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Effect of erinacine A-enriched *Hericium erinaceus* supplementation on cognition: A randomized, double-blind, placebo-controlled pilot study

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ABSTRACT

Population aging has led to an increased interest in various dietary supplements in order to preserve cognitive function. The aim of our study was to examine the effects of *Hericium erinaceus* supplementation (HE) on cognitive function and serum levels of Brain-Derived Neurotrophic Factor (BDNF) and Neuropeptide Y (NPY). An 8-week double-blind comparative study involved 33 subjects, randomly assigned to a HE group and a placebo (P) group. Anthropometric measurements and analyses of selected biochemical parameters were performed before and after the intervention. Simultaneously, faecal samples were collected to determine gut microbiota and chitinase 3-like-1 (CHI3L1) levels. Cognitive function was assessed using two non-verbal speed tests. In the HE group, a significant improvement in cognitive ability was observed when the corresponding cognitive scores at baseline, CHI3L1 level, age, and gender were considered. Moreover, in the HE group there was a significant increase in gut microbiota diversity that was positively correlated to NPY levels. The mechanism that could help to explain the results on the relationship between the active ingredients of the *H. erinaceus* and the cognitive parameters could point to the CHI3L1 activity that may enhance bioavailability of the bioactive ingredients. Supplementing the diet with HE was recognised as a safe and well-tolerated intervention with a neurocognitive benefit.

1. Introduction

Cognitive functioning refers to the performance of mental processes, including thinking, reasoning, perception, learning, memory, understanding, awareness, judgment, and intuition (Fisher et al., 2019). The speed of these mental processes dictates our responses to stimuli, called reaction time. Neural activity associated with processing speed plays a crucial role in cognitive performance and has been extensively investigated as a cognitive marker in various neurocognitive disorders (Lu et al., 2017). With aging, there is a natural decline in processing speed, leading to slower cognitive functioning. This normal (non-pathological, normative) cognitive slowing or age-associated cognitive decline has been shown to begin relatively early in middle age and progress more rapidly with advancing age (Meunier et al., 2014; Murman, 2015). The neuropathological cause of age-related cognitive slowing is related to the speed of neuronal transmissions and to myelination (Chopra et al., 2018). In this generalised slowing, the change of subcortical white matter integrity in the whole brain may play a central role (Coelho et al., 2021; Madden et al., 2017).

Due to increased average life expectancy, we are confronted by a higher number of older adults experiencing both normal age-related cognitive decline as well as a substantial number of individuals experiencing non-normal decline, such as mild cognitive impairment and dementia (Association, 2018). Cognitive (intellectual) ageing has become an important public health challenge attracting the attention of researchers. As preserving cognitive function is an essential component of maintaining a healthy, active, and independent lifestyle, an expanding body of research focuses on appropriate strategies to prevent the progressive cognitive decline (Dysken et al., 2014; Farina et al., 2017; Pinto & Subramanyam, 2009) and slow age-related changes in cognitive functions. These conditions can be delayed or improved by lifestyle changes, such as healthier eating habits (Cohen et al., 2016; Ngandu

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et al., 2015), following a nutritious diet (e.g., MIND diet based on the dietary components of the Mediterranean or DASH diet) (Morris et al., 2015; Singh et al., 2014), caloric restriction (Bishop et al., 2010), regular exercise (Imaoka et al., 2019), and cognitive training practices (Pereira-Morales et al., 2018).

Beneficial effects on cognitive functions of older individuals have been confirmed by the intake of nutraceuticals and/or dietary supplements (Iolascon et al., 2017). However, many of these interventions lack support from the published scientific literature regarding their efficacy or safety (Crawford et al., 2020). Favourable outcomes have also been observed from the consumption of edible mushrooms, which are increasingly valued for their medicinal properties. These mushrooms are not used only as dietary food (functional foods), but also in the form of dietary supplements, nutraceuticals, and mycotherapy products. The use of mushrooms to promote and maintain health and to treat diseases has been known in traditional Chinese medicine since ancient times (Money, 2016). Owing to their health benefits, they are therefore widely used in complementary alternative medicine and complementary integrated medicine (Venturella et al., 2021).

Numerous studies, both *in vitro/vivo* preclinical and clinical, have demonstrated the effects and mechanisms of bioactive compounds derived from *Hericium erinaceus* (Hetland et al., 2020; Venturella et al., 2021). Both the fruiting body and mycelia of *H. erinaceus* contain a remarkable variety of structurally diverse bioactive components that contribute to the prevention, alleviation, and treatment of various diseases. Notably, antioxidative, anti-inflammatory, anticancer, immunostimulant, antidiabetic, antimicrobial, hypolipidemic, and antihyperglycemic properties of *H. erinaceus* have been reported (Khan et al., 2013). Through clinical trials, pharmacological activities and medical evidence of *H. erinaceus* have been demonstrated, showing its effectiveness in improving average cognitive impairment (Mori et al., 2008), treating early Alzheimer's disease (Li et al., 2020), alleviating symptoms of anxiety and depression, and improving sleep quality (Nagano et al., 2010; Okamura et al., 2015; Vigna et al., 2019). As its applications are diverse, *H. erinaceus* is most commonly used for the treatment of neurodegenerative diseases and cognitive impairment (Chong et al., 2021; Kawagishi and Zhuang, 2008; Spelman et al., 2017). Importantly, the bioactive metabolites of *H. erinaceus*, namely the erinacines (a group of cyathin diterpenoids extracted from the mycelium) and hericenones (benzyl alcohol derivatives extracted from the fruiting body), can easily traverse the blood–brain barrier (Venturella et al., 2021). These compounds have been shown to enhance the synthesis of trophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Chiu et al., 2018; Friedman, 2015; Kawagishi et al., 1996; Mori et al., 2008), which exhibit neurotropic and neuroprotective effects (Huang et al., 2021; Roda et al., 2021). The evidences on *H. erinaceus* in stimulating NGF release, regulating inflammatory processes, reducing oxidative stress, and protecting nerve cells from apoptosis, shows its neuroprotective potential (Szućko-Kociuba et al., 2023). Furthermore, the presence of polysaccharides, such as chitin, is also important. Chitin is a β-(1,4)-linked homopolymer of *N*-acetylglucosamine and is one of the most abundant biopolymers found in fungi, serving as a primary component of their cell walls (Shahidi & Abuzaytoun, 2005). Since chitin or chitooligosaccharides are generally resistant to digestion by human enzymes, the presence of chitinase-producing commensal bacterial in gut could facilitate the release and subsequent utilization of bioactive compounds from fungal cells. Moreover, the presence of chitinase-producing bacteria is also necessary for the degradation of chitin and its utilisation as an energy source (Dohnálek et al., 2021). Conversely, certain bioactive compounds found in mushrooms, such as polysaccharides and phenolic compounds, have been shown to significantly affect gut microbiota. These compounds can contribute to the enrichment of the gut microbiota, promote the growth of beneficial bacterial species, including those that produce short-chain fatty acids (SCFAs), increase the *Bacteroidetes/Firmicutes* ratio, and reduce the presence of harmful species (Li et al., 2021). As it is well known, gut microbiota is an important factor influencing mental health since it can affect the gut-brain axis through regulation of different neuroactive modulators such as BDNF (Liaqat et al., 2022). The consumption of *H. erinaceus* has already been shown to promote the growth of beneficial gut bacteria with additional positive effect on cognitive function in elderly mice model. Overall, authors suggest that the modulation of gut microbiota composition could trigger longevity-promoting effects, protecting from age-related cognitive decline (Priori et al., 2024).

