2.1.4 Study Design and Procedure

This pilot study adopted a multi-arm, randomised control, blinded at one level, parallel group, pre-, mid- and post-intervention assessment design to investigate the feasibility of three different fermented foods containing live bacteria in comparison to a control group on emotional health, brain health and gastrointestinal health, as well as self-reported physiological and or psychological symptoms in healthy adults (See Fig. 1 and Table 2). The researcher conducting data collection and analyses was blinded to the group allocation of the participants. The researchers administering the interventions and the participants could not be blinded due to the physical differences of the intervention products. Group sample size was restricted by available funding. Enrolment was on a rolling 'first-come-first-served' basis until target number (n=45) was reached. Participants started the trial immediately upon enrolment. Participants were advised to consume their habitual diet throughout the study period. They were required to visit the laboratory 5 times 1-week apart during a 5-week study period (see Table 2) and were required to avoid caffeine 2 hours before visits 1 – 4.

At visit 1, participants completed diet and lifestyle questionnaires, and a health and well-being questionnaire (MYMOP®) [37] and were familiarised with the cognitive function assessment. They received their first (pre-intervention) stool test kit.

At visit 2, (3-4 days after their first visit) participants returned their filled stool test kit, and undertook the battery of tests for baseline assessments of cognitive function, emotional and gastrointestinal health. Participants were given a 15-day Bristol Stool Scale chart capture document, and 2 x 1-week MYMOP® follow-up documents to take home, complete, and return at visit 3. Another member of the research team provided the participant with a 15-day supply of the product, according to their allocation. Participants in the intervention groups were instructed to consume one portion every day, and all participants were asked to maintain their normal diet and lifestyle. Participants were not asked to exclude any fermented foods from their diet if it was part of their regular food consumption pattern.

The same procedure was applied during visit 3, at day 15 after their second visit, when the participants returned for mid-line assessments and delivering their 15-day Bristol Stool Scale chart capture, and 2 x 1-week MYMOP® follow up documents to take home, complete, and return at visit 3. Notion of any adverse symptoms experienced was made, and the final 15-day supply of the respective product given.

On day 30 from the start of product intake, visit 4, participants returned for end of trial testing (end of intervention). They returned their final 2 completed MYMOP® follow-up documents, and the 15-day Bristol Stool Scale chart and received their second stool test kit which they had to complete the following morning.

Visit 5 took place 3 days later, when participants returned their filled stool test kits.

To encourage compliance, participants were provided with a £30 Amazon voucher upon completion of the intervention. Additionally, they received personalised results reports from the GI Effects Comprehensive Stool Profile.

