fatigue, and a low level of physical activity [1]. Most older people gradually become frail and oscillations between non-frail, pre-frail, and frail are not uncommon [6]. Cognitive impairment is a decline of cognitive functions such as remembering, reasoning, and planning, ranging from mild forms of forgetfulness to severe dementia. Cognition-impaired frailty in human studies was associated with global cognition and perceptual speed, but not with episodic memory [7]. Quality of life in the elderly is particularly affected by impairments in the functioning of the memory system [8]. In order to evaluate the inclusion of cognitive performances in frailty clinical diagnosis [9], it is necessary to first determine whether phenotypic frailty is associated with cognitive impairment [10].

Several epidemiological studies have reported that higher levels of phenotypic frailty increases the risk of cognitive impairment and dementia [11,12,13], and that higher levels of cognitive impairment or dementia increase the risk of phenotypic frailty [14,15,16]. This suggests that frailty may be an early indicator for subsequent cognitive decline. Understanding the mechanisms by which phenotypic frailty is linked to cognitive impairment has implications for the management of those susceptible for both phenotypic frailty and cognitive impairment.

Hericium erinaceus (*H. erinaceus*) is found in Europe, Asia, North America, Oceania, and generally throughout the north temperate latitudes. In Italy, it is considered quite rare; it occurs along the Apennines mountain chain, near Sicily and Sardinia, while in the North only few sporadic sightings have been reported.

H. erinaceus is an edible mushroom widely used as herbal medicine, in all areas mentioned above and in a few East Asian countries. Since 1990, studies on *H. erinaceus* secondary metabolites reported several (about 70) structurally related terpenoids, such as erinacines, hericenones, hericerins, hericenes, hericenols, and erinacerins [17,18,19].

All of the above-mentioned molecules, except erinacines, share a geranyl side chain bonded to a resorcinol framework; that is, they are aromatic compounds containing the 6-alkyl-2,4-dihydroxybenzoic acid unit also known as β-resorcylate [20]. Erinacines are classified as cyathane-type diterpenoids, including 20 members of 24 different diterpenoids in *H. erinaceus* [18]. The standardization of dietary supplements from medicinal mushrooms is still in its early stages, because proper standards and protocols are lost and cannot guarantee product quality [21,22].

Existing data have suggested that there is a neuroprotective effect of dietary supplementation with *H. erinaceus* in mice subjected to middle cerebral artery occlusion [23]. Furthermore, *H. erinaceus* provided a partial recovery of intellectual function of patients with a mild cognitive impairment or against other forms of neurodegenerative diseases, including dementia and Alzheimer's [24,25,26]. It has been shown that erinacines A–I and hericenones C–H are responsible for the neuroprotective effects of stimulating Nerve Growth Factor (NGF) [27,28] and of brain derived neurotrophic factor (BDNF) synthesis in vitro [29,30]. A possible role of polysaccharides in neuroprotection has been suggested as well [25,27,31]. Additionally, the effects of *H. erinaceus* on recognition memory and on hippocampal mossy fiber-CA3 neurotransmission in wild-type middle-aged mice was recently published [32,33]. Among the neurogenic zones, hippocampus is the most interesting area in the adult brain, because it is involved in higher cognitive function, such as memory processes and certain affective behaviors. In particular adult and persistent hippocampal neurogenesis generates new excitatory neurons in the dentate gyrus and contributes in a significant way to plasticity across the life span [34].

In the current report, we have created a frailty index for locomotor and recognition memory performance and we examined the relationship between them in aging, wild-type mice. Furthermore,

we observed the effect of an *H. erinaceus* supplement (He1) containing a known amount of Erinacine A, Hericenone C, and Hericenone D on frailty. Moreover, we assessed the He1 effect on hippocampal and cerebellum neurogenesis in frailty animals, by investigating specific protein markers representative of cell proliferation activity and newborn neurons occurrence.

2. Materials and Methods

2.1. Animals

Fifteen wild-type male mice (strain C57BL-6J), starting at 11 months old, were maintained in single cages in the Animal Care Facility at University of Pavia on a 12-h light/dark cycle. Water and food were provided ad libitum. All experiments were carried out in accordance with the guidelines laid out by the institution's animal welfare committee, the Ethics Committee of Pavia University (Ministry of Health, License number 774/2016-PR).

In vivo experiments were performed at six different experimental times (Figure 1), between 11 and 23.5 months old.

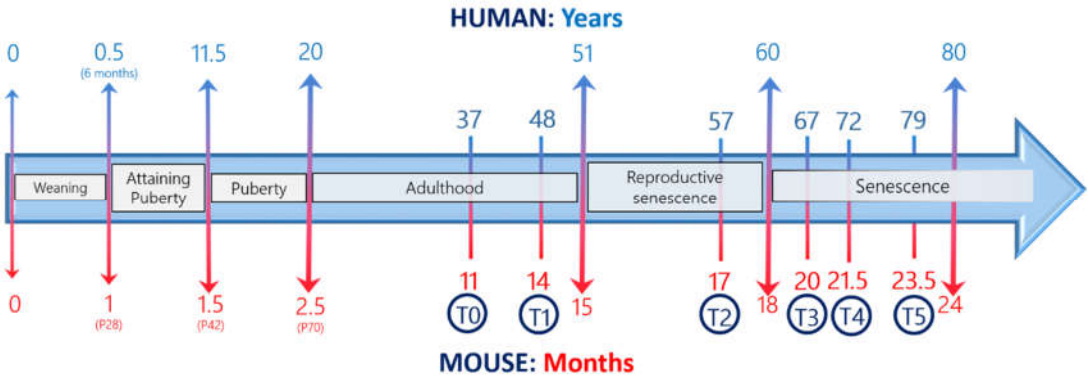


Figure 1. Comparative age between men and mice during their life span and the chosen experimental times (modified by Dutta and Sengupta, 2016).

Seven out of fifteen mice, starting from 21.5 months old, received for two months a drink made by a mixture of He1 mycelium and sporophore as ethanol extracts solubilized in water, in such a way that every mouse received 1 mg of supplement per day. This amount was chosen to mimic the oral supplementation in humans (about 1g/day). Daily consumption of water and supplements was monitored for each mouse.

At each experimental time, mice were weighed; no statistically significant change was recorded either during aging or between the He1 and control groups.

2.2. Apparatus and Procedures

We performed a spontaneous behavioral test to study locomotor activity and recognition memory in mice. For all experiments, researchers were blinded to the group assignment (control and He1). Mice activity was quantified by SMART video tracking system with a selected sampling time of 40 ms/point (2 Biological Instruments, Besozzo, Varese, Italy) and Sony CCD color video camera (PAL). All mice, at different times from T0 until T5, performed two spontaneous tests, Emergence and Novel Object Recognition (NOR) tasks. Emergence and NOR tasks are used to assess recognition memory for the environment and the object, respectively.

2.2.1. Emergence Test

We carried out emergence tests in accordance with procedures described by Brandalise et al., 2017 [32]. In the emergence test, we measured total distance and resting time covered in the familiar

compartment as locomotor parameters, while we measured the number of exits, latency of first exit, and the time of exploration outside as cognitive indicators (Table 1).

Table 1. selected parameters to measure locomotor and cognitive performances in each task. DI = discrimination index between novel/repositioned and familiar object. NOR = novel object recognition.

Test	Locomotor Parameters	Cognitive Parameters
Emergence	Resting Time In (s) Total Distance In (cm)	Exit Number (*n*) Latency of First Exit (s) Time of Exploration (s)
NOR	**(Open Arena)** Resting Time (s) Total Distance (cm) Max Speed (cm/s) Mean Speed (cm/s)	Number of Approaches: DI Time of Approaches: DI

2.2.2. Novel Object Recognition Task

We carried out novel object recognition tasks in accordance with procedures described by Brandalise et al., 2017 [32], consisting of three primary phases: open arena, familiarization, and test. To assess locomotor activity, mice were observed for 15 min while freely exploring the open-field arena in the absence of objects. Locomotor parameters of mean speed, maximum speed, resting time, and the total distance covered in the arena (Table 1) were all considered. During test phase, we measured the number of approaches and the time of approaches to the familiar and the novel objects as cognitive parameters (Table 1). To evaluate the discrimination between novel and familiar objects, we calculated the Mean Novelty Discrimination Index (DI) by using the following formula (1) [35],

$$DI = (n-f)/(n+f) \quad (1)$$

where n is the average time or number of approaches to the novel object and f is the average time or number of approaches to the familiar one (Table 1). This index ranges from −1 to 1, where −1 means complete preference for the familiar object, 0 means no preference, and 1 means complete preference for the novel object.

2.3. The Frailty Index

A variant of Parks's methodology [36,37] was used to calculate the Frailty Index (FI). In Parks's procedure for creating the FI, a graded scale was calculated as follows: values that were 1 standard deviation (SD) above or below the mean reference value were given a frailty value of 0.25; values that differed by 2 SD were scored as 0.5; values that differed by 3 SD were given a value of 0.75, and values that were more than 4 SD above or below the mean received a frailty value of 1. Parameters that differed from T0 reference values by less than 1 SD received a score of 0.

Park's procedure, as described above, was changed in order to obtain more accurate values during aging. The mean value and the standard deviation (SD) for each of the parameters were calculated at T0. The values obtained in each mouse at different times, from T0 to T5, were compared to the mean value at T0, by using the following formula (2):

$$FI = ((Value\text{-}Mean\ Value\ at\ T0))/(SD\ at\ T0)*0.25 \quad (2)$$

This procedure was applied for both Locomotor FI and Cognitive FI. Finally, to obtain LAC (Locomotor And Cognitive) FI we averaged the Locomotor and Cognitive FIs.

2.4. H. erinaceus

The He1 (strain 1 of *H. erinaceus*) was isolated from a basidioma collected in 2013 in Siena province (Region Tuscany, Italy) from a live specimen of Quercus ilex [38]. The basidioma was aseptically cut in small portions (about 1 mm^3) that were placed into Petri dishes with 2% malt extract agar as a culture medium (MEA, Biokar Diagnostics). Chloranphenicol at 50 ppm was added in this first step. Incubation was performed at 24 °C in complete darkness. The strain was maintained in the Italian Culture Collection of Pavia University (MicUNIPV).

2.4.1. Extraction Procedures

Lyophilized mycelium and sporophores of He1 were extracted in 70% ethanol, per the procedure described by Gerbec et al. [39]. In details, one gram of dry substrate was blended with 10 mL of 70% ethanol and left in the thermostat overnight at 50°C. Before withdrawing, the material was stirred for one hour and was centrifuged at 4000 rpm for three minutes. The supernatant was stored at -20°C.