In the present randomised clinical trial, we have thus investigated the effects of daily consumption of erinacine A-enriched *H. erinaceus* supplementation (HE) on cognitive functioning, serum biochemical markers, faecal levels of chitinase, and gut microbiota composition.

2. Methods and materials

2.1. Preparation of erinacine A-enriched dietary supplement

During the R&D project entitled "First food supplement standardised on erinacine A content", also known as the "ErinacinA project," more than 50 strains of *H. erinaceus* were screened for erinacine A content. The most productive strains were later cultivated under optimised growing conditions with more than 2500 cultivation and extraction experiments conducted by MycoMedica Ltd, Kranjska Gora and PharmaHemp Ltd, Ljubljana, Slovenia. As a result, a new food supplement *"H. erinaceus* heteropolysaccharides" was developed being sold under a brand name "GOBA® Hericium erinaceus*"*, containing standardised concentrations of erinacine A. Since HE supplement used in this study is a commercial product for which a patent is currently pending only limited set of data about cultivation is available. Briefly, *H. erinaceus* strain Her. Erin. (fungal culture collection of MycoMedica Ltd, Podkoren, Slovenia) was cultivated in darkness at 24 °C on Potato Dextrose Agar. When fully overgrown mycelium was dispersed in water (one 120 mm petri dish per 1000 mL of sterilized water) using a Waring blender and further used as liquid inoculum.

5 kg of organic cellulose and supplements, containing a vegan, gluten- and allergen-free substrate with moisture content of 65 % (w/w), were packed into polypropylene bags and sterilised at 121 °C for 80 min. Following sterilisation, the substrate was cooled to room temperature and inoculated with 50 mL of liquid inoculum of *H. erinaceus*. The cultivation process took place at a temperature of 24 °C for approximately three months, after which the fungus was harvested. A portion of the harvested fungus was extracted and combined with the initial batch, and then dried under vacuum for 24 h at temperatures below 60 °C. During the drying process, a drying agent containing starch and cellulose was used. Due to vigorous evaporation under vacuum of approximately 60 mbar and elevated temperature (<60 °C) the resulting material achieved moisture content lower than 7 % in less than 24 h. Subsequently, the material was finely powdered, with particle sizes smaller than 250 μm, and further encapsulated into capsules containing 500 mg of material. The samples used in this study included erinacine A-enriched *H. erinaceus* supplement (HE), or corn starch in the case of placebo (P) group. Both substances were filled into hydroxypropyl methylcellulose capsules and administered to the study participants.

2.1.1. Analyses of erinacine A-enriched dietary supplement

For the analysis of erinacine A content in the material, extraction using 70 % ethanol was performed. Quantification was carried out using an Agilent 1260 Infinity II HPLC system with a Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 μm column and a DAD detector set at a wavelength of 340 nm (Agilent, Santa Clara, CA, USA). The employed HPLC method was developed and validated in an ISO 17025 accredited laboratory. Erinacine A standard was obtained from *H. erinaceus* by extract fractionation using Flash/Prep in first dimension, followed by second dimension fractionation using HPLC-UV. The identity of the isolated compound was proven by LC-MS/MS and the purity (against procedural

blank) was determined by LC-CAD (Fig. S1).

Furthermore, HE was analysed for total polyphenols content and antioxidant activity as described before (Atamanchuk and Bisko, 2023). The content of total polyphenols in tested material was 3.17 ± 0.18 mg/g of gallic acid equivalents and antioxidant activity of the material was 81.3 ± 0.43 % (free radical scavenging activity determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay).

The relative amounts of protein (AOAC Official method 954.01), lipid (AOAC Official method 920.39), water (AOAC Official Method 934.01), ash (AOAC Official method 942.05) and fibre (AOAC Official method 978.10) in HE were estimated by proximate analysis. The analyses were conducted at the Biotechnical Faculty, Chair of Nutrition, University of Ljubljana, Slovenia. Carbohydrate content in samples was calculated by difference (100 – water – lipids – proteins – ash). Since these results were not an object of our study, they are presented in Table S2.

2.2. Study design

The study protocol was approved by the Slovenian National Medical Ethics Committee (No. 0120–321/2017–4) and was registered at ClinicalTrials.gov (Identifier: NCT04939961). All the participants signed their informed consent prior to their inclusion in the study. The double-blind randomised, comparative trial was conducted at the University of Primorska, Faculty of Health Sciences Izola, Slovenia, between June and September 2021. The study design is presented in Fig. 1.

