Cover page with official title: The impact of Pozibio and/or Cerbella supplement on the function of the gut, brain, and gut-brain axis, from a physiological and cognitive perspective, when compared with a placebo control in healthy middle-aged and older adults.

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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™.

Omega 3 (Ω-3) polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been shown to improve learning and memory age related cognitive decline in adults. Additionally, ginseng extracts have been shown to improve cognitive performance in healthy volunteers, as well as subjects suffering from vascular dementia and Alzheimer's disease. Numerous studies suggest green tea may confer health benefits due to its pharmacological and biochemical properties. Cerbella™ soft gels contains liquid combination of fish oil (standardised to EPA and DHA), panax ginseng extract (standardised to ginsenosides), green tea extract (standardised to green tea catechins) in a flavoured base of lecithin phospholipids.

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. This electrical communication can be measured in milliseconds, in real-time, giving EEG a high temporal resolution (Mantini et al., 2010). Changes in postsynaptic neural activity become apparent when analysing event-related potentials (ERP's), which is the name given to these changes in the electrical field, and these can be time-locked to sensory, cognitive, and motor events (Hillyard & Anllo-Vento, 1998). EEG technology is used to measure various domains of cognitive function (like attention, memory, mental flexibility and response inhibition; Uddin, 2021; Kiely, 2014), whilst participants undergo different psychological tasks. The tasks chosen for the current study include the Stroop task (Stroop, 1935) and the N-back task (Kirchner, 1958). An eyes open/closed task will also be used to obtain baseline measurements for resting state.

The Stroop task measures the Stroop effect, which is commonly observed as a decreased reaction time, or decreased accuracy, for correctly naming the colour of a word, when the colour is incongruent to the spelling of the word, because words are processed more quickly than colours (Mitchell & Potenza, 2017). The Stroop task is useful for measuring response inhibition and mental flexibility (Li et al., 2017; Bender et al., 2016). Alternatively, the n-back task requires participants to remember a target presented 'n' trials previously, typically requiring participants to respond when: the letter presented matches the letter presented previously (1-back); the letter presented matches the letter presented 2 trials prior (2-back). The n-back task, due to the variable difficulty factor, is used to measure differences in working memory capacity. Typically, ERPs of interest during the Stoop task for the incongruent condition are the N2 (generated by the anterior cingulate cortex, ACC), reflecting conflict monitoring processes (Wang et al., 2021; Heidlmayr et al., 2020); the N400 (generated by the ACC and prefrontal cortex, PFC; Hanslmayr et al., 2008) reflecting interference suppression; and

a larger late positive potential (in the centro-and-temporo-parietal regions; Appelbaum et al., 2009) indicative of conflict detection and resolution (Li et al., 2022; Li et al., 2013; Lansbergen et al., 2007). Alternatively, during the N-back task, the ERP of most interest is the P300 component, with a decreased P300 amplitude typically reflecting increased task difficulty and being more prevalent among older adults (Pergher et al., 2019).

Alongside ERP analysis, band power analysis (which breaks down brain signals into distinct frequency bands like Alpha, Beta, Gamma, Theta, and Delta) will be used to identify differences in attention, task difficulty, and cognitive load (Fink et al., 2005). Alpha and beta activity are thought to reflect attentional processes (Palacios-García et al., 2021; Foxe & Snyder, 2011; Klimesch et al., 1999), and some research suggests that reduced Beta power in older adults can be indicative of mild cognitive impairments (Caravaglios et al., 2016). Information processing and encoding is thought to be characterised by increased Theta activity (Nombela et al., 2014). Pergher et al., (2019) observed increased Alpha and Theta bands in older adults (particularly within the frontal regions) when task difficulty was increased (from 0-back to 3-back during an N-back task), reflecting increased mental load in the harder N-back conditions. During behavioural tasks, congruent and incongruent conditions are believed to produce different changes in beta activity (13-20Hz) within the prefrontal and parietal regions (Nayak & Arayamparambil , 2023; Nombela et al., 2014), demonstrating how spectral analyses of band power can provide insight into cognitive functions like attention, information processing, and memory.