2.4.2. HPLC-UV-ESI/MS Method

HPLC-UV-ESI/MS analyses were carried out on a Thermo Scientific LCQ FLEET system, equipped with a PAD-UV detector working at 254 nm (Thermo Scientific®, San Jose, CA, USA). The chromatographic separation was performed using an Ascentis Express F5 HPLC column (150 × 3.0 mm, 2.7 μm particle size Sigma Aldrich, Milan, Italy) maintained at 40 °C, with a flow rate of 0.3 mL/min and an injection volume of 20 μL. The following gradient method was utilized with water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B): 0–9 min (30–50% B), 9–27 min (50–60% B), 27–54 min (60–100% B), 54–69 min (100–30% B), and 69-75 min (30% B); all solvents are from Sigma Aldrich, Milan, Italy. The HPLC system was interfaced to the ion trap mass spectrometer with an Electro Spray Ionization (ESI) ion source. The compounds were analyzed under positive (ESI+) ion conditions. The ion spray and capillary voltage were set at 5kV and 10V, respectively, in positive ion mode. The capillary temperature was 400 °C. The acquisition was performed both in Full Scan mode (mass range 200–2000 Da) and MS/MS Dependent Scan mode. The data station utilized the Xcalibur MS Software Version 2.1 (Thermo Scientific®, San Jose, CA, USA).

Erinacine A and hericenones C and D were used as standards [40,41]. Stock solutions (1 mg/mL) of erinacine A and hericenones C and D were prepared in 70% ethanol. Standard solutions with the final concentration range of 1–25 μg/mL for erinacine A and 20–100 μg/mL for hericenones C and D were prepared. Linear least-square regression analysis for the calibration curves showed correlation coefficients of 0.9968, 0.9945, and 0.9951, respectively, for erinacine A, hericenones C, and hericenones D with respect to the peak area, demonstrating a good linear relationship in the different ranges tested. Each concentration was analyzed in triplicate.

2.5. Tissue Sampling: Hippocampal and Cerebellar Specimens Preparation

Mice were anesthetized by isoflurane inhalation (Aldrich, Milwaukee, WI, USA) before decapitation.

The brain and cerebellum were immediately excised as previously described [42], washed in 0.9% NaCl, and fixed by immersion for 48 h at room temperature in Carnoy's solution (6 absolute ethanol/3 chloroform/1 acetic acid). The tissues were then kept in absolute ethanol for one hour, followed by acetone for 50 min, and finally embedded in Paraplast X-TRA (Sigma Aldrich, Milan, Italy). Eight micron-thick sections, collected on silane-coated slides, of brain and cerebellar vermis were cut in the sagittal plane.

2.6. Immunohistochemistry: Fluorescence Microscopy Assessment and Quantification of Cell Proliferation and Neurogenesis

To avoid possible staining differences due to small changes in the procedure, the immunoreactions were carried out simultaneously on slides from controls and treated animals. Paraffin-embedded sections were deparaffinized in xylene, rehydrated through a series of graded alcohol treatments and rinsed in phosphate-buffered saline (PBS, Sigma).

PCNA (PC10), a 37 kDa molecular weight protein also known as cyclin, was employed as marker of cell proliferation. In particular, in cells fixed with organic solvents, the PCNA was demonstrated to be strongly associated in the nuclear regions where DNA synthesis is occurring [43]. DCX is considered a marker for neuronal precursors and migrating neuroblasts during neurogenesis recovery [44]. The presence and distribution of PCNA and DCX was assessed using commercial antibodies on murine specimens, focusing on the hippocampus and cerebellum. Brain and cerebellar sections of control and He1 mice were incubated overnight at room temperature with the primary

antibody: (i) mouse monoclonal antibody against PCNA (1:600, Abcam, Cambridge, MA, USA), and (ii) goat polyclonal antibody against DCX (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in phosphate buffer saline (PBS), sections were incubated for one hour with the secondary antibody: (i) Alexa Fluor 488-conjugated anti-mouse (1:100, Molecular Probes, Space, Milano, Italy) and (ii) Alexa Fluor 594-conjugated anti-goat (1:100, Molecular Probes, Space, Milano, Italy),in a dark, moist chamber. Then the nuclei were counterstained for 10 min with 0.1 μg/mL Hoechst 33258 (Sigma Aldrich, Milan, Italy). After PBS washing, coverslips were mounted in a drop of Mowiol (Calbiochem, San Diego, CA, USA).

Sections were observed by fluorescence microscopy with an Olympus BX51 equipped with a 100- W mercury lamp used under the following conditions: 330–385 nm excitation filter (excf), 400 nm dichroic mirror (dm), and 420 nm barrier filter (bf) for Hoechst 33258; 450–480 nm excf, 500 nm dm, and 515 nm bf for the fluorescence of Alexa 488; 540 nm excf, 580 nm dm, and 620 nm bf for Alexa 594. Images were recorded with an Olympus MagnaFire cam and processed with the Olympus Cell F software.

Immunofluorescence quantification was performed by calculating the percentage of PCNA or DCX immunocytochemically positive nuclei or cytoplasm of nervous cells (from the hippocampus and cerebellum) of a total number (about 300) for each animal and experimental condition, in a minimum of 10 randomly selected high-power microscopic fields.

2.7. Statistics

Data are reported as mean ± standard error of the mean (SEM). We performed Bartlett and Shapiro Wilk Tests to establish and confirm the normality of parameters. To verify statistically significant differences, we used a one-way Anova for repeated measures of the aging of mice and a two-way Anova for the effect of *H. erinaceus* supplementation. The statistical analysis for immunofluorescence was carried out using an Unpaired Student's t-test. The differences are considered statistically significant for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Statistical analyses were performed with GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Locomotor and Recognition Memory during Physiological Aging

We first investigated locomotor performance and recognition memory during physiological aging in healthy mice (n = 15) using different spontaneous behavioral tests. Novelty recognition memory for a new environment and for novel objects was tested by way of emergence and NOR tasks, respectively.

We carried out behavioral spontaneous tests in mice at 11 (T0), 14 (T1), 17 (T2), 20 (T3), 21.5 (T4), and 23.5 (T5) months old. For the reader to understand the practical application of these tests, a comparison of the different developmental stages between humans and mice during their life span, according to Dutta and Sengupta is outlined in Figure 1 [45]. To monitor the physiological aging in mice, we choose six experimental times: T0 and T1 corresponding to adulthood phase, T2 to reproductive senescence, and T3, T4, and T5 to senescence phase.

Figure 2A shows the locomotor parameters measured in the open arena during aging. Total distance and resting time decreased from T0 to T1, stabilizing at T3. Mean speed in the open arena changed at T2 and then worsened with the aging. Maximum speed worsened later, in senescence phases T4 and T5.

Figures 2B and 2C show the cognitive parameters measured in emergence and in NOR, respectively. In the emergence test latency to the first exit, the exit number and the exploring time worsened from T0 to T1 and then remained relatively stable, whereas the latency to the first exit worsening again in the senescence phase. In the NOR test, the time of approach and the number of approaches decreased in T2 and even more in senescence phase.

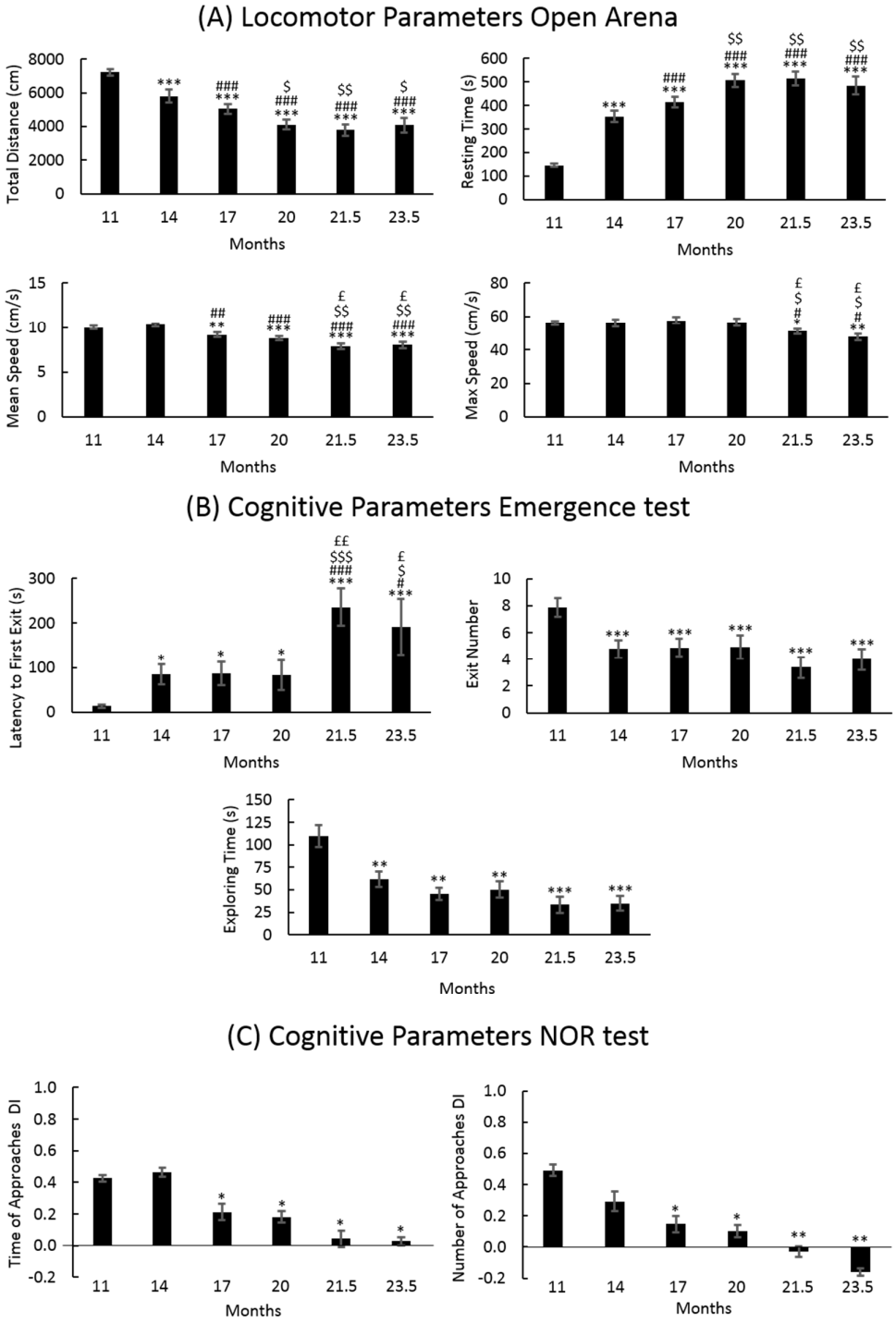
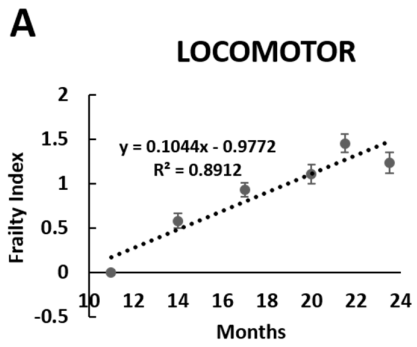


Figure 2. Locomotor and cognitive parameters during aging. (**A**) Locomotor parameters: total distance, resting time, mean speed, and max speed measured in open arena during aging. (**B**) cognitive parameters: latency to first exit, exit number, and exploring time measured in emergence, and (**C**) cognitive parameters: discrimination index (DI) of the time of approaches and of the number of approaches measured in NOR test. Statistical results were performed by Anova for repeated measures: * vs T0, # vs T1, $ vs T2, and £ vs T3. For all symbols reported $p < 0.05$ (*, #, $, £), $p < 0.01$ (**, ##, $$, ££), $p < 0.001$ (***, ###, $$$, £££).