Thirty-six participants were randomly assigned into 2 groups: to the erinacine A-enriched *H. erinaceus* supplementation (HE) group or to the placebo (P) group. Randomisation with stratification was performed using a simple software, free open-source application MinimPy (Saghaei & Saghaei, 2011), accessible through the website (https://sourceforge.net/projects/minimpy). Stratified variables were gender and age. We used two randomly assigned separate sequences for males and females, and three randomly assigned separate sequences for age groups (55–59 years; 60–65 years; 66–75 years).

Therefore, both groups (HE and P) were equal with regard to sex and age. The study was double blind. Capsules were packed and labelled by a research assistant that was blind to the aim of the study. Also, both capsules were similar in colour, size and shape to ensure that they could not be distinguished by the participants or researchers. The entire study consisted of 8 weeks of intervention period, during which subjects ingested two capsules after breakfast, lunch and dinner (6 capsules/day) with daily intake of 3.44 mg of erinacine A. Participants were advised to maintain their level of physical activity and diet, and to maintain their daily life rhythm and habits. Anthropometric, cognitive, and biochemical measurements were performed at baseline (measurement 1) and after 8 weeks of supplement intake (e.g. consumption of either HE or P) (measurement 2). At this two time points, participants provided a stool sample, previously collected at home.

2.3. Study participants

The Caucasian male and female volunteers were recruited through an advertisement posted on internet forums, sent via e-mail lists, or published in local newspapers. Thirty-six asymptomatic adults were recruited into the study. The inclusion criteria were age over 55 years,

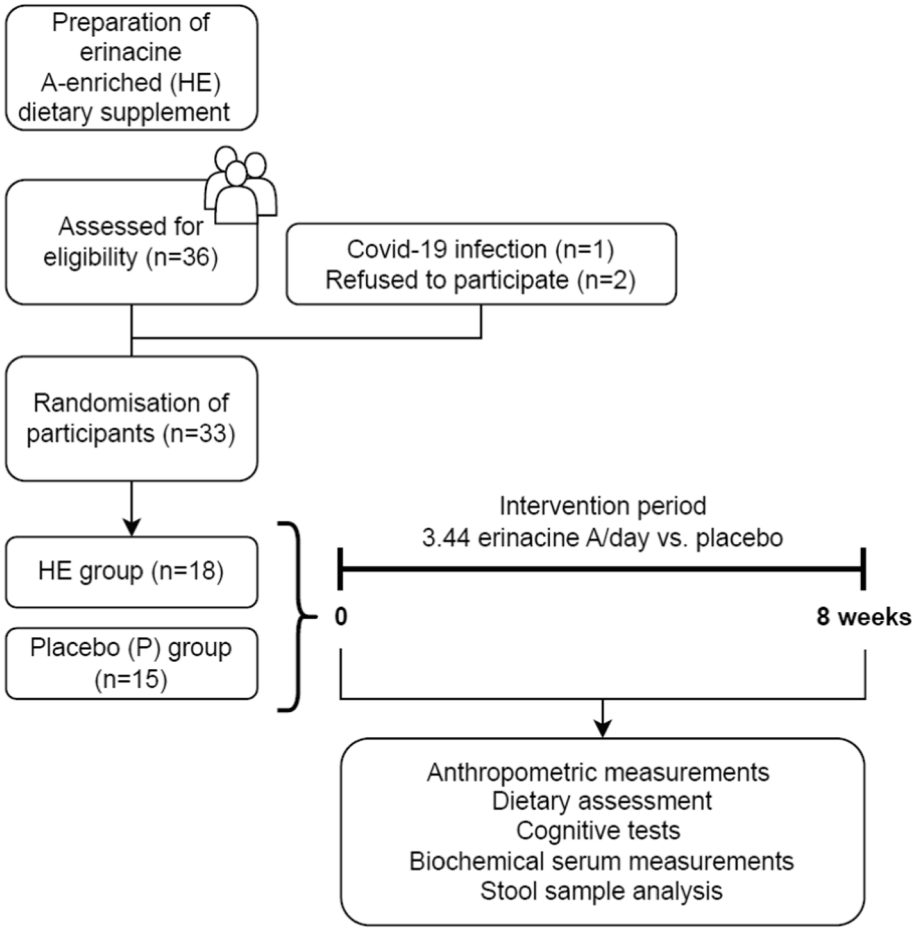

Fig. 1. Participant flow diagram and design of the study. 36 participants were recruited to the study, out of which 33 met the inclusion criteria. All subjects completed 8 weeks of intervention period, with daily intake of 3.44 mg erinacine A or placebo.

absence of chronic, cardiovascular, gastrointestinal, or liver diseases, type 2 diabetes mellitus, or neurodegenerative diseases, and no taking of medications for lipid disorders, anti-inflammatory drugs, antidepressants, or anxiolytics, and absence of other brain diseases like brain injury, brain atrophy, and stroke.

The exclusion criteria were mushroom allergies and use of antibiotics in the past three months. During the study, all participants refrained from taking antibiotics and other dietary supplements.

2.4. Anthropometric measurements and dietary assessment

Anthropometric measurements using a bioelectric impedance analyser Tanita MC-980MA (Maeno-cho, Japan) were performed after an overnight fasting between 7 a.m. and 9 a.m. in standardised conditions by the same examiner. Measured parameters included body weight, body fat, fat free mass, muscle mass, visceral index, total body water, extracellular water, intracellular water, and phase angle. The body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Participants recorded their food intake for three days before visit. Dietary data were analysed using the Open Platform for Clinical Nutrition (OPEN), accessible through the website https://opkp.si/.

2.5. Cognitive tests

A thorough cognitive assessment with two standardised tests was performed at baseline and after an 8-week intervention. Both tests measure cognitive performance: speed (rate of performance) and level (accuracy of response). The primary outcome was a change in cognitive performance as measured by total test score (higher score indicates better performance).

