STUDY PROTOCOL AND STATISTICAL ANALYSIS PLAN

Study title: A Randomized Controlled Trial to Investigate the Neurological, Inflammatory and Metabolic Effects of Acute Mushroom Intervention in Older Adults.

Acronym: OYSACO

Date: 15 August 2022

1. Sample Size Calculation

A power calculation based on a study with a similar design investigating the acute benefits of bioactive rich interventions on cognitive function in participants aged 60-80 years, suggests that 30 participants should give sufficient statistical power (Bell and Williams, 2019). To allow for a 10% attrition rate, 33 older adults has been agreed to be recruited.

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, GP practices). Furthermore, participants will be recruited from the Age UK Berkshire and the local community and if needed from the university of Reading research ageing panel.

Before interested participants are enrolled in our study, they will be invited to attend a screening and familiarization 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 £200 for completing all study visits.

3. Randomisation Process

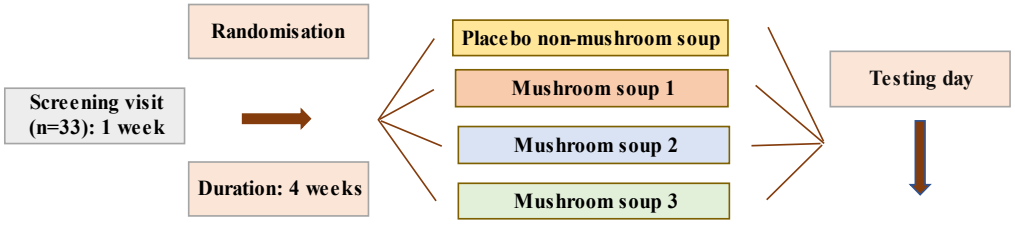
The study will be double blinded since neither the participants nor the researcher will know which intervention meal participants are receiving until the study is over. To achieve this, the Latin Square design (4X4) will be followed, by using blocked randomisation with random permuted blocks of four. Another individual not being involved in this study, will assign a code to each of the four intervention meals and these will be kept in a sealed envelope, concealed to the researcher enrolling and assessing participants. On the testing days, the intervention meal soups will be prepared by research personnel not involved in the conduct of the trial.

4. OYSACO study Protocol

During this study, participants will attend the University of Reading, Psychology department on five separate occasions, with a week interval between each 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 2-hour screening 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.

One week after the screening visit, each participant will attend a further four test sessions, each separated by a week, where they will receive a different dose of freeze-dried oyster mushrooms or a control soup. Participants will be asked to follow a low fibre and flavonoid diet for 48 hours in advance of testing and these four testing days participants have to remain to our unit from 08:00 a.m.- 16:00 p.m. **Figure 1** summarises the OYSACO testing protocol.



	Pre-visit	Post-visit					
	07:00 a.m.	08:00 a.m.	09:00 a.m.	11:00 a.m.	12:00 a.m.	13:00 a.m.	15:00 a.m.
Satiety measure		X	X	X		X	X
Breakfast	X						
Blood draw							X
Cognitive & Mood tests		X		X		X	X
Intervention meal			X				
Lunch					X		
Palatability measure			X				

Figure 1: Study design of the 4-arm OYSACO RCT, investigating the dose-response effects of an acute diet containing ergothioneine-rich Pleurotus mushrooms on the cognitive, metabolic and inflammatory function in healthy older adults.

(*) For the soup interventions, the Pleurotus mushrooms will be in a powdered form at varying doses (equivalent to 0.5 (soup 1), 1 (soup 2) and 2 (soup 3) mushroom servings (with 1 serving~8g powdered mushrooms).

5. Statistical Plan

The present study aims to investigate the beneficial effects of a meal containing *Pleurotus* oyster mushrooms on neurocognitive performance (objective I) and improvements in the blood serum measures of inflammation and metabolism (objective II) in the acute postprandial period (6-hours post-consumption) in older adults aged 60-80 years old.

Participant sample characteristics will be tabulated using descriptive statistics, using SPSS software. Parametric tests will be used throughout having first confirmed that the test data meet the required assumption of normality. Statistical analyses of the cognitive measures will be performed using SPSS linear mixed modelling (LMM), employing an unstructured covariance matrix to model successive repeat measurements. LMM will be applied to model variance relating to both fixed parameters (eg experimental meal) and random parameters such as differences between participants. The analysis will involve the creation of both unadjusted models and adjusted models by controlling for baseline variables such as sex, fruit and vegetable consumption, physical activity level and health status. Baseline performance will be included as a repeated covariate. Subjects will be included as a random factor.

In the primary analysis of the data in each cognitive task, the Visit Order (Visit), Time after receiving the experimental meal (Time), experimental meal (Intervention) and Time X Intervention interaction will be included as fixed factors in the model. A composite score will be also calculated by the summation of the z scores obtained from each cognitive task, to examine the effect of the intervention meal on the overall cognitive performance.

The data obtained from the metabolic and inflammatory markers will be checked for normality before using parametric statistical tests. The blood serum markers obtained

following the intake of the control meal will be compared with the blood serum markers obtained following the intake of the three mushroom containing soups. The SPSS LMM method will be employed, and the data analysis will be similar to the one performed on the cognitive data without the 'Time' being a fixed factor in the model, since the blood sample will be collected at one time point (6 hours post-intervention). Finally, a correlation will be made for the blood biomarkers and cognitive test scores at the 6-hour post-intervention time point.

References

Bell, L., Williams, C.M. (2019). A pilot dose–response study of the acute effects of haskap berry extract (Lonicera caerulea L.) on cognition, mood, and blood pressure in older adults. *Eur J Nutr* 58, 3325–3334. https://doi.org/10.1007/s00394-018-1877-9