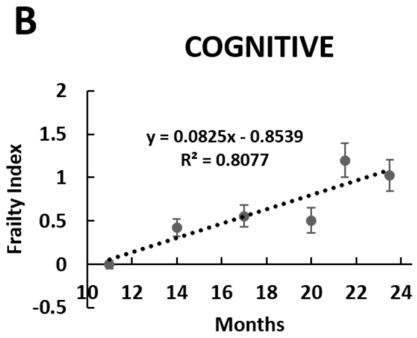
3.1.1. Locomotor Frailty Index

We calculated the Locomotor Frailty Index (FI) for each of the parameters reported in Table 1, then averaged the values for each experimental time. Figure 3, Panel A shows the Locomotor FIs from T0 to T5 and the linear least-square regression analysis, with $R^2 = 0.8912$. Results from a one-way ANOVA are reported in Figure 3. These results suggest that during physiological aging, locomotor performances decline linearly from the adulthood to the senescence stage.

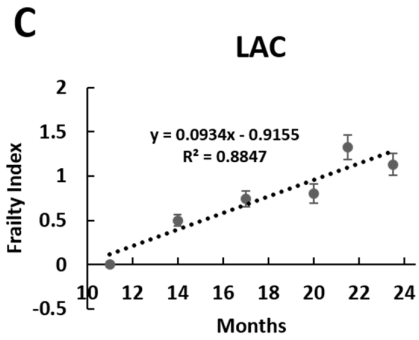
The slope obtained by the linear least-square regression analysis (slope value = 0.1044) indicated that for every three months passed, locomotor activity decreased by about 35.43%, yielding a significantly different Locomotor FI value from the previous one each time.



LOCOMOTOR FI	T1	T2	T3	T4	T5
T0	***	****	****	****	****
T1		***	****	****	***
T2			*	***	**
T3				**	
T4					



COGNITIVE FI	T1	T2	T3	T4	T5
T0	*	**	*	****	****
T1				*	*
T2					*
T3				*	***
T4					



LAC FI	T1	T2	T3	T4	T5
T0	***	****	****	****	****
T1			*	***	***
T2				***	**
T3				***	*
T4					

Figure 3. Locomotor, cognitive, and LAC (locomotor and cognitive) decline during physiological aging in mice. Locomotor (panel (**A**)), cognitive (panel (**B**)), and LAC (panel (**C**)) Frailty Index during physiological aging in mice. Linear regressions of experimental points and statistical results were reported. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3.1.2. Cognitive Frailty Index

Novelty recognition memory was evaluated, resulting in a calculation of the Cognitive Frailty Index (FI) during aging (Figure 3B). Cognitive FI was calculated using a linear least-square regression analysis, with $R^2 = 0.8077$. Results from a one-way ANOVA are reported in Figure 3.

The slope obtained by the linear least-square regression analysis (slope value = 0.0825) indicated that for every 3 months passed, recognition memory declined by about 26.04%, yielding a significantly different Cognitive FI value the previous one each time.

3.1.3. Cognitive Frailty Index

In order to evaluate a global trend of both phenotypic and cognitive decline in physiological aging, we calculated the Locomotor and Cognitive FI (LAC frailty index) by averaging all frailty indices obtained prior to evaluating the locomotor and cognitive performances during aging (Figure 3C). The LAC FI significantly increased from T0 to T5. Similarly, as previously described for Locomotor FI and Cognitive FI, the LAC FI values follow a linear trend ($R^2 = 0.8847$) (Figure 3C). The slope obtained by the linear least-square regression analysis (slope value = 0.0934) indicated that for every 3 months passed, the frailty of the mouse increased by about 30.7%.

3.2. Identification and Quantification of Erinacine A, Hericenone C, and Hericenone D

In order to identify and quantify the bioactive metabolites of interest, we analyzed the mycelium and sporophore extracts of He1 using HPLC-UV-ESI/MS. Erinacine A and hericenones C and D were identified by comparing both retention times and ESI/MS spectra with the authentic standards.

Characteristic ions of Erinacine A in the ESI/MS spectrum (Figure 4) are sodium and potassium adducts of a single molecule as well as a dimer (Table 2). Hericenone C and D spectra (Figure 5) present just [M+H]+ and [M+Na]+ ions (Table 2).

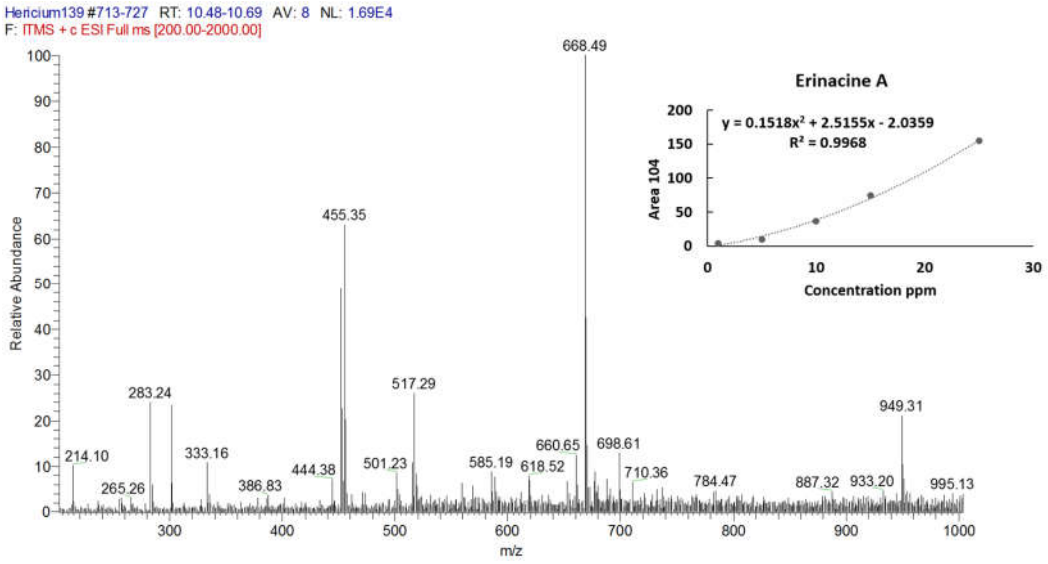


Figure 4. ESI/MS spectrum of Erinacine A. Panel (top, right) reports calibration curves and linear regression curve for Erinacine A.

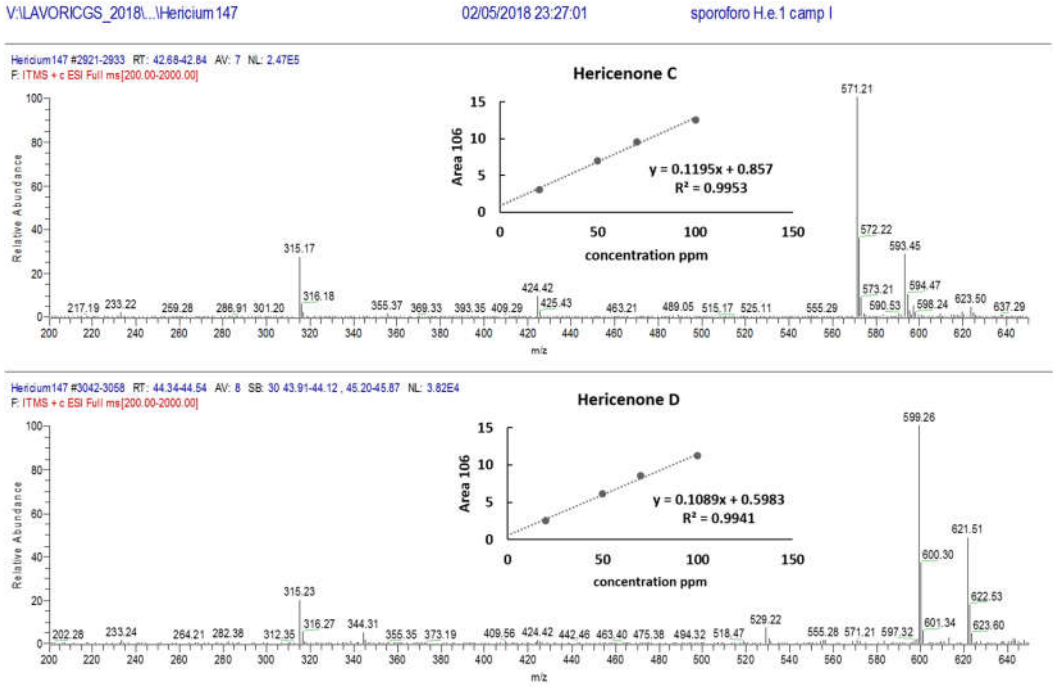
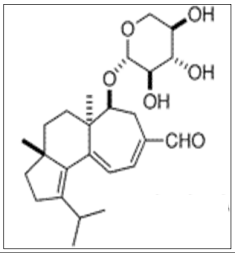
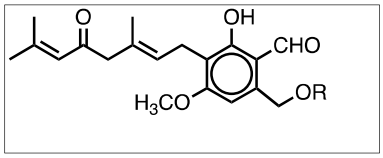
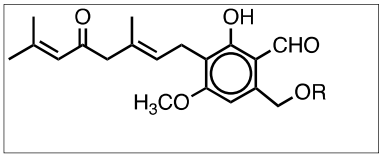


Figure 5. ESI/MS spectra of Hericenone C and D. Panels (top, right) report calibration curves for Hericenone C and D.