2.5.1. Test of perception speed "patterns" (THP)

The THP is a 36-tasks non-verbal speed test to measure the perception speed, mental speed, and in the less capable individuals also biological intelligence (Pogačnik, 2012). In each task, there is one pattern on the left side and four more on the right side, only one of which exactly matches the pattern on the left. The participant must find and mark it as quickly as possible. The test is limited to 4.5 min. The reliability calculated on various samples according to the KR-20 formula was 0.86 to 0.71 (Pogačnik, 2012).

2.5.2. Test of series (TN-10-A)

The TN-10-A is a non-verbal speed test for measuring fluid intelligence, which is relatively independent of education, experience and culture (Pogačnik, 2006), and reflects information processing capacity. The result is primarily influenced by the ability to recognise relationships and legitimacy, working memory, the speed of information processing, and mental and perceptual speed. The test thus measures a broad grouping of mental abilities. It consists of 45 series of tasks with progression in difficulty. Each task contains a string of 15 characters on the left side and five suggested answers on the right side, only one of which continues the series correctly. The time for the test accomplishment is 10 min, meaning that mental speed affects the scores. The reliability reported for the TN-10 is 0.86 to 0.90 and by the test–retest method (48 months) 0.90 (Pogačnik, 2006).

2.6. Biochemical serum measurements

For venous blood withdrawal, the participants were instructed to restrain from eating for 12 h prior to the blood withdrawal. The blood was collected into 5 mL vacuum blood serum collection tubes, and serum samples were obtained as previously described (Tuck et al., 2009). The serum samples were stored in polypropylene tubes at -80 °C until further analysis. To determine the levels of serum glucose (GLU), Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), total cholesterol (TC), triacylglycerols (TAG), Alanine Aminotransferase (ALT), C-reactive Protein (CRP), and uric acid (UC), a Cobas c111 analyser (Roche, Basel, Switzerland) was used. Analyses were conducted using fresh Cobas reagents and carried out following the manufacturer's instructions.

Serum concentrations of Neuropeptide Y (NPY) and Brain-Derived Neurotrophic Factor (BDNF) were determined in duplicate using human ELISA kits (EMD Millipore Corporation, Missouri, USA). The assays were performed according to the manufacturer's instructions. The optical density of each well was measured using a microplate reader (Tecan, Mannedorf, Switzerland). Assay sensitivity was 15 pg/ml for BDNF and 2 pg/ml for NPY. Inter-assay and intra-assay CVs were typically < 10 %.

2.7. Stool sample analysis

Participants were asked to collect stool samples at home and store them at -20 °C before taking them to the laboratory, where they were kept at -80 °C until analysis. Stool samples were weighed and divided for faecal chitinase 3-like-1 (CHI3L1) measurement and bacterial DNA extraction. CHI3L1 faecal extraction, were conducted as described before (Volkers et al., 2022) with some modifications. Briefly, Phosphate Buffered Saline (PBS) was added to adjust weight-to-PBS ratios and vortexed into a homogeneous mixture. Samples were then centrifuged at 13,000*g* for 10 min and the resulting supernatants were used in the commercial ELISA kit (R&D Systems, Human Chitinase 3-like-1 DuoSet ELISA, Minneapolis, MN, USA), following manufacturer's instructions.

For the assessment of the bacterial community composition, DNA was extracted using the commercial QIAamp DNA Stool Mini kit (Qiagen N.V., Venlo, The Netherlands) and analysed as described before (Petelin et al., 2022; Šik Novak et al., 2022). Briefly, amplification of V4 16S rRNA was performed with modified 515F and 806R. Primer 806R was elongated with the sequence of P1 adapter and 515F primer was elongated with the sequence of linker, barcode and adapter at their 5′-end in order to produce amplicons compatible for sequencing with Ion S5™ System (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was amplified with a unique barcode in triplicates to reduce PCR bias. Negative control was performed with a unique barcode as well. PCR products were checked on agarose gel and equal volume of pooled triplicates were joined in a final library. Final library was cleaned with Agencourt AMPure XP magnetic beads (Beckman Coulter) using bead-to-DNA ratio of 0.7:1. Quality of DNA library and DNA concentration was determined on Agilent 2100 Bioanalyser using High Sensitivity DNA Assay kit (Agilent Technologies, Santa Clara, CA, USA). Ion 530™ chip with DNA library and Ion S5™ calibration standard was prepared with Ion OneTouch™ 2 system using the kit Ion 520™ & Ion 530™ Kit-OT2 and sequencing was performed on Ion S5™ System (Thermo Fisher Scientific, Waltham, MA, USA). Reads were analysed with QIIME2 v.2021.8. DADA2 (qiime dada2 denois-pyro plugin) was used for denoising and determination of amplicon sequence variants (ASVs). Taxonomy classification was made with feature classifier plugin based on naive Bayes classifier trained on SILVA reference database, version 138.1. Microbiota diversity was quantified using Shannon index, which reflects the richness of bacterial community (Kim et al., 2017).

2.8. Statistics

Statistical analysis was performed using SPSS version 26.0 (IBM Corp., Armonk, NY). The normality of the variables was tested using the Shapiro–Wilk test. Data are presented as the mean value with the standard deviation, the median value with the minimum and maximum, or a percentage. The effects of the interventions within each group were analysed using Student's paired samples *t*-test or the Wilcoxon signed-rank test, whereas the comparison of mean changes between the two groups was done using an independent *t*-test or Mann–Whitney *U* test. Moreover, as age and sex have an impact on observed cognitive parameters, a univariate analysis of covariance (ANCOVA) with age and

sex as a covariate was performed to compare cognitive data among the groups. The effects of the interventions were analysed using ANCOVA with the change at week 8 as the dependent variable, adjusted to the corresponding values at baseline, and stratified for age and sex. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Baseline characteristics of study participants

In this clinical study, 36 subjects started the experiments; one subject dropped out due to Covid-19, and two after the follow-up period. Therefore, the final sample size was 33 subjects (67 % F; 33 % M), aged 55–75. The baseline characteristic of the participants in the study are presented in Table 1. At baseline, groups did not differ significantly with respect to age, gender, BMI, and energy intake.

