STUDY PROTOCOL AND STATISTICAL ANALYSIS PLAN

Study title: A Randomized Controlled Trial to Investigate the Cognitive, Mood, Anti-inflammatory and Metabolic Effects of Chronic Oyster Mushroom Intervention in Older Adults.

Acronym: OYSCOG

Date: 01 October 2023

The Pleurotus oyster species is a common edible mushroom rich in ergothioneine, a bioactive compound with known neurocognitive benefits. The aim of the OYSCOG is to investigate the chronic effect (12-weeks) of the equivalent 1 portion of ergothioneine-rich Pleurotus oyster mushrooms (in dried powdered form) on cognition and mood, with examination of the potential inflammatory, metabolic, and neurological related mechanisms that may underlie these effects.

1. Sample Size Calculation

A power calculation based on similar research investigating the chronic benefits of other mushroom interventions on cognitive function suggests that 72 participants should give sufficient statistical power (with a=0.05, beta=0.80, cohen's d=0.60). This calculation was based on the average cohen's d value obtained from 7 RCTs examining the effect of Lion's Mane (Mori et al., 2009; Saitsu et al., 2019; Li et al., 2020; Grozier et al., 2022), Reishi mushroom (Tsuk et al., 2017; Wang et al., 2018) or vitamin D enriched mushroom (Zajak et al., 2020) on global cognitive performance.

To allow for a 10% attrition rate, 80 healthy older adults aged 60-80 years old will be recruited primarily from the University of Reading Ageing Research Panel and the local community. Given the parallel design of the RCT, the cohort will be half split to receive either the control intervention (N=40) or the oyster mushroom intervention (N=40).

2. Recruitment and Screening

Participants will be initially recruited using opportunity sampling. The study will be advertised via email and posters will be placed around public areas (eg university area, sports hall, library, churches, parkruns, GP practices). Furthermore, participants will be recruited from the Age UK Berkshire and the local community.

Before interested participants are enrolled in our study, they will be invited to attend a familiarisation session to our unit, to determine if they meet the eligibility criteria and they will be given an opportunity to become familiar with the cognitive task battery to reduce the influence of practice effects. Participants will be compensated with £100 for completing all study visits.

3. Inclusion/Exclusion criteria

Inclusion criteria

-Aged between 60-80 years old

-Have normal vision and hearing

-Have healthy status

-Have normal body mass index (cutoff: BMI<=30)

Exclusion criteria

-Smokers

-Vegans/vegetarians

-Being diagnosed with psychiatric/neurological condition (eg., stroke, schizophrenia, depression, cognitive impairment, dementia)

-Being diagnosed with a learning/behavioural disorder (eg., dyslexia, autism, ADHD)

-Being diagnosed with a metabolic disease (eg., type I/II diabetes and cardiovascular disease), or suffer from unmediated hypertension or thrombosis related disorders

-Being anaemic

-Taking disease medication such as anticoagulants, antiplatelet medication, dementia medication, antidepressants, antiepileptic medication, or thyroid medication

-Currently taking regular vitamin supplements (including prebiotics/probiotics)

-Have food allergies

-Having a difficulty in completing computer-based cognitive tasks

4. Randomisation Process

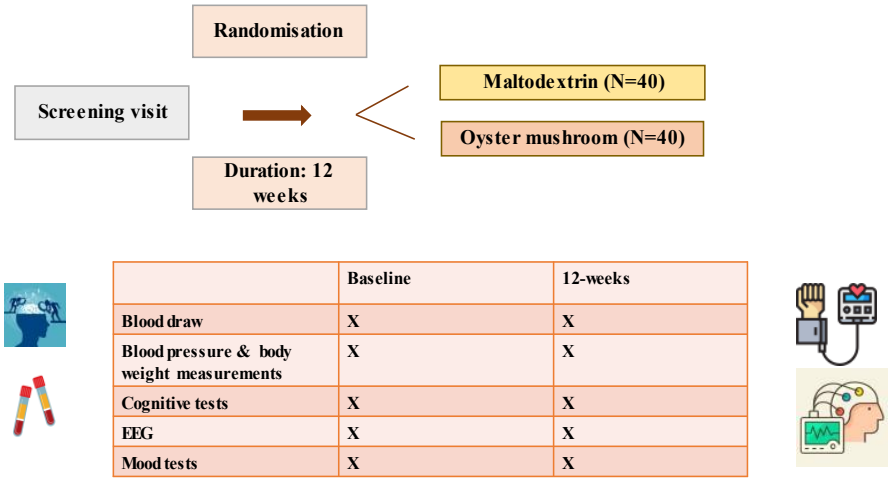
The study will be double blinded since neither the participants nor the researcher will know which intervention meal participants are receiving until the study is over. To achieve this, the Latin Square design will be followed, by using blocked randomisation with random permuted blocks of two. Another individual not being involved in this study, will assign a code to the placebo and mushroom intervention and these will be kept in a sealed envelope, concealed to the researcher enrolling and assessing participants.