Table 2. shows the Erinacine A, Hericenones C and D molecular formula, molecular weight, chemical structure, characteristic structure, and content in 1 g of dried He1 mycelium and sporophore.

	Molecular Formula	Molecular Weight (g/moL)	Chemical Structure	Characteristic Ions (m/z)	Content (μg/g)
Erinacine A	$C_{25}H_{36}O_6$	432.56		455 $[M+Na]^+$ 452 $[2M+K+H]^{2+}$ 668 $[3M+K+H]^{2+}$ 949 $[2M+HCOOH+K]^+$	150 in mycelium
Hericenone C	$C_{35}H_{54}O_6$	570.81	 R = Palmitoyl	571 $[M+H]^+$ 593 $[M+Na]^+$	500 in basidioma
Hericenone D	$C_{37}H_{58}O_6$	598.87	 R = Stearoyl	599 $[M+H]^+$ 621 $[M+Na]^+$	<20 in basidioma

Erinacine A was detected using HPLC/ESI-MS. By comparing the retention time and molecular ion or mass spectra, we confirmed the peak identification. We quantified it by comparing the peak areas with those of the standard (Figure 6). The calibration curve was constructed by injecting

standard mixture solutions at five concentrations (1, 5, 10, 15, and 25 μg/mL). The linear least-square regression analysis for the calibration curve showed a correlation coefficient of r = 0.9968. The level of Erinacine A present in mycelium of He1 calculated by the calibration curve was 150 μg/g (Table 2).

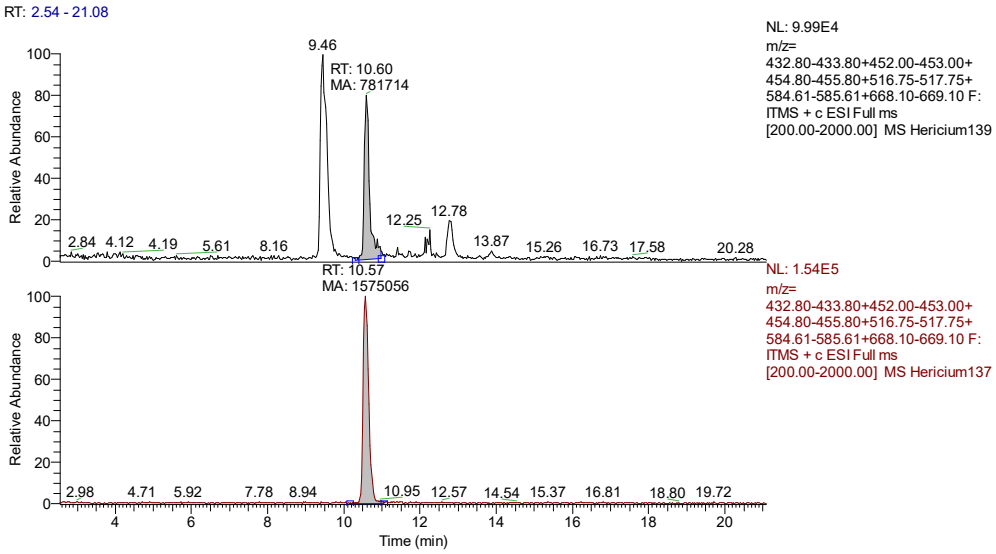


Figure 6. MS (Mass Spectrum) traces of He1 mycelium and Erinacine A (Rt 10,57) standard. Peak area of Erinacine A is pointed out.

The hericenones C and D chromatographic conditions produced a good resolution of adjacent peaks. UV detection provided sufficient sensitivity for each analyte, allowing proper quantification of both compounds by comparing the peak areas in the UV trace with those of the standards (Figure 7). Calibration curves were constructed by injecting the standard mixture solutions at four concentrations (20, 50, 75, 100 μg/mL.). Linear least-square regression analysis for the calibration curves showed correlation coefficients of r = 0.9945 and r = 0.9951, respectively, for hericenones C and D. The levels of hericenones C and D present in He1 basidioma, calculated by calibration curves, were 500 μg/g and less than 20 μg/g, respectively (Table 2).

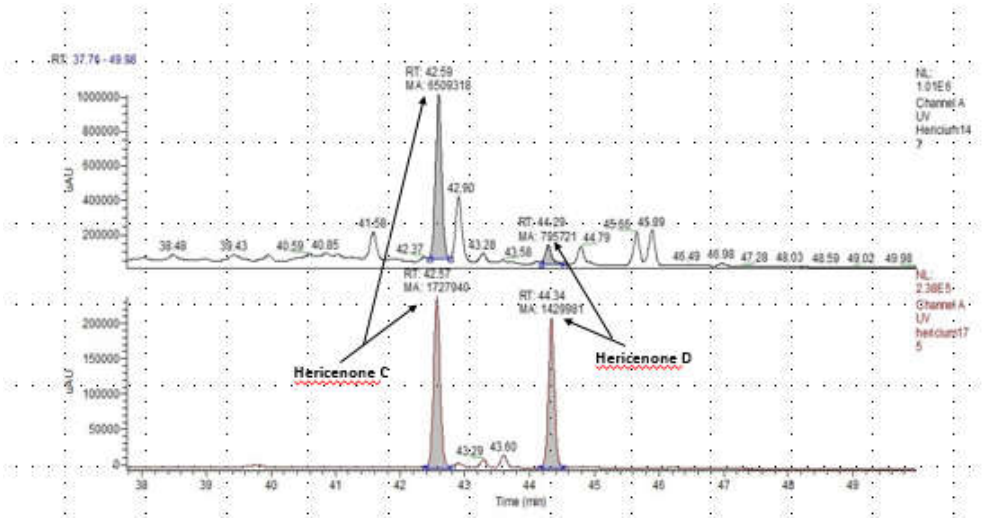


Figure 7. UV (Ultra Violet) traces of He1 sporophore (top) and Hericenone C (Rt 42.57) and D (Rt 44.34) standards (bottom). Peak areas corresponding to Hericenone C and D are pointed out.

3.3. He1 Supplementation Improved Recognition Memory Performances during Aging

We investigated cognitive and locomotor performances after oral *H. erinaceus* supplementation on frail mice. Seven mice with a T4 LAC FI score measured of more than 1.30 received a mixture of components made by He1 mycelium and basidioma for two months until T5.

Figure 8 shows the frailty index before (T4) and after He1 supplementation (T5). He1 supplementation improved recognition memory in mice during aging, characterized by a Cognitive FI decrease from 1.71 ± 0.21 to 0.72 ± 0.22. Locomotor performances before and after He supplementation were not significant different. Considering together locomotor and memory performances by means of the LAC index, He1 regressed aged-related frailty, but this change in LAC index was completely driven by the improve in memory function.

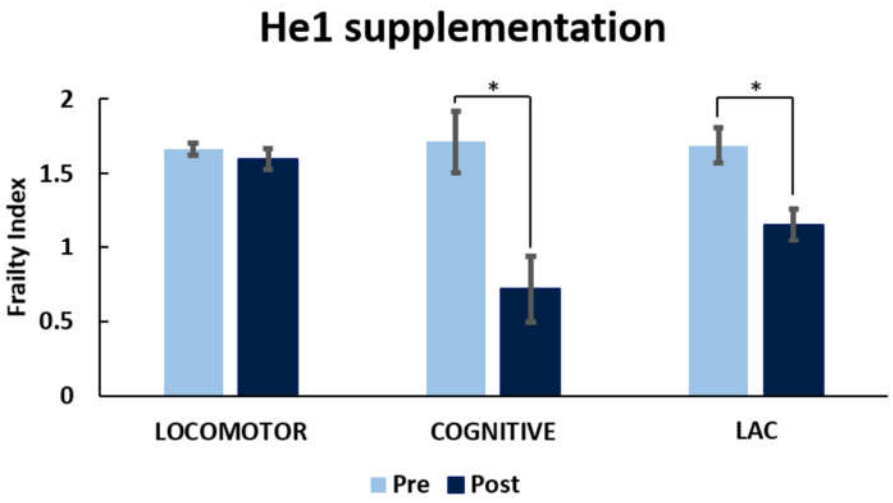


Figure 8. *H. erinaceus* improved recognition memory during mice aging. Value measured pre-supplementation (pre) and post-supplementation (post) on locomotor, recognition memory, and LAC (Locomotor And Cognitive) FI. $p < 0.05$ (*).

3.4. He1 Supplementation Improved Hippocampal and Cerebellum Proliferation and Neurogenesis

To examine the molecular mechanism involved in the He1 effect on aging mice, we performed immunocytochemical studies on different cerebral tissues, i.e. the hippocampus and cerebellum, testing both the proliferating the cell nuclear antigen (PCNA) and doublecortin (DCX) as specific markers of active proliferation and neurogenesis, respectively. We performed immunocytochemistry on He1 frail mice and on untreated (control) animals at T5.

Preliminary data seem to demonstrate that cell proliferating activity achieved the highest expression in both brain areas in He1 mice compared to control animals. Specifically, the PCNA nuclear immunolabelling appeared more marked in the hippocampal dentate gyrus (DG) granule cells and in the CA3 pyramidal neurons, while the immunopositive cells (possibly granular or glial cells) predominantly localized in the width of the outer molecular layer in the cerebellar cortex (Figure 9A). Notably, clusters of PCNA-positive cells, possibly newborn granule cells, were observed suggesting the occurrence of a recovered proliferation wave (Figure 9A). Accordingly, quantitative analysis demonstrated that the PCNA labelling frequency detected in He1 treated mice (22.89% ± 6.09) reached significantly higher values compared to those measured in controls (10.80% ± 3.09, $p < 0.05$) in the hippocampus. Similarly, the PCNA labelling frequency detected in He1 mice (25.60% ± 6.66) reached significantly higher values compared to those measured in controls (8.19% ± 4.43, $p < 0.05$), in the cerebellum (Figure 9B).