3.2. Effects of HE on anthropometric and biochemical parameters

The consumption of HE or P had no major effect on anthropometric or basic biochemical parameters. No significant differences were found between the groups, neither in the initial nor in the final values (with the respect to anthropometric and biochemical parameters, Table 2). BMI, visceral fat, glucose, total cholesterol, CRP, and liver enzyme ALT did not change significantly in either group. Notably, the serum concentration of TAG showed a significant increase in the P group ($p \leq 0.05$). In the HE group, there was an increase in serum LDL cholesterol and a decrease in HDL cholesterol and UA. Similar but not significant changes in these parameters were observed in the placebo group. When changes from baseline were compared, no significant differences between groups were detected.

3.3. Effects of HE on cognitive function

The main focus of the present study was to test the effect of erinacine A-enriched HE supplement on the cognitive functioning of individuals. We were interested in whether the consumption of the supplement during an 8-week intervention had an effect on improving certain cognitive abilities. Initial cognitive test scores did not differ significantly between the groups, nor did final scores or changes from baseline (Table 3). Although a slight increase in THP and TN scores were observed in the HE group after the intervention, no significant differences in participants' cognitive performance were found using a simple parametric tests.

Since gender differences in cognitive functioning had consistently been reported in some cognitive tasks (Gurvich et al., 2020), it is important to consider age and sex differences when examining cognitive performance. Looking at the group difference between the HE group and the placebo group, adjusted for baseline values and stratified by age and gender, a statistically significant improvement in cognitive function in the THP test/score ($F = 4.6$, $p < 0.05$) was observed in the HE group at the end of the intervention (8 weeks). We thus noted a significant beneficial effect of the intervention with HE supplementation for the cognitive outcome when age, sex, and baseline THP score have been included in the model.

Table 1
Baseline characteristics of the subjects included in the clinical trial.

	HE group (N = 18)	P group (N = 15)	p value
Gender (% F, M)	67 % F, 33 % M	67 % F, 33 % M	1
Age (years)	63.1 ± 7.0	62.5 ± 7.6	0.841
BMI (kg/m^2)	25.0 ± 3.8	25.7 ± 3.2	0.568
Energy intake (kcal/day)	1967 ± 799	1833 ± 523	0.569

Abbreviation: BMI, body mass index; F, female; HE, erinacine A-enriched *H. erinaceus* supplementation; M, male; P, placebo.
Values are expressed as means ± SD. P-value denotes difference between groups using an independent samples *t*-test or Mann–Whitney *U* test.

3.4. Effects of HE on neurotrophic markers

In addition to cognitive function, we focused on the effect of HE on serum levels of the neurotrophic factors BDNF and NPY. In the HE group, we observed an increase in BDNF levels from baseline (7.47 ± 4.86) to 8 weeks (8.49 ± 3.28). This suggests that the intervention (HE) had a positive effect on increasing serum BDNF levels. In contrast, the placebo group demonstrated a notable decrease in BDNF serum levels over the same period. The baseline BDNF level in the placebo group was 9.29 ± 3.65, which decreased to 7.01 ± 2.85 after 8 weeks. This decrease was found to be statistically significant ($p < 0.05$) according to the results presented in Table 3. In summary, the study findings imply that the intervention (HE) had a positive impact on increasing BDNF serum levels, whereas the placebo group experienced a significant decrease in BDNF levels over the 8-week period. Importantly, the difference in the intervention effect between the HE and placebo groups reached statistical significance ($p < 0.05$). For NPY, there were no differences within or between the groups.

3.5. Effects of HE on faecal CHI3L1 levels and relation to cognitive and neurotrophic markers

Due to the possibility of the chitinase effect on the bioavailability of bioactive components of HE supplement, CHI3L1 levels in the stool samples of each participant were measured. There were no statistically significant differences in CHI3L1 levels between the groups and they were not observed either when the value after the intervention was compared with the initial value within the group (Table 3). Mean values from data before and after the intervention for each participant were then calculated. We investigated the potential effect of CHI3L1 faecal concentrations on cognitive abilities and neurotrophic markers.

After including the mean values of CHI3L1 as a covariate into our model, along with other relevant parameters (baseline values, age, gender), a comprehensive analysis revealed a statistically significant impact of the HE intervention on the parameters THP ($F = 5.3$, $p = 0.040$), TN ($F = 9.3$, $p = 0.010$), and NPY ($F = 5.3$, $p = 0.039$). This underscores the significant influence of HE on these parameters, even when accounting for the covariate CHI3L1. Conversely, in the P group, CHI3L1 did not exhibit a statistically significant effect on any of the parameters studied, emphasizing a differential impact compared to the HE group. Notably, similar results were replicated when incorporating the post-intervention value of CHI3L1 instead of the average value in stool into the model. This consistency strengthens the robustness of our findings, further supporting the notion that HE exerts a significant effect on THP, TN, and NPY parameters in contrast to the placebo group.

3.6. Effect of HE on gut microbiota composition

The data of gut microbiota are presented for 32 subjects that is one less than for other study results; one subject dropped out due to the missing faecal sample in one of the time points. The relative abundance of bacteria phyla before the intervention was the same across the groups and was the highest for *Firmicutes* (51.5 ± 17.4 vs. 49.6 ± 15.4 %). There were also no statistically significant differences in genera distribution (with relative abundance ≥ 1 % that were found in more than 10 % of faecal samples) between the groups (Table S1).

The statistically significant differences in the HE group after the intervention were observed only for three genera with a relative abundance of less than 1 %. These genera were as follows: *Intestinimonas* ($p = 0.040$), *Gastranaerophilales* ($p = 0.038$), and *Tyzzerella* ($p = 0.028$). A higher abundance of *Flavobacteriaceae* family ($p = 0.042$) in the HE group was also observed. When comparing the relative abundance of phyla and genera that were present at a relative abundance of more than

Table 2
Anthropometric and biochemical parameters (means ± standard deviations) at baseline and after intervention (week 8).