Self-report data pertaining to overall health and wellbeing will be collected pre and post intervention using the following questionnaires: the Gastrointestinal Symptom Rating Scale (GSRS), the 6-Item Short Form Health Survey (SF-36), the Warwick-Edinburgh Mental Health Well-being Scale (WEMWBS), and the Pittsburgh Sleep Quality Index (PSQI). In addition, the Mini Mental State Exam (MMSE) questionnaire will be used to assess global cognitive function, which participants will again complete pre-and-post intervention.

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We are aiming to recruit a cohort (n = 120) of middle-aged and older adults (>60 years) who will be randomized into 1 of 4 groups:

1) PoZibio™ (1 x 167mg capsule daily) and matching Cerbella placebo (1 x soft gel daily),
2) Cerbella (1 x soft gel daily) and PoZibio placebo (1 x 167mg capsule daily)
3) PoZibio™ (1 x 167mg capsule daily) and Cerbella (1 x soft gel daily),
4) Placebo (1 x capsule daily) and placebo (1 x soft gel daily).

Participants will be asked to consume a total of 2 capsules/soft gels every day for 60 days. Subjects will be asked to take these supplements in the morning with their breakfast. Subjects will be split between Aberystwyth (Wellbeing and Health Assessment Research Unit) and Trimsaran (Trimsaran Community Centre). Biological samples are being collected including capillary blood, stool, and urine samples.

Eligibility will be assessed online (in REDCap) or over the phone, using a medical screening questionnaire.

2) Statement of Purpose

A randomised, double-blinded, placebo controlled, parallel human clinical trial of heat-treated Lactobacillus paracasei (post-biotics) and/or Cerbella supplementation in healthy middle-aged and older subjects is proposed, to assess the potential for improved physiological and cognitive health. We would like to explore if consumption of Cerbella™ soft gels or PoZibio™ capsules, in healthy middle aged and older subjects (>60 years), is beneficial in terms of cognitive function and overall health and well-being when compared to a placebo control over 60 days. The placebo will be matched to the active product by taste and texture.

3) Investigational Product

4) Description and 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 with the European Directive 2001/18/EC. This product is not hazardous. 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. Sacco S.r.l. is ISO 22000 and FSSC 22000 (food safety) certified since 2014. 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.

Studies have been conducted previously using *Lactobacillus paracasei* postbiotics in human cohorts(1-4)

1. Calame W, van Olderen D, Calabretta V, Bottari L, Paparo L, Bruno C, et al. Baseline Concentrations of Various Immune Biomarkers Determine Their Increase after Consumption of a Postbiotic Based on Cow's Milk Fermented with Lactobacillus paracasei CBA L74 in Both Newborns and Young Children. Applied Sciences. 2022;12(4):2009.
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3. Corsello G, Carta M, Marinello R, Picca M, De Marco G, Micillo M, et al. Preventive Effect of Cow's Milk Fermented with Lactobacillus paracasei CBA L74 on Common Infectious Diseases in Children: A Multicenter Randomized Controlled Trial. Nutrients. 2017;9(7):669.
4. Nocerino R, Paparo L, Terrin G, Pezzella V, Amoroso A, Cosenza L, et al. Cow's milk and rice fermented with Lactobacillus paracasei CBA L74 prevent infectious diseases in children: A randomized controlled trial. Clinical Nutrition. 2017;36(1):118-25.

The supplements are suitable for vegetarians and vegans. The supplements are produced and stored in a food grade facility.

Cerbella

This is a follow-up trial. A pilot study to determine the feasibility, safety, and tolerability of Cerbella™ has already been published (Carmichael et al., 2018). Green tea is widely consumed, and green tea extracts are generally regard as safe (GRAS) ingredients. The U.S. Food and Drug Administration (FDA) classifies intake of up to three grams of omega-3 fatty acids from fish daily as GRAS. Panax Ginseng is considered to be relatively safe even in large amounts.
The supplements are *not* vegetarian or vegan. The supplements are produced and stored in a food grade facility.

3.1) 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 pozibio supplements are vegan and the cerbella are *not* vegetarian or vegan, and they are produced and stored in a food grade facility. The pozibio 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 for both supplements.