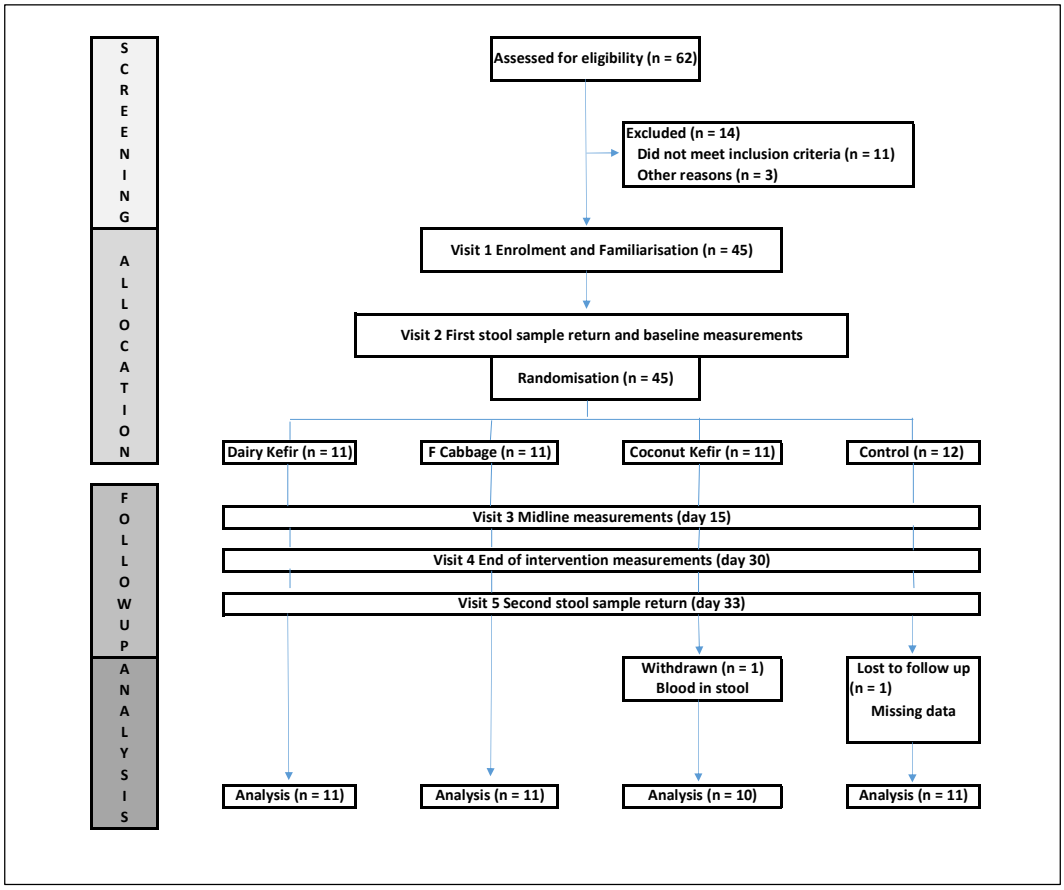

Fig 1 Flow chart of the study design

2.1.5 Data Collection

At the enrolment session, subjects completed a detailed diet and lifestyle questionnaire. Details of intake of fermented foods with live bacteria (other than the intervention products) and probiotic supplementation was captured at visits three and four. Participants were asked to complete a 7-day plant food diary.

2.1.5.1 MYMOP®

For quality of life measures the Measure Yourself Medical Outcome Profile [37] questionnaire (MYMOP®) was used, an individualised health-related quality of life evaluation instrument that is problem-specific. It enables an individual to measure change in self-chosen symptoms during an intervention. It required participants to specify one or two symptoms, psychological or physical, that concerns them most.

The symptoms were rated on a 7-point Linkert scale, ranging from 0 (as good as it could be) to 6 (as bad as it could be). The second part of the questionnaire uses the same scale to assess whether the symptom(s) is limiting or preventing a daily activity (physical, social or mental). General feeling of wellbeing was rated too. A MYMOP® follow up questionnaire was used to rate the same symptom(s), activity and general feeling of wellbeing on a weekly basis during the intervention.

2.1.5.2 Cognitive Function

Three tests from the Cambridge Neuropsychological Test Automated Battery [38] were used to assess cognitive function. The computerised neurocognitive tests were presented on a touch-screen iPad running the CANTAB eclipse software. Subjects were seated at a comfortable height, approximately 0.5 m from the monitor, and were instructed to carry out the tasks by touching the screen. Each test started with a detailed visual display explanation and a guided practice session. Participants completed the battery in the following order:

(1) Delayed matching to sample (DMS): This task tests visual memory in a 4-choice delayed recognition memory paradigm, assessing both simultaneous visual matching ability and short-term visual recognition memory for non-verbalizable patterns.

(2) Rapid Visual Information Processing (RVP): A visual continuous performance task, using digits rather than letters, measuring sustained attention and working memory.

(3) Cambridge gambling task (CGT): Measures executive function for risk-taking behaviour and decision-making under uncertainty.

2.1.5.3 Emotional Health

The 12-item short form of the profile of mood states (POMS) [39] was used to measure subjective psychological symptoms, scoring six subscales of mood – Tension, Depression, Anger, Fatigue, Confusion, Vigour, as well as to calculate Total Mood Disturbance (TMD) index. The 21 item self-report questionnaire Depression, Anxiety and Stress Scale (DASS-21) [40] was used to measure the self-perceived negative emotional states of depression, anxiety and stress. The depression scale assessed dysphoria, hopelessness, devaluation of life, self-deprecation, and lack of interest/involvement, anhedonia and inertia. The anxiety scale assessed autonomic arousal, skeletal muscle effects, situational anxiety, and subjective experience of anxious affect, and the stress scale assessed difficulty in relaxing, nervous arousal, and being easily upset/agitated, irritable/over reactive and impatience.

2.1.5.4 Gastrointestinal Health

2.1.5.4.1 Faecal biomarkers and gut microbiome

The GI Effects Stool Profile, a proprietary multi-component laboratory assessment was used to measure digestive function, intestinal inflammation, as well as the gastrointestinal microbiota. Across 3 consecutive days before visit 2 (pre-intervention) and visit 5 (post-intervention) participants collected fresh stool samples (selecting from different parts of the stool), using a specialist kit (Genova Diagnostics, Asheville, North Carolina). The stool samples were collected at home and stored at refrigerated temperatures. The completed kits were shipped (at ambient temperature) within 24 – 48 hours of the last (3rd day) of sample collection to the clinical lab for analyses.