	HE group (N = 18)			P group (N = 15)		
Measure	Baseline	Week 8	Difference	Baseline	Week 8	Difference
BMI (kg/m^2)	25.0 ± 3.8	25.0 ± 3.9	0.0 ± 0.4	25.7 ± 3.2	25.8 ± 3.0	0.1 ± 0.6
Visceral fat	8.7 ± 3.0	9.0 ± 3.2	0.3 ± 0.5	9.1 ± 2.1	8.9 ± 2.2	−0.2 ± 0.8
GLU (mmol/L)	5.41 ± 0.77	5.49 ± 0.91	0.08 ± 0.41	5.34 ± 0.47	5.35 ± 0.57	0.01 ± 0.43
TC (mmol/L)	5.48 ± 0.99	5.92 ± 0.95	0.08 ± 0.49	5.30 ± 1.26	5.37 ± 1.22	0.07 ± 0.47
LDL (mmol/L)	4.35 ± 1.08	4.51 ± 1.00 [a]	0.17 ± 0.30	3.91 ± 1.28	3.98 ± 1.18	0.07 ± 0.41
HDL (mmol/L)	1.67 ± 0.32	1.60 ± 0.35 [a]	−0.07 ± 0.12	1.61 ± 0.31	1.54 ± 0.38	−0.08 ± 0.19
TAG (mmol/L)	1.42 ± 0.71	1.44 ± 0.72	0.02 ± 0.30	1.22 ± 0.58	1.41 ± 0.57 [a]	0.19 ± 0.30
ALT (Ukat/L)	0.31 ± 0.11	0.32 ± 0.12	0.01 ± 0.10	0.42 ± 0.35	0.38 ± 0.37	−0.04 ± 0.45
UA (μmol/L)	356 ± 65	332 ± 68 [a]	−24 ± 46	321 ± 72	310 ± 80	−11 ± 35
CRP (mg/L)	1.65 ± 1.14	1.84 ± 1.45	0.19 ± 1.03	1.38 ± 0.88	1.28 ± 0.59	−0.10 ± 0.92

Abbreviations: BMI, body mass index; GLU, glucose; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TAG, triacylglycerols; ALT, alanine aminotransferase; UA, uric acid; CRP, C-reactive protein; HE, erinacine.
A-enriched *H. erinaceus* supplementation; P, placebo.
[a]Denotes a significant ($p < 0.05$) difference when compared with the initial value within the group using a Student's paired samples *t*-test or Wilcoxon signed-rank test.

Table 3
Cognitive and neurotrophic markers, and CHI3L1 concentrations (means ± standard deviations) at baseline and after intervention (8 week).

	HE group (N = 18)			P group (N = 15)		
Measure	Baseline	Week 8	Difference	Baseline	Week 8	Difference
THP score	20.3 ± 3.3	21.5 ± 5.0	1.2 ± 4.1 [†, #]	19.8 ± 3.5	19.7 ± 6.3	−0.1 ± 4.6
TN score	15.4 ± 4.1	16.1 ± 3.9	0.7 ± 4.0 [#]	17.9 ± 3.2	18.2 ± 3.4	0.27 ± 4.0
BDNF [pg/mL]	7.47 ± 4.86	8.49 ± 3.28	1.02 ± 3.38	9.29 ± 3.65	7.01 ± 2.85[a]	−2.28 ± 4.16[b]
NPY [pg/mL]	9.67 ± 4.36	11.10 ± 6.84	1.43 ± 5.52 [#]	13.17 ± 7.76	13.35 ± 5.92	0.19 ± 6.75
CHI3L1 [ng/mL]	1.96 ± 3.15	4.36 ± 6.7	2.40 ± 3.56	4.53 ± 6.93	3.66 ± 5.04	−0.87 ± 1.89

Abbreviations: THP, Test of Perception Speed Pattern; TN, Test of series; BDNF, Brain-Derived Neurotrophic Factor; NPY, Neuropeptide Y; CHI3L1, Chitinase 3 like-1; HE, erinacine A-enriched *H. erinaceus* supplementation; P, placebo.
[a]Denotes a significant ($p < 0.05$) difference when compared with the initial value within the group using a Student's paired samples *t*-test or Wilcoxon signed-rank test.
[b]Denotes a significant ($p < 0.050$) difference in changes between the *H. erinaceus* and placebo groups. Changes were calculated by subtracting the initial values from the final values.
[(†)] Calculated from ANCOVA, adjusting for baseline values, and stratified for age and sex.
[(#)] Calculated from ANCOVA, adjusting for baseline values and levels of CHI3L1, and stratified for age and sex.

1 %, there were no statistically significant differences detected between the HE and P groups. However, a difference in *Flavobacteriaceae* family ($p = 0.003$) was observed between the groups after the intervention. As gut microbiota composition is influenced by a set of various factors, the effect of HE supplementation was valued also regarding age and gender of study participants. Accordingly, there was a statistically significant higher abundance of *Actinobacteria* ($p = 0.023$) and *Bacteroidetes* ($p = 0.016$) phyla after the intervention in women.

The Shannon diversity index per group at the genus level is shown in Table 4. The Shannon index significantly increased after the period of HE supplementation ($t = 1.84$, $p = 0.042$), whereas a significant decrease in the P group ($t = -2.16$, $p = 0.025$) was observed. At baseline, Shannon index did not differ between the groups ($p = 0.098$). However, changes after the intervention period were significantly different ($t = 2.84$, $p = 0.008$). Furthermore, the increase in diversity was positively correlated to higher levels of NPY ($r = 0.59$, $p = 0.012$).

4. Discussion

In this double-blind, placebo-controlled clinical trial we aimed to investigate the effects of erinacine A-enriched HE supplement on cognitive function, serum biochemical markers, faecal levels of CHI3L1, and gut microbiota composition in healthy adults (aged 62.9 ± 7.1). To emphasize, there were no significant differences between the groups in terms of age, anthropometric, and biochemical parameters (Table 1, 2). In the light of available information, this is the first study revealing diverse range of effects after the *H. erinaceus* supplementation. During the 8-week intervention, participants supplemented their normal diet with a daily intake of 3.44 mg of erinacine A.