5. OYSCOG study Protocol

During this 2-arm parallel double-blind study, participants will attend the University of Reading, Psychology department on three separate occasions; a) a familiarisation visit; b) a baseline visit, a week after the familiarisation visit and c) a post-visit, 12-weeks after the baseline visit.

In the pre-screening phase, the participants interested in our study will be sent a link to REDCap containing online versions of a Health and Lifestyle Questionnaire and the Epic Norfolk Food Frequency Questionnaire (FFQ). Then, participants, will be contacted to attend a 3-hour familiarisation session at our department, during which the participant's weight, height and blood pressure will be checked, along with a finger-prick, to ensure that the participants are not anaemic. Furthermore, participants will complete the Raven's Progressive Matrices (RPM) measure of fluid intelligence and will perform the cognitive battery tasks twice to control for practice effects in the run up to the test session days. Also, the head and cap size measurements will be taken in a subset of participants (N=20 from each experimental group) that agreed to undertake the EEG at the testing visits.

At the end of the familiarisation visit, participants will be allocated to one of the treatment groups and they will receive a 12-week supply of the intervention powder in sachets. After the familiarisation visit, participants will be asked to follow a low flavonoid diet for 24-hours in advance of the two testing days. In both the baseline and post-visit testing sessions, participants will be instructed to perform several cognitive and mood related tasks. In addition to the behavioural tests, blood pressure, heart rate and body weight will be measured, and electroencephalogram (EEG) measurements will be recorded in a subset of participants using electrodes placed on the scalp for detecting electrical brain activity. Finally at the end of each testing visit, a blood sample will be taken. Each test visit will last approximately 2 hours. **Figure 1** summarises the OYSCOG testing protocol.



	Baseline	12-weeks
Blood draw	X	X
Blood pressure & body weight measurements	X	X
Cognitive tests	X	X
EEG	X	X
Mood tests	X	X

Figure 1: Study design of the parallel 2-arm OYSCOG RCT, investigating the chronic effects of ergothioneine-rich Pleurotus mushrooms on cognition, cognition, inflammation, and metabolism.

The cognitive-mood battery used will contain the following tasks:

- Positive and Negative Affect Schedule (PANAS-X)- In this mood task, participants are asked to rate their emotion on a 5-point Likert scale, in response to negative emotions (e.g., guilt, fear), positive emotions (e.g., attentiveness, joy) and other affect states (e.g., surprise, shyness).

- Depression, Anxiety and Stress Scale - 21 items (DASS-21)- This 21-item measure assesses symptoms of depression, anxiety and stress. Each item is scored from 0-3 where higher scores indicate higher levels of distress in anxiety (scores range from 0 to >20), depression (0 to >28) or stress (0 to >34).

- Rey Auditory Verbal Learning Task (RAVLT)- This episodic memory task consists of 5 consecutive free recalls of the same 15 nouns presented as an auditory list (list A), followed by recall of a further 15 nouns presented as an interference list (list B) which is recalled only once. List A is subsequently recalled straight after list B and then again after a longer delay of around 30 minutes.

- Task Switching Task (TST) - In this executive function task, participants view 8 spaced radii of a circle above and below a bold line and a stimulus digit selected from 1-9 (except 5) appears clockwise in each segment, either above or below the bold line. Depending on the stimulus position in the segments, participants perform different tasks with each being switched every 4 trials.

- Corsi block tapping task (CBTT)- In this spatial working memory task, participants are shown 9 arranged blocks positioned on a computer screen and are asked to reproduce a given sequence by clicking on the blocks (using the mouse) in the same sequence as they see them.

- Simple and complex finger tapping task (SFT & CFT)- In this psychomotor function task, participants tap on a key as quickly as possible with the index finger of their dominant hand for 1 minute. They then tap out a specific sequence using 4 fingers, again for 1 minute.

•RAVLT word recognition- In this delayed memory task, participants are shown a sequential list of 50 nouns containing the words from lists A and B from the previously

described RAVLT task plus 20 additional words not previously heard, and are asked to indicate those from list A.

•N-back- In this working memory and attention task, participants are shown a series of stimuli, and they will have to respond to indicate whether the stimuli match the target presented at the beginning of the task. This task will be performed while also taking EEG measurements (using Brain Products software and 16 active electrodes), to examine changes in brain activity.

• Subjective mental fatigue- At the end of the cognitive battery participants are asked to rate their mental fatigue level on a 9-point Likert scale.

6. Statistical Plan

All statistical analyses will be performed by using SPSS software. Initially, extreme outliers will be identified and excluded using boxplots (using 3*IQR rule), and subjects with missing data will be excluded from analysis. The main analysis will use a mixed ANOVA to investigate a) the main effect of time (within subject factor, baseline vs 12-weeks), b) the main effect of intervention (between subject factor, placebo vs oyster mushroom group) and c) the intervention*time interaction. For the outcome measures that show significant changes at baseline, a 1-way ANOVA will be applied by treating the baseline scores as a covariate to examine the main effect of intervention. In all analyses, a Bonferroni correction will be examined for post-hoc pairwise comparisons regardless of the significance of the overall F test statistic. Significant comparisons will be reported (*$p<0.05$, **$p<0.01$, ***$p<0.001$).