**Cover page with official title: A supplement on cognitive function and brain activity in middle age and older healthy adults (ID, INSERT)**

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## 1) Introduction

Nowadays, the oral use of probiotics is widespread, in foods (i.e., yogurt), drinks (i.e., kombucha) and supplements. Heat-treated probiotics (essentially pasteurised or killed), cell-free supernatants, and purified key components can confer beneficial effects, mainly immune effects, protection against bacterial infections, and maintenance of gut health, which can positively impact on mental health and cognitive ability. Post-biotics, as they are called, have an advantage for food industry applications as they can easily be supplemented in several food lines/products and are shelf stable. PoZibio™ capsules contains the probiotics *Lactobacillus paracasei* which is a species of lactic acid bacteria often used in the fermentation of dairy products. It's found in the human intestinal tract and mouth, but also in foods such as yogurt and naturally fermented vegetables and milk. This has been heat-killed in PoZibio™.

Understanding whether cognition, in middle aged and older healthy adults, may be positively affected by the oral consumption of postbiotics, is important for helping us better understand the neuroprotective and enhancing properties of postbiotics and their future implementation into food and supplements. Electroencephalography (EEG) is a low cost, non-invasive method to record an electrogram of the spontaneous electrical activity of the brain. The bio signals detected by EEG have been shown to represent the postsynaptic potentials of pyramidal neurons in the neocortex and allocortex. It shall be combined with 3 psychological tasks (Stroop, Go/No-Go, and Flanker), to measure a variety of cognitive domains including attention, processing speed, accuracy, and response inhibition (Davidson et al., 2003; Feil et al., 2003; Redick et al., 2011). An eyes open/closed task will obtain baseline measurements for resting state. The Mini Mental State Exam (MMSE) questionnaire and geriatric depression scale (GDS) shall be used as screening tools to assess global cognitive function and depressive symptomology in participants prior to taking part. The EQ-5D questionnaire shall be used to obtain an overall profile of the health state and quality of life of participants.

We will collect venous blood samples (3 x 4.5mL) for the investigation into the chemical composition using metabolomics at AberInnovation and Aberystwyth University, the quantification of short box chain fatty acids as well as clinical biochemistry (Chem21 to include lipid panel tests) at Bronglais hospital.

## 2) Statement of Purpose

A randomised, placebo controlled parallel human clinical trial of heat-treated *Lactobacillus paracasei* (post-biotics) in healthy middle aged and older subjects is proposed, to assess the potential for clinically relevant benefits in terms of cognitive function. We are aiming to recruit a cohort (n = 30) of middle aged and older adults (>50 years) who will be randomised into PoZibio™ (2 x capsules daily) or placebo (2 x capsules daily) supplementation for 6 weeks. Subjects will be asked to take both capsules in the morning with their breakfast. The placebo will be matched to the active product by taste and texture.

## 3) Investigational Product

### 3.1) Description

The investigational product will contain a capsule with heat-treated *Lactobacillus paracasei* (inactivated cells count 50 billion counts (50 x 10^9 CFUs/ capsule)). The format will be a

167 mg capsule bottle containing 60 capsules. Subjects will be asked to take the 2 capsules daily. The placebo will be matched to the active product by taste and texture.

### 3.2) Quality

This product is free of the following components and their products thereof: cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk (including lactose), nuts, celery, mustard, sesame seeds, sulphur dioxide and sulphite, lupin and molluscs, in compliance with Regulation (EC) No. 1169/2011. This product does not contain added colorants. Cultures are Kosher, Halah approved. The supplements are vegan, and they are produced and stored in a food grade facility. The freeze-dried culture is packaged inside waterproof and airproof pouches, consisting of three layers (in order, going inwards): polyester, aluminium, and polyethylene. The packaging material used is food grade.

### 3.3) Dose

The dose of investigational product and placebo will be 2 x 1 ml capsules daily.

## 4) Investigational Product Safety

The species *Lactobacillus paracasei* is included in EFSA's Quality Presumption of Safety (QPS) list. Total inactivated cells count > 1.5E+11 cells/g. The microbial strains are not genetically modified (GMO), in accordance to the European Directive 2001/18/EC. This product is not hazardous. Sacco S.r.l. is ISO 22000 and FSSC 22000 (food safety) certified since 2014. This product is considered safe with respect to bovine spongiform encephalopathy (BSE) or transmissible spongiform encephalopathies (TSEs) transmissions in accordance to Regulation EMA 410/01 rev. 3.