We hypothesised that the administration of HE exerts a positive effect on the rapid and accurate processing of information in the brain. The speed of information processing serves as an indicator of central nervous system efficiency and represents a fundamental aspect of intellect and cognitive function, particularly in the terms of memory efficiency. In this study, the applied cognitive test also includes perceptual speed, i.e., the ability to perceive shapes in the field of view quickly and accurately, involving sensory and motor processes as well as various types of cognitive operations, such as comparison, substitution, or transformation. The results revealed beneficial effects on cognitive performance observed as improved perceptual speed in the HE group, when adjustments for age, gender, and baseline THP score were

Table 4
Gut microbiota diversity at baseline and after intervention (8 week).

	HE group (N = 17)			P group (N = 15)		
	Baseline	Week 8	Difference	Baseline	Week 8	Difference
Shannon diversity index	2.75 ± 0.45	2.88 ± 0.34[a]	0.13 ± 0.28	2.99 ± 0.31	2.82 ± 0.44[a]	−0.16 ± 0.30[b]

Abbreviations: HE, erinacine A-enriched *H. erinaceus* supplementation; P, placebo.
[a]Denotes a significant ($p < 0.05$) difference when compared to the initial value within the same group.
[b]Denotes a significant ($p < 0.050$) difference in changes between the *H. erinaceus* and placebo groups. Changes were calculated by subtracting the initial values from the final values.

considered, that is the way information is organised, interpreted, and consciously experienced. Interestingly, *H. erinaceus* has been previously reported to promote recovery of the sensory function after crush injury and improve peripheral nerve regeneration (Wong et al., 2015).

The improvements in cognitive function observed in the HE group could be associated to altered serum levels of BDNF. In a recent study on elderly hearing-impaired patients (Chan et al., 2022), BDNF levels were elevated by the consumption of *H. erinaceus* that was however more effective for patients aged ≧ 65. It is known that BDNF is highly expressed in the hippocampus (Bathina & Das, 2015), a region critical for the maintenance of the cognitive function (Luo et al., 2017). One of the most empowering results about BDNF is its potent effects on synapses, making it an ideal and essential regulator of cellular processes that underlie cognition, playing a key role in modulating synaptic transmission and plasticity (Bekinschtein et al., 2007). Thus, as BDNF is one of the key molecules modulating brain plasticity and promoting axonal and dendritic growth, the altered BDNF levels in the HE group could underlie the changes in structural connectivity detected in cognitive testing. Increased BDNF levels, which were observed in the HE group, but not in the P group in our study, could support the proposed neurotropic and neuroprotective effects (Venturella et al., 2021) of the bioactive metabolites of *H. erinaceus*. Researchers found that the hericenones and erinacines present in *H. erinaceus* can easily cross the blood–brain barrier, and they have been reported to stimulate the production of trophic factors, such as BDNF (Chiu et al., 2018; Rupcic et al., 2018). Erinacine A has been shown to increase the expression of signalling proteins, including NGF and BDNF, in the central nervous system in animal models (Shimbo et al., 2005). In a recent pilot study (Vigna et al., 2019) involving seventy-seven volunteers who suffered from overweight or obesity and had one or more mood disorders, BDNF levels were tested before and after two months of daily supplementation with *H. erinaceus*. An increase in circulating pro-BDNF levels was observed. As reviewed by Chong et al., 2021, based on neurotrophic and neurogenic pathophysiology of disease, *H. erinaceus* may be a potential alternative medicine also for the treatment of depression. Results of experiments on animal models have also revealed that *H. erinaceus* supplementation increased neurogenesis in the dentate gyrus of the hippocampus (Brandalise et al., 2017) and improved hippocampus-dependent recognition memory with enhancement of neurotransmission and neurogenesis in the hippocampus and cerebellum (Ratto et al., 2019).

The expression of neurotropic factors, including BDNF, has been shown to be regulated by NPY (Wirth et al., 2005) and conversely, BDNF overexpression can significantly increase NPY mRNA and protein levels (Croce et al., 2013). This suggests a cooperative relationship between BDNF and NPY in regulating the nutritional microenvironment and supporting neuronal development and survival. In the present 8-week intervention with a daily intake of HE supplement, no significant effect on serum levels of NPY was observed. However, subsequent analyses revealed that the intervention had effect on cognitive performance and NPY levels, accounting for corresponding values at baseline, age, gender, and faecal CHI3L1 levels. Therefore, we hypothesise that higher levels of faecal CHI3L1 could lead to a more pronounced effect of supplementation due to the localisation of chitinase producing bacteria. To our knowledge, this is the first study investigating the effect of HE intervention in relation to faecal CHI3L1 concentration. Until recently it was generally considered that due to the natural absence of chitin, chitinases are lacking in mammalian cells. However, the presence of mammalian chitinases and chitinase-like proteins including CHI3L1 (also known as YKL-40 or human cartilage glycoprotein 39 (HCgp-39)) was observed (Hakala et al., 1993), whereas the exact mechanisms and functions of the protein in the human gut are still being studied and are not yet fully understood. CHI3L1 is a member of glycoside hydrolase family 18 and is expressed by a multitude of cells, including colonic epithelial cells (CECs), macrophages, and neutrophils (Krause et al., 1996; Mizoguchi, 2006; Renkema et al., 1998; Volck et al., 1998). Faecal CHI3L1 has been recognised as one of the intestinal inflammatory markers in relation to inflammatory bowel disease, with a cut-off value of 13.7 ng/g to differentiate healthy individuals (Aomatsu et al., 2011). In our study, there was no evidence of inflammation before or after the intervention, as indicated by mean faecal CHI3L1 of 4.03 ± 5.91 ng/mL and 3.16 ± 5.34 ng/mL, respectively. Although CHI3L1 lacks enzymatic activity it possesses a binding ability to chitin and chito-oligosaccharides (Fusetti et al., 2003). It has been shown that chitinase-producing bacteria produce chitin-binding molecules such as CBP21, that can be involved in the bacterial adhesion to CECs (Vaaje-Kolstad et al., 2005). Moreover, CHI3L1 can enhance the CBP21-mediated bacterial adhesion to CECs *in vitro* (Kawada et al., 2008). We assume that a slight increase in CHI3L1 that was detected in the HE group after the intervention (Table 4) could promote the binding and accumulation of chitin-degrading commensal bacteria. The degradation of chitin in *H. erinaceus* cells could increase the bioavailability of compounds and thus pronounce the effect of intervention on cognitive function. However, further research is needed to better understand the exact role and mode of action of CHI3L1 and interaction to gut microbiota during the intervention.