Genova Diagnostics evaluated samples, and data was collected from the GI Effects Comprehensive Digestive Stool Analysis Report. Inflammation and Immune biomarkers: Calprotectin, Eosinophil Protein X (EPX), Faecal secretory IgA; Gastrointestinal Microbiome biomarkers: Total Short Chain Fatty Acids (SCFA), n-Butyrate Concentration, n-Butyrate percentage, Acetate percentage, Propionate percentage, Beta-glucuronidase, 24 commensal bacteria (PCR), and Zonulin Family Peptide.

2.1.5.4.2 Self-reported stool consistency

The Bristol Stool Scale [41] is a diagnostic medical tool designed to classify the form of human stool into seven categories. It provides insights into gut health, particularly the transit time of stool through the colon.

Type 1-2: Hard, lumpy stools indicate slow transit and constipation.

Type 3-4: Smooth, sausage-like stools are considered normal and healthy.

Type 5-7: Soft to watery stools suggest rapid transit and is often linked to diarrhoea or incomplete digestion.

Participants were given a Bristol Stool Scale chart for 15 days at the start (Day 1) and midway (Day 15). They were asked to self-rate their stool consistency each day for 30 days at home and return the completed charts midway (Day 15) and at the end (Day 30).

2.2 Data Analyses

2.2.1 MYMOP®

All domains (symptom severity, activity, and wellbeing) can be analysed individually or as a total score, the profile score, that equals the mean of the sub

scores recorded [37]. From the MYMOP® data, we collected participant-generated symptom changes, activity scores, the general wellbeing scores, and calculated the profile scores for each participant.

2.2.2 Cognitive Function

From the Delay Matching to Sample the total number of times a participant chose the correct answer on their first box choice (min 0, max 20) (DMSTC) was used for analysis; the total number of times a participant chose the correct answer on their first box choice for trials where the response stimuli appeared on the screen after a 0, 4 and 12 second delay after the target stimulus was shown (min 0, max 5) (DMSTC0, DMSTC4, DMSTC12); the percentage of assessment trials during which the participant chose the correct box on their first box choice (DMSPC); and the mean latency between the presentation of the response stimuli options and the participant selecting the correct box on their first attempt (DMSML). How good the participant was at detecting target sequences (min 0, max 1) (RVPA); the total number of correct hits (min 0, max 54) (RVPTH); the total number of false alarms (min 0, max 546) (RVPTFA); and the response latency on correct hits, measured in milliseconds (min 100, max 1900) (RVPML); and the total number of target sequences that were not responded to within the allowed time (min 0, max 54), were analysed for the Rapid Visual Processing (RVPTM). From the Cambridge Gambling Task the delay aversion total, which allows for the dissociation between risk taking and impulsivity by determining whether participants simply just place a bet at the first opportunity (min -0.9, max 0.9) (CGTDAVT); the mean decision time from presentation of the boxes to the participant's selection of the colour on which to bet, measured in milliseconds (min 0) (CGTDMMT); decision making quality, which is the proportion (0-1) of all trials where the participant chose the majority box colour (min 0, max 1) (CGTDMGMT); and total risk taking, which is the mean proportion (0-1) of current points gambled, in which the number of boxes in each colour differed and the participant chose the majority box colour (CGTRTKMT) were analysed.

2.2.3 Emotional Health

The Profile of Mood State (POMS) [39] questionnaire was answered on a 5-point Linkert scale to evaluate six transient distinct mood sates namely, tension, depression, anger, fatigue, confusion and vigour, and scored accordingly - 0 = not at all, 1 = slightly, 2 = moderate, 3 = very, 4 = extremely. Total Mood Disturbance (TMD) was calculated by summing the totals for the negative subscales (tension, anger, depression) and then subtracting the totals for the positive subscale (vigour).

For the Depression and Stress and Anxiety scale (DASS21) subjects were assessed based on a 4-point Linkert scale - 0 = did not apply to me at all – NEVER, 1 = applied to me to some degree, or some of the time – SOMETIMES, 2 = applied to me to a considerable degree, or a good part of time – OFTEN, 3 = applied to me very much,

or most of the time – ALMOST ALWAYS. The scores for each subscale of the DASS-21 questionnaire, depression, anxiety and stress, were categorised into five standard ranges – Normal, Mild, Moderate, Severe, and Extremely Severe.

2.2.4 GI Health

The following data were extracted from the Genova Diagnostics GI Effects Stool test to assess several characteristics of the individual's GI health [35].