## 5) Study Design

### 5.1) Objectives of the Study

#### 5.1.1) Primary Outcome Measure:

- Cognitive Control (Selective attention, processing speed, mental flexibility)

Cognitive Control (Selective attention, processing speed, mental flexibility) measured using the Stroop task in E-Prime
[Time Frame: Improved score (faster response time and improved accuracy) from baseline score at 6 weeks after pozibio]

- Cognitive Control (Selective attention, processing speed, mental flexibility)

Cognitive Control (Selective attention, processing speed, mental flexibility) measured using the Stroop task in E-Prime
[Time Frame: Improved score (faster response time and improved accuracy) at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Response inhibition (core construct in cognitive control and self-regulation)

Measured using the Go/No-go task in E-Prime
[Time Frame: Improved (fewer commission errors) score from baseline score at 6 weeks after pozibio]

- Response inhibition (core construct in cognitive control and self-regulation)

Response inhibition (core construct in cognitive control and self-regulation) measured using the Go/No-go task in E-Prime
[Time Frame: Improved (fewer commission errors ) score at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Selective attention and response inhibition (core constructs in cognitive control and self-regulation)

Selective attention and response inhibition (core constructs in cognitive control and self-regulation) measured using the Flanker task in E-Prime
[Time Frame: Improved score (faster response time and improved accuracy) from baseline score at 6 weeks after pozibio]

- Selective attention and response inhibition (core constructs in cognitive control and self-regulation)

Selective attention and response inhibition (core constructs in cognitive control and self-regulation) measured using the Flanker task in E-Prime
[Time Frame: Improved score (faster response time and improved accuracy) at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Electroencephalogram (EEG) during the Stroop task

Assessing event related potentials (ERP's) in the P3 component and the N2 component across the frontal and parietal regions
[Time Frame: After 6 weeks of pozibio, no delay of the P3 component and more N2 components when compared with baseline]

- Electroencephalogram (EEG) during the Stroop task

Assessing event related potentials (ERP's) in the P3 component and the N2 component across the frontal and parietal regions
[Time Frame: After 6 weeks of pozibio, no delay of the P3 component and more N2 components when compared with 6 weeks of placebo]

- Electroencephalogram (EEG) during the Flanker task

Assessing event related potentials (ERP's) in the P3 component and the N2 component across the frontal and parietal regions
[Time Frame: After 6 weeks of pozibio, no delay of the P3 component and more N2 components when compared with baseline]

- Electroencephalogram (EEG) during the Flanker task

Assessing event related potentials (ERP's) in the P3 component and the N2 component across the frontal and parietal regions
[Time Frame: After 6 weeks of pozibio, no delay of the P3 component and more N2 components when compared with 6 weeks of placebo]

- Electroencephalogram (EEG) during the go/no-go task

Assessing event related potentials (ERP's) in the P3 component and the N2 component across the frontal and parietal regions
[Time Frame: After 6 weeks of pozibio, no delay of the P3 component and more N2 components when compared with baseline]

- Electroencephalogram (EEG) during the go/no-go task

Assessing event related potentials (ERP's) in the P3 component and the N2 component across the frontal and parietal regions

[Time Frame: After 6 weeks of pozibio, no delay of the P3 component and more N2 components when compared with 6 weeks of placebo]

- Electroencephalogram (EEG) during the stroop, Flanker and go/no-go tasks

Assessing alpha and delta activity
[Time Frame: After 6 weeks of pozibio, increased alpha and delta activity when compared with baseline]

- Electroencephalogram (EEG) during the stroop, Flanker and go/no-go tasks

Assessing alpha and delta activity
[Time Frame: After 6 weeks of pozibio, increased alpha and delta activity when compared with 6 weeks of placebo]

- EuroQol 5 Dimension 5L

EuroQol 5 Dimension 5 5L questionnaire: Generic quality of life. Mobility- Level 1-5 Self-Care- Level 1-5, Usual Activities- Level 1-5, Pain/Discomfort- Level 1-5, Anxiety/Depression- Level 1-5, EQ-VAS: Numerical value between 0-100 representing how the patient perceives their overall health to be.
[Time Frame: Reduced score from baseline EuroQol 5 Dimension 5 score at 6 weeks after pozibio]

- EuroQol 5 Dimension 5L

EuroQol 5 Dimension 5 5L questionnaire: Generic quality of life. Mobility- Level 1-5 Self-Care- Level 1-5, Usual Activities- Level 1-5, Pain/Discomfort- Level 1-5, Anxiety/Depression- Level 1-5, EQ-VAS: Numerical value between 0-100 representing how the patient perceives their overall health to be.
[Time Frame: Reduced EuroQol 5 Dimension 5 score at 6 weeks after pozibio when compared with placebo after 6 weeks]