Furthermore, edible mushroom polysaccharides (EMPs) are considered as prebiotics as they can be selectively fermented by colonised microbiota in the gastrointestinal tract (Ma et al., 2021). The potential regulation effects of EMPs of different origin on gut microbiota was shown, e.g., decreased *Firmicutes/Bacteroidetes* ratio and enriched faecal microbiota diversity (Zhao et al., 2019). The effect of *H. erinaeus* on microbiota was previously tested *in vitro* (Mitsou et al., 2020), using animal models (Diling et al., 2017; Yang et al., 2021), and to our knowledge in only one short-term pilot clinical study (Xie et al., 2021). As shown by Xie et al. (2021), short-term supplementation of *H. erinaeus* increased the alpha diversity and relative abundance of the SCFAs producing bacteria, and downregulated some pathobionts. Despite our expectations, only few changes in the gut microbiota composition were observed after the intervention. This could be attributed to the study duration while the results of the short-term dietary interventions in humans showed that alternations in gut microbiota are transient and do not persist (David et al., 2014; Leeming et al., 2019). Due to the variety of factors influencing the gut microbiota composition, participants in our study were advised not to change eating, sleeping, or activity habits. However, the effect of HE supplementation on gut microbiota composition was shown to be influenced by age and gender of study participants. These results indicate the importance of considering the influence of multiple factors that can significantly impact the composition of the microbiota. After the intervention, the statistically significant differences in the HE group were observed only for three genera with a relative abundance of less than 1 %. Notably, after the intervention a higher abundance of *Flavobacteriaceae* family inside the HE group and also when comparing the HE group to the P group has been detected. Because members of *Flavobacteriaceae* family have been reported to digest insoluble chitin and many other polysaccharides (Kharade & McBride, 2014) the higher relative abundance of this family in the HE group could be connected to higher release of bioactive compounds and possibly improving the effect on cognitive parameters. Moreover, it was shown that the increase in relative abundance of the *Flavobacteriaceae* family was associated to normal body weight and a healthy metabolic profile, thus indicating a good metabolic health (Palmas et al., 2021). Gut microbiota diversity had previously been linked to cognitive function in older adults (Canipe et al., 2021), but other benefits, such as enhanced nutrient utilisation and a more effective immune system, were shown. Following the intervention, a statistically significant difference ($t = 2.84$, $p = 0.008$) in bacterial diversity between the groups was observed. Accordingly, the higher diversity was positively correlated to higher levels of NPY in the HE group.

However, the relatively small sample size in the present study may be considered a limitation. We also note that our sample of participants reflected the sex imbalances inherent in each group. The lack of greater

homogeneity within our sample could have a negative impact on the validity of the results obtained from our sample. Due to the presence of wide range of bioactive compounds in mushrooms the results may also be more relevant if placebo capsules contained the same material as experimental group, without erinacine A. In addition, the daily intake of supplements was self-administered, so the possibility of non-adherence and misreports of intake could not be excluded. Finally, it remains unclear why serum BDNF levels decreased in the P group after the intervention. The significant decrease in BDNF levels in the P group may have several explanations, including stress, poor diet, genetics, environmental factors and physical activity. One possible explanation is seasonal variation, as the first measurements were taken in spring/summer and the second in autumn. There is evidence that BDNF levels fluctuate with the seasons, increasing in spring/summer and decreasing in autumn/winter. This could be due to the fact that sun exposure influences vitamin D production, which is known to be associated with BDNF. Sun exposure also influences circadian rhythms, which affects neurotrophic factors. In addition, there are conflicting studies on the effects of physical activity on peripheral BDNF levels, although the mechanisms are unclear. Further research is needed to fully understand the relationship between intervention and BDNF levels.

5. Conclusion

As the elderly population increases, it is important to understand the mechanisms by which cognitive aging can be prevented, alleviated, or treated. These study results contribute to our understanding of the multiple effects of *H. erinaceus* supplementation and provide insight into its potential preventive effect in cognitive health. In this study, we also tried to find a relationship between the active ingredients of *H. erinaceus* and cognitive parameters, pointing to CHI3L1 activity as a possible mechanism for higher bioavailability of the active ingredients from mushrooms. The observed improvements in cognitive function and changes in circulating BDNF levels support the proposed neurotropic and neuroprotective effects of the bioactive metabolites present in *H. erinaceus*. However, to further support these effects of HE it is important to study the underlying neuroprotective mechanism, gene expression and proteins, and the effects of this medicinal mushroom on neuroplasticity. The addressed limitations will be taken into consideration and mitigated when preparing future study designs.

CRediT authorship contribution statement

Maša Černelič Bizjak: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Zala Jenko Pražnikar:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Saša Kenig:** Writing – review & editing, Investigation. **Matjaž Hladnik:** Writing – review & editing, Investigation. **Dunja Bandelj:** Writing – review & editing, Investigation. **Andrej Gregori:** Writing – review & editing, Conceptualization. **Katja Kranjc:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ethics statement

This study was conducted in accordance to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the Slovenian National Medical Ethics Committee (No. 0120-321/2017-4). The protocol for the present clinical study was registered at Clinicaltrials.gov (NCT 04939961).

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Appendix A. Supplementary data

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