In He1 mice, hippocampus showed a more marked DCX labelling compared to control animals. In particular, DCX immunolabelling appeared more intense in the dentate gyrus (DG) granule cells

in the hippocampus (Figure 10A, panel a and b). In the cerebellar cortex the immunopositivity was less expressed, nonetheless mainly localized in cells present in the molecular layer (Figure 10A, panel c and d). Quantitative analysis showed that the DCX labelling frequency in He1 mice achieved significantly higher values compared to those measured in control animals in hippocampus (8.45% ± 3.02 vs 0.22% ± 0.45, respectively, with $p < 0.05$). In the cerebellum the DCX labelling frequency displayed a trend of increase in He1 mice but did not reached the threshold for the statistically significant difference compared to controls (4.68 ± 3.06 vs 0.26% ± 0.79, respectively, with $p = 0.073$) (Figure 10B). It should be noted that the DCX labelling frequency in control animals is only about 0.2% in both hippocampus and cerebellum. As the PCNA labelling identifies DNA repair as well as duplication, and DCX positivity links to the presence of newborn neurons, the increased expression of this cytochemical marker may be the manifestation of different biological responses involving the recovery of cell proliferation and neurogenesis, potentially highlighting the occurrence of an upswing phase owed to the neurobiological effects exerted by the oral supplementation with He1.

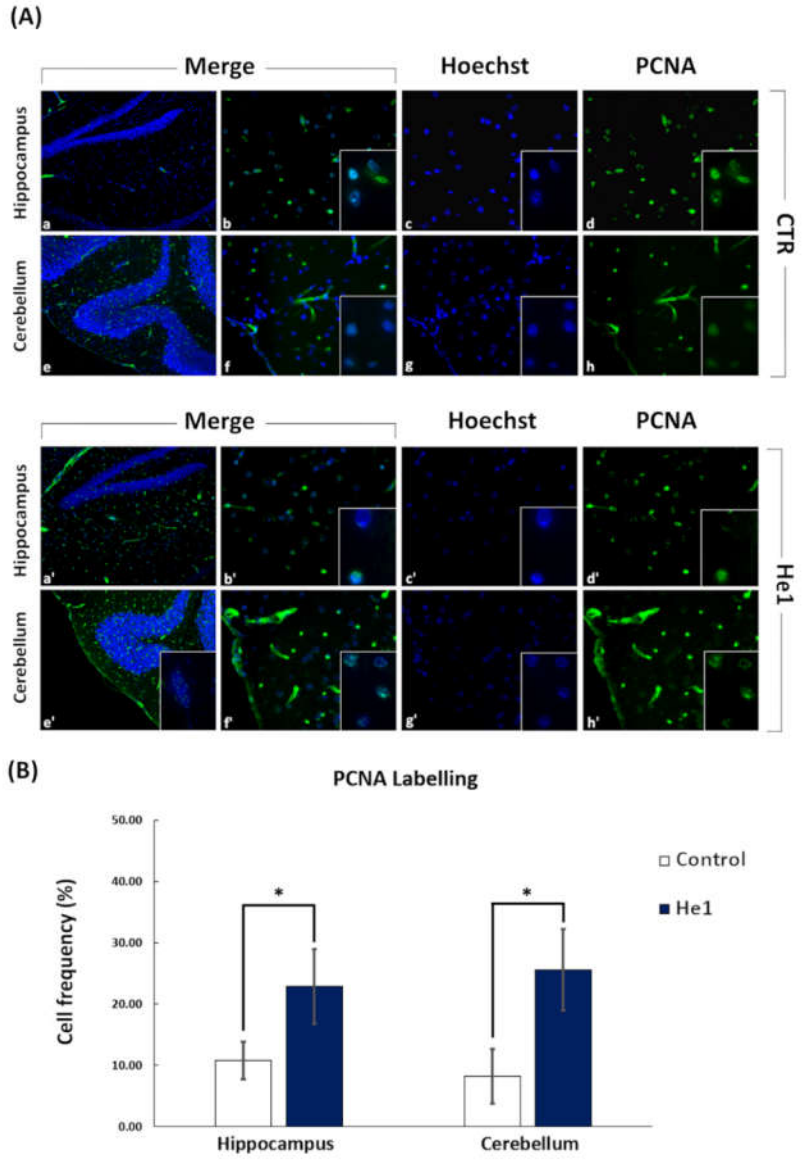


Figure 9. Panel (**A**) shows cell proliferating activity immunocytochemically detected by PCNA (Proliferating Cell Nuclear Antigen) labelling, observed at T5 after 2 months oral supplementation with He1 in both hippocampus and cerebellum, (a'–d' and e'–h', respectively), compared to control

untreated mice (CTR) (a–h). Cell proliferation was significantly enhanced in He1 mice, with the labelling appearing more intense in the hippocampal DG granule cells and in CA3 pyramidal neurons (a'–d') and in cerebellar molecular layer (e'–h'), compared to controls (a-d and e-h, respectively), predominantly localized in the DG granule cells and in CA3 pyramidal neurons, as also in the width of the cerebellar. Objective magnification: 20 x (a, e and a', e'); 40 x (b-d, f-h and b'-d', f'-h'); 100 x (insert in b-d, f-h, b'-d', f'-h'). Panel (**B**) shows changes in the percentage of PCNA labelling index of hippocampal and cerebellar cells in He1 mice. $p < 0.05$ (*).

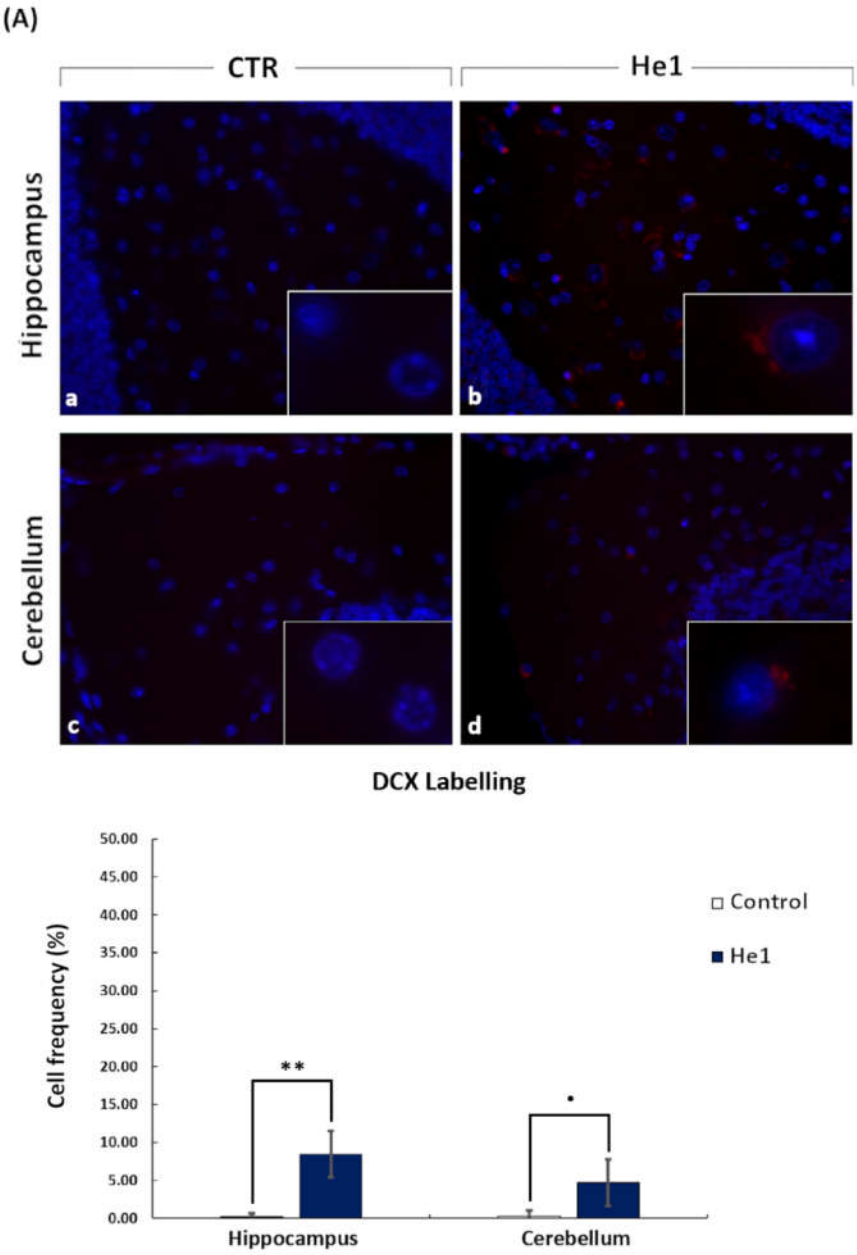


Figure 10. Panel (**A**) shows doublecortin (DCX) immunocytochemistry, observed at T5 after 2 months oral supplementation with He1 in hippocampus and cerebellum (b and d), compared to control mice (a and c). Objective magnification: 40 x (a-d); 100 x (inserts). Panel (**B**) shows the cell frequency percentage of DCX labelling in the hippocampal dentate gyrus and cerebellar molecular layer in control and He1 mice. $p < 0.01$ (**), $p = 0.07$ (•).

4. Discussion

The aim of this study was to develop an index to monitor locomotor and cognitive frailty in aging mice in vivo and to study the effect of *H. erinaceus* extracts containing a known amount of erinacines A and hericenones C and D. The mechanism of action of He1 was evaluated by immunocytochemistry.

We developed a frailty index score (LAC FI) based both on locomotor and cognitive decline during aging. To achieve a translational approach, we chose to monitor locomotor indicators that compare well to phenotypic frailty indicators in humans, such as gait speed and the level of physical activity [1]. We therefore measured the mean and maximum speed, resting time, and the total distance covered in an open arena test.

In concordance with Fried's phenotype model [1] and with Parks observations on activity level [36], the current paper demonstrates that the locomotor performances of mice progressively worsened during aging. The locomotor FI increased significantly during aging, with a linear progression from 11 to 23.5 months. We have developed a simplified, non-invasive method to monitor the development of frailty during mice aging, using a FI measured according to Whitehead [37].

Researchers use often the Stenberg Item Recognition Paradigm (SIRP) to measure cognitive impairment during aging in humans [46,47], during which a small group of items, called the positive set, is presented for the subject to memorize. After a delay, a single item is presented that may (familiar) or may not have (novel) been shown before and the subject is asked to answer "yes" or "no" to indicate their recognition of the item. The NOR and the emergence test in mice assess the same ability to recognize a familiar and a novel object [48] or a new environment. We measured exit numbers, the latency of the first exit and the time of exploration in the emergence test and the number and time of approaches in the NOR test [32] as cognitive parameters. We preferred to use different parameters, as suggested by Ennaceur et al., because it has been recognized that this methodology supports the validity and interpretation of the data of a behavioral experiment [49].