3.2) Dose

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

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5) Study Design

5.1) Objectives of the Study

5.1.1) Primary Outcome Measure:

- 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 60 days after intervention (pozibio, cerbella or combination)]

- 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 60 days after intervention (pozibio, cerbella or combination)]

- 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 60 days of intervention (pozibio, cerbella or combination), 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 60 days of intervention (pozibio, cerbella or combination), no delay of the P3 component and more N2 components when compared with baseline and the placebo group (at day 60)]

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

Assessing alpha and delta activity
[Time Frame: After 60 days of intervention (pozibio, cerbella or combination), increased alpha and delta activity when compared with baseline]

- Mini Mental State Exam (MMSE)

We will measure Mini Mental State Exam results to determine if interventions impact cognition.
[Time Frame: increased cognition from baseline at 60 days after intervention (pozibio, cerbella or combination)]

- 36-Item Short Form Health Survey (SF-36)

We will measure 36-Item Short Form Health Survey results to determine if interventions impact health.
[Time Frame: increased health from baseline at 60 days after intervention (pozibio, cerbella or combination)]

- Pittsburgh Sleep Quality Index (PSQI)

We will measure Pittsburgh Sleep Quality Index results to determine if interventions impact sleep.
[Time Frame: improved sleep from baseline at 60 days after intervention (pozibio, cerbella or combination)]

- Gastrointestinal Symptom Rating Scale (GSRS)

We will measure Gastrointestinal Symptom Rating Scale results to determine if interventions impact gastrointestinal health.
[Time Frame: increased gastrointestinal health from baseline at 60 days after intervention (pozibio, cerbella or combination)]

- Warwick-Edinburgh Mental Wellbeing Scale (WEMWS)

We will measure Warwick-Edinburgh Mental Wellbeing Scale results to determine if interventions impact mental wellbeing.
[Time Frame: increased mental wellbeing from baseline at 60 days after intervention (pozibio, cerbella or combination)]

5.1.2) Secondary Outcome Measures:

- Fecal mucin

Fecal mucin in stools as a marker of inflammation.
[Time Frame: Reduced Fecal mucin from baseline at 60 days after intervention (pozibio, cerbella or combination)]

- Calprotectin

Calprotectin in stools as a marker of inflammation
[Time Frame: Reduced Calprotectin at 60 days after intervention (pozibio, cerbella or combination) when compared with placebo after 60 days]

- Leaky gut markers in plasma

We will measure LBP, sCD14 and Zonulin in plasma to determine if interventions impact leaky gut markers.
[Time Frame: reduced LBP, sCD14 and Zonulin tests from baseline at 60 days after intervention (pozibio, cerbella or combination)]

- Intervention (pozibio, cerbella or combination)
- Cytokines

Circulating cytokines such as TNF-alpha or IL-6 in plasma
[Time Frame: changes in circulating cytokines at 60 days after intervention (pozibio, cerbella or combination) when compared with placebo after 60 days]

- 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 intervention (pozibio, cerbella or combination) at 60 days 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 intervention (pozibio, cerbella or combination) at 60 days compared with that after placebo at 60 days]

- Changes in metabolomic fingerprint in plasma

Changes in polar and non-polar chemistry in plasma measured using Flow Infusion Electrospray Ionisation Mass Spectrometry (FIE-MS).
[Time Frame: Changes in concentration of polar and non polar chemistry after the intervention (pozibio, cerbella or combination) at 60 days compared with that after placebo at 60 days]

- Changes in metabolomic fingerprint in urine

Changes in polar and non-polar chemistry in urine measured using Flow Infusion Electrospray Ionisation Mass Spectrometry (FIE-MS).
[Time Frame: Changes in concentration of polar and non polar chemistry after the intervention (pozibio, cerbella or combination) at 60 days compared with that after placebo at 60 days]

- Changes in microbiome in stools

Using whole genome sequencing, changes in microbiome diversity indices and phylogenetic abundances in stools
[Time Frame: Changes in microbiome diversity indices and phylogenetic abundances in stools after the intervention (pozibio, cerbella or combination) at 60 days compared with that after placebo at 60 days]

5.2) Subject Selection

120 participants, over 60 years, mixed gender, mixed ethnicity

- **Inclusion Criteria:**
  - Subjects over 60 years of age
  - Subjects able to provide written informed consent PRIOR to performing any study
  - procedures.
  - Subjects who are able to commit to visits to WARU or Trimsaran Community Centre
  - Subjects who are willing to complete a series of questionnaires including the: Pittsburgh Sleep Quality Index (PSQI), Mini Mental State Exam (MMSE), 36-Item Short Form Health Survey (SF-36), Warwick-Edinburgh Mental Wellbeing Scale (WEMWS), and the Gastrointestinal Symptom Rating Scale (GSRS).
  - Subjects who are willing to provide capillary bloods, stool, and urine samples, and commit to EEG appointments.
- **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 14 days of screening.
  - 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 who are taking antibiotics, and/or having diarrhea and vomiting in past 30 days
  - Any subjects to whom PI feels not to be eligible based on critical conditions