### 5.1.2) Secondary Outcome Measures:

- C-reactive protein

C-reactive protein in plasma as a marker of inflammation
[Time Frame: Reduced C-reactive protein from baseline at 6 weeks after pozibio]

- C-reactive protein

C-reactive protein in plasma as a marker of inflammation
[Time Frame: Reduced C-reactive protein at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Kidney function tests

Kidney function tests including sodium/potassium/creatinine/urea
[Time Frame: Reduced Kidney function tests from baseline at 6 weeks after pozibio]

- Kidney function tests

Kidney function tests including sodium/potassium/creatinine/urea
[Time Frame: Reduced Kidney function tests at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Total Cholesterol

Total Cholesterol
[Time Frame: Reduced total Cholesterol from baseline at 6 weeks after pozibio]

- Total Cholesterol

Total Cholesterol
[Time Frame: Reduced total Cholesterol at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Total Triglyceride

Total Triglyceride
[Time Frame: Reduced total Triglyceride from baseline at 6 weeks after pozibio]

- Total Triglyceride

Total Triglyceride
[Time Frame: Reduced total Triglyceride at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Liver function tests (LFTs)

Aspartate aminotransferase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Gamma-glutamyltransferase (GGT), Prothrombin time (PT), Albumin,
[Time Frame: Reduced Liver function test from baseline at 6 weeks after pozibio]

- Liver function tests (LFTs)

Aspartate aminotransferase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Gamma-glutamyltransferase (GGT), Prothrombin time (PT), Albumin,
[Time Frame: Reduced Liver function test results at 6 weeks after pozibio when compared with placebo after 6 weeks]

- Changes in short chain fatty acids concentrations in plasma

Changes in short chain fatty acids concentrations in plasma measured using Gas Chromatography-Flame Ionization Detection
[Time Frame: Increased concentration of total short chain fatty acids after the pozibio at 6 weeks compared with the baseline]

- Changes in short chain fatty acids concentrations in plasma

Changes in short chain fatty acids concentrations in plasma measured using Gas Chromatography-Flame Ionization Detection
[Time Frame: Increased concentration of total short chain fatty acids after the pozibio at 6 weeks compared with that after placebo at 6 weeks]

### 5.2) Subject Selection

- 30 participants, over 50 years, mixed gender, mixed ethnicity.

- **Inclusion Criteria:**
  - Subjects over 50 years of age.
  - Subjects with Mini-Mental State Exam (MMSE) of 25-30 inclusive (global cognitive function).Subjects who are able to undergo EEG and to commit to visits to WARU/P5.
  - Subjects who are able to provide venous blood samples.
  - Subjects able to provide written informed consent PRIOR to performing any study procedures.

- **Exclusion Criteria:**
  - Subjects with diagnosis of Alzheimer's disease or other dementia.
  - Subjects taking medication for the treatment of dementia (such as acetylcholinesterase inhibitors (Aricept, Excelon), memantine (Namenda) or other medications with similar mechanisms of action) or medical foods (such as Cerefolin, Souvenaid, Axona) for the treatment of dementia.
  - Subjects who are already regularly taking probiotics, post-biotics, nutraceutical and/or vitamin supplements related to *PoZibio* ™ within 30 days of screening.
  - Subjects with Geriatric Depression Scale > 6.
  - Subjects with a Mini Mental State Exam score below 25.
  - Subjects who are pregnant or lactating.
  - Subjects with medical condition or disease that is life threatening.
  - Subjects who smoke cigarettes or use other products containing nicotine.
  - Subjects diagnosed with diabetes.
  - Subjects taking warfarin.
  - Subjects who have a diagnosed or suspected mental health condition, or who have any concerns surrounding their mental health.
  - Subjects who have immediate family members with diagnosed mental health condition or suspected mental health concerns.

- **Covid Exclusion criteria**
  - Showing (or anyone within the household) any COVID-19 symptoms (see COVID-19 basic health screen)*
  - Higher risk or vulnerable from coronavirus or live with someone at a higher risk of a severe illness from COVID-19 (over 70, undergoing cancer treatment, high risk of getting infections).
  - Had a letter from the NHS advising you to shield (isolate).

- Had been at risk of exposure to COVID-19 such as travel, contact with someone with COVID-19, been exposed to the virus, or has been asked to self-isolate by the track and trace system.
- Serious health conditions that require daily long-term medication.