Locomotor and Cognitive Frailty Index scores were interpolated by a straight line. The slope of the linear regression indicates that locomotor performances decreases at a steeper rate than cognitive performances during the mouse's life span. Therefore, the data, therefore, indicate that locomotor frailty is associated with lower performance in recognition memory. These data suggest that when mice meet frailty criteria, they should be seen as mice at risk of cognitive decline.

Standardization, efficacy, and the mechanism of action of medicinal mushroom products is a pressing problem [21]. Thanks to the comparison with standard measures, we have identified and quantified erinacines A in the mycelium, and hericenones C and D in the sporophore of He1 using the HPLC-UV-ESI/MS technique. It is worth noting that the content of 0.15 mg/g of erinacine A present in mycelium in this *Hericium* strain is comparable to that reported by Krzyczkowski et al. under the use of the most favorable combination of nutrients [50]. By monitoring the temperature and ventilation during the processing, Chen et al. subsequently obtained the highest content of erinacine A, suggesting a carbon-to-nitrogen ratio of 6 and a pH value of 4 may be important parameters in promoting the biosynthesis of erinacine A [51].

Basidioma of *H. erinaceus* contains a considerable quantity of bioactive molecules such as hericenones. The quantity of hericenones C and D in our sample of *H. erinaceus* was 0.5 mg/g and 0.02 mg/g, respectively, similar to that reported by Lee et al. in some wild-type and local varieties of *H. erinaceus* strains [52].

To be able to best generalize our results to humans, we decided to use an amount of the mycelium and basidioma extracts to mimic the supplementation used in humans. It should be noted that in vitro and in vivo effects of erinacine A and hericenones on NGF synthesis [53], on reducing amyloid burden [51], on reducing amyloid plaques, and on recovering from impairments in Morris water maze tasks [54] were obtained by using a daily millimolar concentration starting from 1-until 30 mg/Kg body weight [55]. In our experimental condition, we used 100-fold lower concentration.

Using the same experimental condition, we previously described the effect of *H. erinaceus* on improving recognition memory and the increase in spontaneous and evoked excitatory synaptic

current in mossy fiber-CA3 synapses [32,33]. Similarly, Rossi et al. showed that two months of *H. erinaceus* treatment increased locomotor performances in mice [33]. The current paper confirms that He1 supplementation may increase recognition memory performance in mice during aging and may also revert the cognitive decline in frail mice.

Hippocampal neurogenesis is pivotally involved in higher cognitive function and, new excitatory dentate gyrus (DG) granule cells, generated by adult hippocampal neurogenesis, contributes significantly to neural plasticity throughout the entire life duration. Our data showed in hippocampus the recovery of cell proliferation in DG granule cells and CA3 pyramidal neurons and the presence of progenitor cells in DG granule cells. These data are in accordance with a recent in vivo study by Ryu et al. [56], supporting the notion that *H. erinaceus* extract administration promotes hippocampal neurogenesis in the adult mouse brain.

There is, currently, a dearth of literature available on the cerebellum area. An in vitro investigation showed the ability of *H. erinaceus* to promote the normal development of cerebellar cells, demonstrating a regulatory effect on the myelin genesis process [57]. Recently, Trovato and colleagues [58] demonstrated in vivo the neuroprotective action of *H. erinaceus* through the up-regulation of lipoxin A4 and modulation of stress responsive vitagene proteins. Our data supports the occurrence of a cell proliferation upswing in the cerebellum, as evidenced by the presence of several PCNA-immunopositive cells, possibly granular cells. Traditionally, the adult cerebellum is commonly considered as a "non-neurogenic" area. Nonetheless, our present results concerning cerebellar cortex demonstrated the presence of DCX-immunopositive cells localized in the molecular layer of He1-treated mice, suggesting the occurrence of newborn immature nervous cells.

In accordance with our preliminary results, recent investigations hypothesized the existence of stem cell populations within the cortex of the adult cerebellum that express stem cell markers and that can give rise to neuronal progeny when expanded in vitro and subsequently transplanted back into the murine cerebellum [59]. Therefore, further in-depth experiments, testing additional molecular markers, need to be carried out to confirm these data, initially identifying the PCNA-immunopositive proliferating cell type and then corroborating the occurrence of a "non-canonical" cerebellar neurogenic process in adult mice.

5. Conclusions

In conclusion, we suggest that during aging, the two form of vulnerability in locomotor and cognitive performances develop in parallel and therefore, we need to integrate motoric and cognitive evaluations. As suggested by Lauretani et al., an investigation of the "brain-muscle loop" in a simultaneous assessment of all aspects that may progressively lead to loss of independence is imperative [60]. Furthermore, *H. erinaceus* is a seemingly good candidate to regress recognition memory decline during aging, possibly through an increase in neurogenesis in the hippocampus and cerebellum. These findings rise the possibility that *H. erinaceus* extracts could be a new therapeutic strategy for preventing or treating neurodegenerative diseases such as dementia and Alzheimer's, as suggested by other authors [24,25,26]. Future studies should investigate the mechanisms involved in this at a cellular level.

Author Contributions: D.R., A.O., C.D.I. carried out the behavior experimental tasks and the experimental analysis. F.C., B.M., V.C., C.G. carried out HPLC-UV-ESI/MS. E.C.P., F.C., B.F., F.D.L. carried out the immunoistological experiments and analysis. D.R., E.R., P.R. wrote the manuscript. M.G.B., H.K., E.S. critically analyzed and reviewed the manuscript.

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References

1. Fried, L.P.; Tangen, C.M.; Walston, J.; Newman, A.B.; Hirsch, C.; Gottdiener, J.; Seeman, T.; Tracy, R.; Kop, W.J.; Burke, G.; et al. Frailty in older adults: Evidence for a phenotype. *J. Gerontol. A Biol. Sci. Med. Sci*. **2001**, *56*, 46–56.
2. Bandeen-Roche, K.; Xue, Q.L.; Ferrucci, L.; Walston, J.; Guralnik, J.M.; Chaves, P.; Zeger, S.L.; Fried, L.P. Phenotype of Frailty: Characterization in the women's health and aging studies. *J. Gerontol. A Biol. Sci. Med. Sci.* **2001**, *61*, 262–266, doi:10.1093/gerona/61.3.262.
3. Hogan, D.B.; Mackinght, C.; Bergman, H. Models, definitions, and criteria of Frailty. *Aging Clin. Exp. Res.* **2003**, *15*, 1–29.
4. Ferrucci, L.; Cavazzini, C.; Corsi, A.; Bartali, B.; Russo, C.R.; Lauretani, F.; Ferrucci, L.; Cavazzini, C.; Corsi, A.M.; Bartali, B.; et al. Biomarkers of frailty in older persons. *J. Endocrinol. Invest*. **2002**, *25*, 10–15.
5. Taffet, G.E. Physiology of aging. In *Geriatric Medicine*; Springer: New York, NY, USA, 2003; pp. 27–35.
6. Espinoza, S.E.; Jung, I.; Hazuda, H. Frailty transitions in the San Antonio Longitudinal Study of Aging. *J. Am. Geriatr. Soc*. **2012**, *60*, 652–660, doi:10.1111/j.1532-5415.2011.03882.x.
7. Boyle, P.A.; Buchman, A.S.; Wilson, R.S.; Leurgans, S.E.; Bennett, D.A. Physical frailty is associated with incident mild cognitive impairment in community-based older persons. *J. Am. Geriatr. Soc*. **2010**, *58*, 248–255, doi:10.1111/j.1532-5415.2009.02671.x.
8. Brodziak, A.; Brewczyński, A.; Bajor, G. Clinical significance of knowledge about the structure, function, and impairments of working memory. *Med. Sci. Monit*. **2013**, *19*, 327–338, doi:10.12659/MSM.883900.
9. Sternberg, S.A.; Wershof-Schwartz, A.; Karunananthan, S.; Bergman, H.; Mark-Clarfield, A. The identification of frailty: A systematic literature review. *J. Am. Geriatr. Soc*. **2011**, *59*, 2129–2138, doi:10.1111/j.1532-5415.2011.03597.x.
10. Robertson, D.A.; Savva, G.M.; Kenny, R.A. Frailty and cognitive impairment-a review of the evidence and causal mechanisms. *Ageing Res. Rev*. **2013**, *12*, 840–851, doi:10.1016/j.arr.2013.06.004.
11. Auyeung, T.W.; Lee, J.S.W.; Kwok, T.; Woo, J. Physical frailty Predicts future cog-nitive decline a four-year Prospective study in 2737 cognitively normal olderadults. *J. Nutr. Health Aging* **2011**, *15*, 690–694.
12. Mitnitski, A.; Fallah, N.; Rockwood, K. A multistate model of cognitive dynamics in relation to frailty in older adults. *Ann. Epidemiol*. **2011**, *21*, 507–516, doi:10.1016/j.annepidem.2011.01.006.
13. Samper-Ternent, R.; Al-Snih, S.; Raji, M.A.; Markides, K.S.; Ottenbacher, K.J. Relationship between frailty and cognitive decline in older Mexican Americans. *J. Am. Geriatr. Soc*. **2008**, *56*, 1845–1852, doi:10.1111/j.1532-5415.2008.01947.x.
14. Aranda, M.P.; Ray, L.A.; Snih, S.A.; Ottenbacher, K.J.; Markides, K.S. The pro-tective effect of neighborhood composition on increasing frailty among older Mexican Americans: A barrio advantage? *J. Aging Health* **2011**, *23*, 1189–1217, doi:10.1177/0898264311421961.
15. Doba, N.; Tokuda, Y.; Goldstein, N.E.; Kushiro, T.; Hinohara, S. A pilot trial to predict frailty syndrome: The Japanese Health Research Volunteer Study. *Exp. Gerontol.* **2012**, *47*, 638–643, doi:10.1016/j.exger.2012.05.016.
16. Raji, M.A.; Al Snih, S.; Ostir, G.V.; Markides, K.S.; Ottenbacher, K.J. Cognitive status and future risk of frailty in older Mexican Americans. *J. Gerontol. Biol Sci Med Sci* **2010**, *65*, 1228–1234, doi:10.1093/gerona/glq121.
17. Kawagishi, H.; Ando, M.; Mizuno, T. Hericenone A and B as cytotoxic principles from the mushroom Hericium erinaceum. *Tetrahedron Lett*. **1990**, *31*, 373–376, doi:10.1016/S0040-4039(00)94558-1.
18. Friedman, M. Chemistry, Nutrition, and Health-Promoting Properties of Hericium erinaceus (Lion's Mane) Mushroom Fruiting Bodies and Mycelia and Their Bioactive Compounds. *J. Agric. Food Chem*. **2015**, *63*, 7108–7123, doi:10.1021/acs.jafc.5b02914.
19. He, X.; Wang, X.; Fang, J.; Chang, Y.; Ning, N.; Guo, H.; Huang, L.; Huang, X.; Zhao, Z. Structures, biological activities, and industrial applications of the polysaccharides from Hericium erinaceus (Lion's Mane) mushroom: A review. *Int. J. Biol. Macromol*. **2017**, *97*, 228–237, doi:10.1016/j.ijbiomac.2017.01.040.
20. Cookson, R.; Barrett, T.N.; Barrett, A.G. β-Keto-dioxinones and β,δ-diketo-dioxinones in biomimetic resorcylate total synthesis. *Acc. Chem. Res.* **2015**, *48*, 628–642, doi:10.1021/ar5004169.
21. Wasser, S.P. Medicinal mushroom science: Current perspectives, advances, evidences, and challenges. *Biomed. J*. **2014**, *37*, 345–356, doi:10.4103/2319-4170.138318.