- Subjects who have a diagnosed or suspected mental health condition, or who have any concerns surrounding their mental health
- Subjects who are vegetarian/vegan
  - **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

Upon first point of contact, participants shall be provided with an information sheet detailing the study, followed by a REDCap link for completion of eligibility screening.

Online Eligibility Session

- Provide basic details (name, DOB, gender, preferred contact method), and answer a few medical health questions, within REDCap (compliant with GDPR standards), to assess eligibility. *For participants without access to a valid email address or home PC, these details will be obtained over the phone with a member of the WARU team.*
- Receive invitation for an in-person induction session (via email or over the phone).

Online/remote Induction Session

- Participants shall be posted their consent form to complete, along with written instructions for how to use the urine and stool home sampling kits. For participants without access to an email address, they will also be posted a paper copy of the participant information sheet.
- Participants shall be posted their urine and stool home sampling kits.
- The researcher will schedule the participants day 0 and day 60 appointments via phone or email and provide the opportunity for participants to ask questions.
- Measure height, weight, waist, and hip circumference.

Two Days before day 0 Assessments

- Participants with access to a PC and valid email address will be sent a REDCap link for completion of the self-report questions.
- Participants without access to a PC or valid email address shall be posted paper copies of the questionnaires at the same time as their consent form and urine/stool home sampling kits, which participants can complete at home the day before their assessments and bring with them, OR they can complete them with the researcher whilst their EEG cap is fitted.

Experimental visit 1 and 2- week 0 EEG and Behavioural Tasks
Visit 1 week 0:

- Stool samples will have been collected within the 12-hour period prior to this appointment, urine samples will have been collected that morning.
- Collection of capillary blood samples.

Visit 2 week 0- within 48 hours of visit 1

- Participant to arrive with clean, dry hair.
- EEG cap preparation (participants without access to PC at home can complete paper versions of the questionnaires in this time (duration = 25-30 minutes)
- Completion of the behavioural tasks for cognitive assessments within E-Prime (an 'eyes open/closed task', the Stroop task, and the Go/No-go task).
- Thorough instructions and practice rounds will be provided, and the opportunity to ask questions.
- Collection of supplements from the WARU team.
- Visits 1 and 2 may be combined

Day 60 EEG and Behavioural Tasks

- Repeat the activities explained above:

After the trial:

- Optional completion of a feedback questionnaire (this can be completed digitally or provided during their day 60 appointment).
- Participants informed of which group they were randomly assigned to.
- Participants provided with their vouchers (via email or post) to compensate their participation.
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All study materials (including pozibio and cerbella supplements) will be stored at the Well-Being and Health Assessment Research Unit (WARU) at Aberystwyth University.

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. The supplements are NOT suitable for vegetarians or vegans.

EEG is non-invasive, however placing the EEG electrodes along the scalp can be time consuming (approximately 30 minutes), and participants are required to sit relatively still during the application and testing process (the application and testing process combined take approximately 1.5 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 [for Trimsaran cohort: Trimsaran leisure centre; for Aberystwyth cohort: the Carwyn James Building, Penglais Campus], for them to use. For participants not wishing to use washing facilities, hats are advised to cover their head for their own comfort after the EEG. Participants are also reminded that capillary blood draws can also cause localised soreness.

7) Benefits to participant

Participants will receive a £20 Amazon voucher for each batch of testing sessions (day 0 and day 60), equating a total of £40 for completion of the trial which they will be provided via email. They will also allow researchers to gain important insight into the Cerbella™ and Pozibio™ supplements, and the extent to which they support or improve overall physical and mental health in older, healthy populations, which may be applied to other cohorts such as those suffers 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. Files are backed up and all data storage is using the AU network. Data is collected directly into REDCap. However if paper versions are used, 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, microbiome and leaky gut markers will be analysed at the University of Florida. Statistics will be conducted by the Aberystwyth University researchers.