*If the potential participant has had COVID-19 previously (and are fully recovered and not within isolation) then they are eligible to join the study.

### 5.3) Study Design

This is a randomised, placebo controlled, parallel study, where participants are asked to take the capsules (PoZibio™ or Placebo) twice a day for a period of 6 weeks. Participants will be required to make five visits to the Well-being and Health Assessment Research Unit (WARU) and Psychology department at Aberystwyth University; however, several visits can be combined if needed. EEG data analysis will be conducted at Aberystwyth University. Chemical composition using metabolomics will be conducted at AberInnovation and Aberystwyth University, and the quantification of short chain fatty acids as well as clinical biochemistry (Chem21 to include lipid panel tests) will be analysed at Bronglais hospital.

**Pre-Induction**

Participants are welcomed with tea or coffee and talked through how safe working practices are being conducted during coronavirus (COVID-19). Participants are informed that they will be completing the Mini Mental State Exam (MMSE) to assess their overall cognitive function, and the Geriatric Depression Scale (GDS) to assess for any depressive symptomatology, as part of the screening process. They are reminded that these screening questionnaires are not diagnostic and are not intended for clinical assessment purposes; instead, they are to ensure consistency across the participant sample, whereby the inclusion and exclusion scores are taken from pre-existing literature. Participants are informed that the MMSE should take approximately 10 minutes to complete, and the GDS will take approximately 5 minutes to complete, and that they will be told their scores after they have completed both questionnaires. If they meet the:

- **Inclusion Criteria:**
  Their scores will be retained, but their name will be pseudonymised using their randomised, unique code, and only the researchers closely affiliated with the project will have access to their data
- **Exclusion criteria:**
  They will no longer be able to continue with the study, their data will be deleted, and they shall be provided a participant exclusion form with what to do next.

Participants are provided with the chance to ask any questions. If they are happy to continue, they are asked to read and sign the consent form. After screening ends, participants are offered water and the opportunity to use the bathroom whilst their scores are calculated.

**If they meet the inclusion criteria, then:**

- A record of their scores is retained in an excel spreadsheet next to their anonymous ID code.
- They are informed that they have met the inclusion criteria.

- They are thanked for their time and walked to the door.

**If they do not meet the inclusion criteria, then:**

- A note of their scores is made in the participant exclusion feedback form identifying which (or both) of the exclusion criteria they met.
- The participant is talked through the participant exclusion feedback form and provided with the chance to ask questions.

- They are thanked for their time, and if they are happy to leave, then they are walked to the door.
- Their personal data is deleted in compliance with GDPR.

**Testing day 1**

Participants arrive at WARU on their pre-organised day and time after a 12 hour fast for a venous blood draw (3 tubes), followed by tea and toast. They are asked to complete an EQ-5D (5L) questionnaire. Their height and weight, waist, and hip circumference are measured. They are then free to leave, unless they have chosen to combine testing day 1 and 2 together, in which case they are guided to the P5 building where the activities outlined under testing day 2 commence.

**Testing day 2 (can be combined with day 1 if needed)**

If participants opt not to combine testing days 1 and 2 together, then they are met at the main reception and guided to the P5 building, where they are encouraged to go to the toilet prior to testing. They are then taken to P4, where the EEG laboratory is located. They shall then undergo their first EEG and cognitive assessment. They are first asked to participate in an eyes open/closed task for measurements of their EEG resting state. Following this, three cognitive tasks (the Stroop task, the Go/No-go task, and the Flanker task) are digitally completed in a computer program called E-Prime. Practice rounds are provided for each task, with the researcher present, to allow for any questions before each experiment begins. After each task, the EEG program is stopped, and a new data file is created, before recording for the following task takes place. This provides an opportunity in between tasks for a sip of water, or a toilet break, if necessary, although the EEG cap must remain in place, and the nearest accessible toilets are in P5. Altogether, the tasks shall take approximately 60-70 minutes to complete, with an additional 45 minutes allocated for the application of the EEG cap. Participants are encouraged to bring their own bottled drinks, and snacks are made available. They are also granted access to the 'break out' room on the second floor of P5, prior to and following their participation, where there are chairs and tea/coffee machines available. Following their participation, participants are re-directed back to WARU for the collection of their capsules. Participants are reminded before taking part that they will not receive any scores post EEG testing, indicating how well they performed, but that the overall general findings of the study can be made available to them, following the end of the study, by contacting WARU@aber.ac.uk.