22. Wasser, S.P. Medicinal Mushrooms in Human Clinical Studies. Part I. Anticancer, Oncoimmunological, and Immunomodulatory Activities: A Review. *Int. J. Med. Mushrooms* **2017**, *19*, 279–317, doi:10.1615/IntJMedMushrooms.v19.i4.10.
23. Hazekawa, M.; Kataoka, A.; Hayakawa, K.; Uchimasu, T.; Furuta, R.; Irie, K.; Yoshida, M.; Fujioka, T.; Egashira, N.; Oishi, R.; et al. Neuroprotective effect of repeated treatment with Hericium erinaceum in mice subjected to middle cerebral artery occlusion. *J. Health Sci.* **2010**, *56*, 296–303, doi:10.1248/jhs.56.296.
24. Mori, K.; Inatomi, S.; Ouchi, K.; Azumi, Y.; Tuchida, T. Improving effects of the mushroom Yamabushitake (Hericium erinaceus) on mild cognitive impairment: A double-blind placebo-controlled clinical trial. *Phytother. Res.* **2009**, *23*, 367–372, doi:10.1002/ptr.2634.
25. Kawagishi, H.; Zhuang, C. Compounds for dementia from Hericium erinaceum. *Drug Future* **2008**, *33*, 149–155, doi:10.1358/dof.2008.033.02.1173290.
26. Tsai-Teng, T.; Chin-Chu, C.; Li-Ya, L.; Wan-Ping, C.; Chung-Kuang, L.; Chien-Chang, S.; Chi-Ying, H.F.; Chien-Chih, C., Shiao, Y.J. Erinacine A-enriched Hericium erinaceus mycelium ameliorates Alzheimer's disease-related pathologies in APPswe/PS1dE9 transgenic mice. *J. Biomed. Sci.* **2016**, *23*, 49, doi:10.1186/s12929-016-0266-z.
27. Mori, K.; Obara, Y.; Hirota, M.; Azumi, Y.; Kinugasa, S.; Inatomi, S.; Nakahata, N. Nerve growth factor-inducing activity of Hericium erinaceus in 1321N1 human astrocytoma cells. *Biol. Pharm. Bull*. **2008**, *31*, 1727–1732, doi:10.1248/bpb.31.1727.
28. Kawagishi, H.; Shimada, A.; Hosokawa, S.; Mori, H.; Sakamoto, H.; Ishiguro, Y.; Sakemi, S.; Bordner, J.; Kojima, N.; Furukawa, S. Erinacines E, F, and G, stimulators of nerve growth factor (NGF)-synthesis, from the mycelia of Hericium erinaceum. *Tetrahedron Lett.* **1996**, *37*, 7399–7402, doi:10.1016/0040-4039(96)01687-5.
29. Chiu, C.H.; Chyau, C.C.; Chen, C.C.; Lee, L.Y.; Chen, W.P.; Liu, J.L.; Lin, W.H.; Mong, M.C. Erinacine A-Enriched Hericium erinaceus Mycelium Produces Antidepressant-Like Effects through Modulating BDNF/PI3K/Akt/GSK-3β Signaling in Mice. *Int. J. Mol. Sci*. **2018**, *19*, pii: e341, doi:10.3390/ijms19020341.
30. Rupcic, Z.; Rascher, M.; Kanaki, S.; Köster, R.W.; Stadler, M.; Wittstein, K. Two New Cyathane Diterpenoids from Mycelial Cultures of the Medicinal Mushroom Hericium erinaceus and the Rare Species, Hericium flagellum. *Int. J. Mol. Sci*. **2018**, *19*, pii: E740, doi:10.3390/ijms19030740.
31. Wong, K.H.; Naidu, M.; David, P.; Abdulla, M.A.; Abdullah, N.; Kuppusamy, U.R.; Sabaratnam, V. Peripheral Nerve Regeneration Following Crush Injury to Rat Peroneal Nerve by Aqueous Extract of Medicinal Mushroom Hericium erinaceus (Bull.: Fr) Pers. (Aphyllophoromycetideae). *Evid. Based Complement. Alternat. Med*. **2011**, 580752, doi:10.1093/ecam/neq062.
32. Brandalise, F.; Cesaroni, V.; Gregori, A.; Repetti, M.; Romano, C.; Orrù, G.; Botta, L.; Girometta, C.; Guglielminetti, M.L.; Savino, E.; et al. Dietary Supplementation of Hericium erinaceus Increases Mossy Fiber-CA3 Hippocampal Neurotransmission and Recognition Memory in Wild-Type Mice. *Evid. Based Complement. Alternat. Med*. **2017**, 3864340, doi:10.1155/2017/3864340.
33. Rossi, P.; Cesaroni, V.; Brandalise, F.; Occhinegro, A.; Ratto, D.; Perrucci, F.; Lanaia, V.; Girometta, C.; Orrù, G.; Savino, E. Dietary Supplementation of Lion's Mane Medicinal Mushroom, Hericium erinaceus (Agaricomycetes), and Spatial Memory in Wild-Type Mice. *Int. J. Med. Mushrooms* **2018**, *20*, 485–494, doi:10.1615/IntJMedMushrooms.2018026241.
34. Kempermann, G.; Song, H.; Gage, F.H. Neurogenesis in the adult hippocampus. *Cold Spring Harb. Perspect. Biol*. **2015**, *7*, doi:10.1101/cshperspect.a018812.
35. Silvers, J.M.; Harrod, S.B.; Mactutus, C.F.; Booze, R.M. Automation of the novel object recognition task for use in adolescent rats. *J. Neurosci. Methods* **2007**, *166*, 99–103, doi:10.1016/j.jneumeth.2007.06.032.
36. Parks, R.J.; Fares, E.; Macdonald, J.K.; Ernst, M.C.; Sinal, C.J.; Rockwood, K.; Howlett, S.E. A Procedure for Creating a Frailty Index Based on Deficit Accumulation in Aging Mice. *J. Gerontol. Biol. Sci. Med. Sci.* **2012**, *67*, 217–227, doi:10.1093/gerona/glr193.
37. Whitehead, J.C.; Hildebrand, B.A.; Sun, M.; Rockwood, M.R.; Rose, R.A.; Rockwood, K.; Howlett, S.E. A clinical frailty index in aging mice: Comparisons with frailty index data in humans. *J. Gerontol. Biol. Sci. Med. Sci.* **2014**, *69*, 621–632, doi:10.1093/gerona/glt136.
38. Cesaroni, V.; Brusoni, M.; Cusaro, C.M.; Girometta, C.; Perini, C.; Picco, A.M.; Rossi, P.; Salerni, E.; Savino, E. Phylogenetic comparison between Italian and worldwide Hericium species. *IJMM* **2019**, submitted.
39. Gerbec, B.; Tavčar, E.; Gregori, A.; Kreft, S.; Berovic, M. Solid State Cultivation of Hericium erinaceus Biomass and Erinacine: A Production. *J. Bioproces. Biotech*. **2015**, *5*, 1–5, doi:10.4172/2155-9821.1000210.