2.2.4.1 Inflammatory and Immune biomarkers

Calprotectin. A calcium-binding protein with antimicrobial properties. It accounts for 60% of neutrophil cytosolic content and is found in monocytes and macrophages. Calprotectin is released from the intestinal mucosa into the stool in intestinal inflammation. The reference range for calprotectin for individuals aged between 10 and 59 years is <=50 mcg/g of stool [42].

Eosinophil Protein X (EPX), also known as eosinophil-derived neurotoxin (EDN). One of the four basic eosinophil granule proteins, and a marker of eosinophil activity. Eosinophils are specialized white blood cells that proliferate and accumulate in areas of inflammation. They are scarce in a steady state large intestine and increase only under intestinal inflammation. The reference range for EPX/EDN is <=4.6 mcg/g of stool [43].

Faecal secretory IgA (fSIgA). fSIgA is the most abundant class of antibody found in the human intestinal lumen and is used to assess gastrointestinal barrier function. The reference range for fSIgA is <=885 mcg/g of stool [43].

2.2.4.2 Gastro-Intestinal metabolites

The GI microbiome biomarkers provide information regarding the health, function and diversity of the GI tract microbial cells. They indicate how well the microbiome is performing the metabolic functions that are shared with the human host.

Total short chain fatty acids (SCFA's). SCFA's are organic acids, of which propionate, acetate, and n-butyrate are the most abundant (≥95%). They maintain intestinal barrier function, provide fuel for colonocytes, regulate colonic absorption of water, electrolytes and nutrients, salvage unabsorbed carbohydrates, support commensal bacteria, and modulate anti-inflammatory antimicrobial activities. Optimal levels of SCFA's have not been established. Higher levels are considered beneficial. The reference range for Total SCFA's is >=23.3 micromole/g of stool [44].

n-Butyrate concentration and percentage. n-Butyrate is the primary fuel source for colonocytes, and an inadequate level is associated with poor colonic health. The

reference range for n-Butyrate concentration is >= 3.6 micromole/g of stool; and n-Butyrate percentage is 11.8 – 33.3% [45].

Acetate percentage. Acetate is the most abundant SCFA in the colon and makes up more than half of the total SCFA's. The reference range for Acetate percentage is 48.1 – 69.2% [46].

Propionate percentage. Propionate has anti-inflammatory effects. The reference range for Propionate percentage is <= 29.3% [46]

Beta-glucuronidase. β-glucuronidase is an enzyme which is produced by colonocytes and by some intestinal bacteria. It breaks down complex carbohydrates and increases bioavailability of plant polyphenols. The reference range for ß-glucuronidase is 368 – 3,266 U/g of stool [43].

2.2.4.3 Commensal Bacteria

24 Commensal gut bacteria (at genus or species levels) using PCR methodology was available from the Genova Diagnostics GI Effects Stool test at the time of conducting this intervention trial (Table 3).

2.2.4.4 Bristol Stool Scale

From the self-reported data, we analysed frequency and category change trends over the 30-day intervention period.

2.2.5 Intervention products' metagenomics

The samples were analysed using metagenomics based on sequencing amplicons from the 16S rRNA genes from lactic acid bacteria, by Campden BRI (Chipping Campden) Ltd, UK, UKAS laboratory No. 1079. DNA was extracted using the 'PowerFood' DNA extraction system. Resulting extractions were quantified using a fluorometer (Qubit 3.0) and dilutions prepared for PCR. Primers designed to pick up the V3-V4 variable region of the 16S rRNA gene, checked using a Bioanalyser from Agilent, and then cleaned in a column-based system. Samples were sequenced on an Illumina MiSeq instrument. The reads were compared against databases of 16S ribosome genes using the Metagenomics package from Illumina's Basespace program. The raw data was received from Campden BRI in excel.

Study Activities	Screening	Visit1 familiarisation	Visit 2 baseline	Visit 3 (day 15) mid-line	Visit 4 (day 30) end	Visit 5 post testing
Informed Consent	x					
Health Questionnaire	x					
Randomization		x				
Plant Food Diary		x				
Diet and Lifestyle Questionnaire		x				
Brain Health						
Cognitive Function		x	x	x	x	
Emotional Health						
POMS			x	x	x	
DASS-21			x	x	x	
Gastrointestinal Health						
Bristol Stool Scale			x receive	x return/receive	x return	
Stool Test Kit receive		x			x	
Stool Test Kit return			x			x
Quality of Life						
MYMOP®		x	x follow-up	x follow-up	x follow-up	
Product receive			x	x		
Adverse Symptom Assessment				x	x	

Table: 2 Schedule of the trial assessments

Legend*: POMS (Profile of Mood State); DASS-21 (Depression, Anxiety, and Stress Scales-21); MYMOP® (Measure Yourself Medical Outcome Profile*