**Testing day 3**

After the 6-week supplementation period, participants are asked to complete the activities that they undertook during testing day 2. A staff member is told to meet the participant in the P5 lobby to walk them over to P4. This is the final assessment of EEG and cognitive testing. If participants decide to combine testing days 3 and 4 together, then a staff member is made available to walk participants down to WARU.

Note: All participants are encouraged to try to combine testing days 3 and 4 together (if possible), to ensure that cognitive testing and the venous blood draw occur within the same 48-hour window to when the last capsules of the 6-week trial are taken.

**Testing day 4 (can be combined with day 3 if needed)**
Within 48 hours after testing day 3, still following the supplementation regime, participants are invited to WARU after a 12 hour fast for their final venous blood draw (3 tubes), followed by tea and toast. They are then asked to digitally complete the EQ-5D (5L) and MMSE questionnaires. Their height and weight, waist, and hip circumference measurements are taken. There is an optional feedback questionnaire at the end.

## 6) Participant Risks

The supplements have already been tested for any adverse effects in a human cohort, however if any negative effects occur, participants are asked to refrain from continuing in the study.

EEG is non-invasive, however placing the EEG electrodes along the scalp can be time consuming (approximately 45 minutes), and participants are required to sit relatively still during the application and testing process (the application and testing process combined take approximately 2 hours). The application of the EEG cap requires EEG gel (saline solution) to be applied to the scalp, which makes hair messy upon removal of the cap. Participants are informed that towels will be placed along their shoulders to avoid the solution meeting their clothes, and these towels (in addition to paper towels) are provided to remove any excess gel from their hair. Participants are reminded that hair washing facilities are available in P5 for them to use, but that this would require a short walk from P4 back to P5. Therefore, participants are advised to bring a hat to cover their head for their own comfort. Participants are also reminded that venous blood draws can also cause localised soreness, stress, and dizziness.

## 7) Benefits to participant

There is no financial gain for participants if they decide to join this study. They will allow us to gain important insight into the PoZibio $^{TM}$ supplement to improve cognitive performance in healthy volunteers, which may be applied to other cohorts such as those suffering from vascular dementia and Alzheimer's disease.

## 8) Privacy/confidentiality

Participants are informed that only the researchers involved with the study will be able to look at the information they provide. Specific details and personal identifiers will only be available to the researchers. At the end of the study, any information relating to participants will be made pseudonymous (coded without their name associated). Participants will not be identifiable in any publication that may arise from this research. Electronic files will be kept in a logical manner and will always be kept grouped within specific folders and password-protected.

protected. Files are backed up and all data storage is using the AU network. Once the raw data has been extracted from a paper version onto a computer, the paper will be destroyed via a paper shredder or in confidential waste bags to ensure the participant's confidentiality. There may be times when keeping paper forms are necessary (consent forms), but in this case the paper versions will be kept in a locked filing cabinet. All files that are stored on the WARU share drive will always be protected with a secure password. Setting passwords will automatically encrypt the document and will not allow any unauthorised access to the data. The Gatekeeper of the passwords delivers the passwords by encrypted emails. All biofluids are stored in a locked freezer. Key is kept by the gatekeeper. EEG recordings will be stored on a password protected PC in the EEG lab. Due to the nature of the datafiles and the location of the software, it is not possible to store these files in a password protected folder on OneDrive. The lab PC is password-protected, files are stored under random codes and the PC itself is not connected to any network or the internet. EEG recordings will be removed (by deletion) from the lab PC after five years but may be removed sooner due to disk space issues. Only authorised staff members have access to the EEG lab, and the key for the storage cupboard affiliated with the EEG lab is stored within the locked EEG lab.

## 9) Safety Monitoring

- Participant:
  If a participant, or a member of their family/household become unwell during the study, then they are pre-warned to alert a member of the research team immediately using the contact information they have been provided. Participation in the study will be suspended immediately until further discussion with the research team has taken place. If they become unwell at any point and need medical assistance, they are advised to contact 111 and seek advice from the NHS health sector or their doctor's surgery. We have a duty of care towards them and can help monitor their health remotely over 14 days and will help in any way we can.
- Data:
  As above

## 10) Data analysis and statistics

EEG data analysis will be conducted at Aberystwyth University. Chemical composition using metabolomics will be conducted at AberInnovation and Aberystwyth University, and the quantification of short chain fatty acids as well as clinical biochemistry (Chem21 to include

lipid panel tests) will be analysed at Bronglais hospital. Statistics will be conducted by the Aberystwyth University researchers.