40. Kawagishi, H.; Ando, M.; Sakamoto, H.; Yoshida, S.; Ojima, F.; Ishiguro, Y.; Ukai, N.; Furukawa, S. Hericenones C, D and E, stimulators of nerve growth factor (NGF)-synthesis, from the mushroom Hericium erinaceum. *Tetrahedron Lett.* **1991**, *32*, 4561–4564, doi:10.1016/0040-4039(91)80039-9.
41. Kawagishi, H.; Ando, M.; Sakamoto, H.; Yoshida, S.; Ojima, F.; Ishiguro, Y.; Ukai, N.; Furukawa, S. Erinacines A, B and C, strong stimulators of nerve growth factor (NGF)-synthesis, from the mycelia of Hericium erinaceum. *Tetrahedron Lett*. **1994**, *35*, 1569–1572, doi:10.1016/S0040-4039(00)76760-8.
42. D'Angelo, E.; Rossi, P.; DeFilippi, G.; Magistretti, J.; Taglietti, V. The relationship between synaptogenesis and expression of voltage-dependent currents in cerebellar granule cells in situ. *J. Physiol. Paris* **1994**; *88*, 197–207.
43. Shi, J.; Zhong, X.; Song, Y.; Wu, Z.; Gao, P.; Zhao, J.; Sun, J.; Wang, J.; Liu, J.; Wang, Z. Long non-coding RNA RUNX1-IT1 plays a tumour-suppressive role in colorectal cancer by inhibiting cell proliferation and migration. *Cell Biochem. Funct*. **2019**, *37*, 11–20, doi:10.1002/cbf.3368.
44. Ayanlaja, A.A.; Xiong, Y.; Gao, Y.; Ji, G.; Tang, C.; Abdikani Abdullah, Z.; Gao, D. Distinct Features of Doublecortin as a Marker of Neuronal Migration and Its Implications in Cancer Cell Mobility. *Front. Mol. Neurosci.* **2017**, *10*, 199, doi:10.3389/fnmol.2017.00199.
45. Dutta, S.; Sengupta, P. Men and mice: Relating their ages. *Life Sci.* **2016**, *152*, 244–248, doi:10.1016/j.lfs.2015.10.025.
46. Sternberg Task. Dual Mechanisms of Cognitive Control, Washington University in St. Louis. Available online: http://pages.wustl.edu/dualmechanisms/sternberg-task#sbergdesc (accessed on 26 March 2019).
47. Nosofsky, R.M.; Little, D.R.; Donkin, C.; Fific, M. Short-term memory scanning viewed as exemplar-based categorization. *Psychol. Rev*. **2011**, *118*, 280–315, doi:10.1037/a0022494.
48. Brodziak, A.; Kołat, E.; Różyk-Myrta, A. In Search of Memory Tests Equivalent for Experiments on Animals and Humans. *Med. Sci. Monit*. **2014**, *20*, 2733–2739, doi:10.12659/MSM.891056.
49. Ennaceur, A.L.; Michalikova, S.; Bradford, A.; Ahmed, S. Detailed analysis of the behavior of Lister and Wistar rats in anxiety, object recognition and object location tasks. *Behav. Brain. Res*. **2005**, *159*, 247–266.
50. Krzyczkowski, W.; Malinowska, E.; Herold, F. Erinacine A biosynthesis in submerged cultivation of Hericium erinaceum: Quantification and improved cultivation. *Eng. Life Sci*. **2010**, *10*, 446–457.
51. Chen, C.C.; Tzeng, T.T.; Chen, C.C.; Ni, C.L.; Lee, L.Y.; Chen, W.P.; Shiao, Y.J.; Shen, C.C. Erinacine S, a rare sesterterpene from the mycelia of Hericium erinaceus. *J. Nat. Prod*. **2016**, *79*, 438–441, doi:10.1016/j.bbr.2004.11.006.
52. Lee, D.G.; Kang, H.W.; Park, C.G.; Ahn, Y.S.; Shin, Y. Isolation and identification of phytochemicals and biological activities of Hericium ernaceus and their contents in Hericium strains using HPLC/UV analysis. *J. Ethnopharmacol*. **2016**, *184*, 219–225, doi:10.1016/j.jep.2016.02.038.
53. Kawagishi, H.; Mori, H.; Uno, A.; Kimura, A.; Chiba, S. A sialic acid-binding lectin from the mushroom Hericium erinaceum. *FEBS Lett*. **1994**, *340*, 56–58.
54. Tzeng, T.T.; Chen, C.C.; Chen, C.C.; Tsay, H.J.; Lee, L.Y.; Chen, W.P.; Shen, C.C.; Shiao, Y.J. The cyanthin diterpenoid and sesterterpene constituents of Hericium erinaceus mycelium ameliorate Alzheimer's disease-related pathologies in APP/PS1 transgenic mice. *Int. J. Mol. Sci*. **2018**, *19*, 598, doi:10.3390/ijms19020598.
55. Li, I.C.; Lee, L.Y.; Tzeng, T.T.; Chen, W.P.; Chen, Y.P.; Shiao, Y.J.; Chen, C.C. Neurohealth Properties of Hericium erinaceus Mycelia Enriched with Erinacines. *Behav. Neurol*. **2018**, 1–10, doi:10.1155/2018/5802634.
56. Ryu, S.; Kim, H.G.; Kim, J.Y.; Kim, S.Y.; Cho, K.O. Hericium erinaceus Extract Reduces Anxiety and Depressive Behaviors by Promoting Hippocampal Neurogenesis in the Adult Mouse Brain. *J. Med. Food*. **2018**, *21*, 174–180, doi:10.1089/jmf.2017.4006.
57. Kolotushkina, E.V.; Moldavan, M.G.; Voronin, K.Y.; Skibo, G.G. The influence of Hericium erinaceus extract on myelination process in vitro. *Fiziol. Zhurnal* **2003**, *49*, 38–45.
58. Trovato, R.; Siracusa, R.; Di Paola, M.; Scuto, M.L.; Ontario, O.; Bua, P.; Di Mauro, M.A.; Toscano, C.C.T.; Petralia, L.; Maiolino, A.; et al. Redox modulation of cellular stress response and lipoxin A4 expression by Hericium Erinaceus in rat brain: Relevance to Alzheimer's disease pathogenesis. *Immun. Ageing* **2016**, *13*, 23, doi:10.1186/s12979-016-0078.
59. Ahlfeld, J.; Filser, S.; Schmidt, F.; Wefers, A.K.; Merk, D.J.; Glaß8, R.; Herms, J.; Schüller, U. Neurogenesis from Sox2 expressing cells in the adult cerebellar cortex. *Sci. Rep.* **2017**, *7*, 6137, doi:10.1038/s41598-017-06150-x.

60. Lauretani, F.; Meschi, T.; Ticinesi, A.; Maggio, M. "Brain-muscle loop" in the fragility of older persons: From pathophysiology to new organizing models. *Aging Clin. Exp. Res.* **2017**, *6*, 1305–1311, doi:10.1007/s40520-017-0729-4.

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4. CONCLUSIONS

The purpose of my PhD project was to deepen the knowledge about the effects of oral supplementation of *H. erinaceus* on cognitive functions.

To date, only few papers have been published about the effect of *Hericium* supplementation in humans central nervous system.

During my first year of PhD, I have had the opportunity to collect sporophores of genus *Hericium*, such as *H. alpestre*, *H. coralloides* and *H. erinaceus*. These are three of the four species present in Italy. *H. erinaceus* sporophore and its strain (H.e.1) were sampled and isolated respectively in 2013, and it was available for analytical analyses since the beginning of my PhD project. Regarding to the other species, *H. coralloides* (H.c.1) and *H. alpestre* (H.a.1) grew very slowly on cultural media so, one of the future perspectives could be to use natural substrata such as little pieces of trunk or smudged needles added to cultural media to improve their growth.

Chemical analyses were performed on samples in order to identify the presence of specific bioactive molecules known from literature to be able to pass the blood-brain barrier and exert a neuroactive effect on the central nervous system. Thanks to the collaboration with Professor H. Kawagishi of the Shizuoka University in Japan that provided us the three standard molecules of erinacine A, hericenone C and hericenone D, we were able to detect these molecules in our samples.

The HPLC-ESI-MS/MS was used also as an analytical screening method, because gave me a direct feedback about the quality of samples in order to identify the best growing condition obtaining the highest production of bioactive molecules of interest.

During my second year of PhD, I had a collaboration with Professor M. Brusoni of the University of Pavia in order to perform molecular analysis on the *Hericium* strains collected in field.

Molecular data confirmed the identity of all the strains and do not reveal any difference between strains of *H. erinaceus* collected in Mediterranean area and in other regions. Bayesian phylogenetic analysis produced a phylogram that permitted good discrimination between *Hericium* species. It provides an updated phylogeny within the genus *Hericium* and a better understanding of affinity among the species analyzed. The main *Hericium* clade includes: the *H. erinaceus* group; the *H. alpestre* / *H. coralloides* group, where the two species cluster separately.

The data obtained have been published in the International Journal of Medicinal Mushrooms (Cesaroni *et al.*, 2019).

The experience on cultivation procedures obtained during the first PhD year allowed me to improve the quality of the process, such as type of grains used for the production of spawn and procedures

for the sterilization (time, cycle of resting out of autoclave, weight of bags etc.). Moreover, I was able to quantify the bioactive metabolites present in the two strains of *H. erinaceus*, both in mycelia and wild-type sporophores.

During the third year of my PhD, in order to achieve the final goals, I worked to set up the cultivation of *H.erinaceus* (strain H.e.2) in the greenhouse of the Botanical Garden of the University of Pavia (Italy). Even if the value of biological efficiency was quite low (23,14 %), the data obtained allowed us to select the best performing strain for cultivation: H.e.2 cultivated sporophore contained the higher amount of hericenones C and D, compared to H.e.1 strain. In the next future, further experiments will be necessary in order to improve the efficiency of sporophores production.

All the data obtained by quantitative analysis of the two strains on the array of described metabolites, allowed us to prepare a supplement with the highest amount of neuroactive molecules as a blend made by H.e.1 mycelium and H.e.2 sporophore, that could be used in future *in vivo* experiments.

Thanks to data obtained from *in vivo* tests (Brandalise *et al*., 2017 and Rossi *et al*., 2018), it was possible to confirm the dual-component hypothesis of memory recognition, where ("knowledge" and "remember") are placed in two different brain areas, such as hippocampus and parahippocampus. This result confirms and reinforces this new paradigm about memory in Neuroscience and Neurobiology.

During physiological aging in mice we tuned and monitored a frailty index for both locomotor and cognitive performances. Data displayed that locomotor frailty anticipate the cognitive decline in recognition memory, suggesting that by monitoring locomotor performances we can intercept cognitive decline in memory. Two months *H. erinaceus* oral supplementation reverted the cognitive frailty during aging, suggesting that *H.erinaceus* could be a potential candidate for preventing and/or treating neurodegenerative diseases, such as Alzheimer's and dementia.

It should be noted that we used a translational approach with the purpose to transfer all the results obtained in preclinical model to clinical trials on humans. For this reason, we used a blend with an amount of mycelium and sporophore extract to mimic the dose used in humans. Furthermore, we chose to monitor in mice locomotor parameters (mean and maximum speed, resting time and the total distance covered in an open arena) that compared well to phenotypic frailty parameters in humans (gait speed and the level of physical activity).

Up to now, in all the *in vivo* experiments it was tested a mixture of the strain H.e.1 with a standardized amounts of erinacine A and hericenones C and D or a commercial product provided by

the AVD Reform, Parma, Italy. Since we were able to select a blend containing the two best performing strains for the production of mycelium (H.e.1) and, in particular, for the development of the sporophores (H.e.2), in the next experiments it will be interesting to test the effect of this mixture.

Thanks to the interdisciplinary scientific approach followed during my PhD, I was able to draw the guidelines for a better use and standardization of *H. erinaceus* dietary supplements on cognitive functions. Starting from all stages of the production chain (strain selection, extraction, chemical analysis, and finally pre-clinical test on animal models), it should be possible to transfer the acquired knowledge “from bench to bedside” on humans.

I hope that my PhD project will be useful to solve at least some critical points on standardization and creation of guidelines for dietary supplements production from medicinal mushrooms, and that pave the way for new clinical trials on humans based on data obtained in animal models.

5. AKNOWLEDGMENTS

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Another important collaboration was the one with Professor Kawagishi of the Shizuoka University (Japan), that provided us three standard molecules of the bioactive metabolites present in *H. erinaceus*, giving us the possibility to quantify these molecules in the Italian samples.

Thanks to the availability of the instruments at Centro Grandi Strumenti of the University of Pavia and the help of Dott. Barbara Mannucci, we were able to perform specific chemical analyzes and obtain interesting results for the future.

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