PROTOCOL

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Title of study: Influence of vitamin D and a probiotic on inflammation and gut bacteria

Study identifiers

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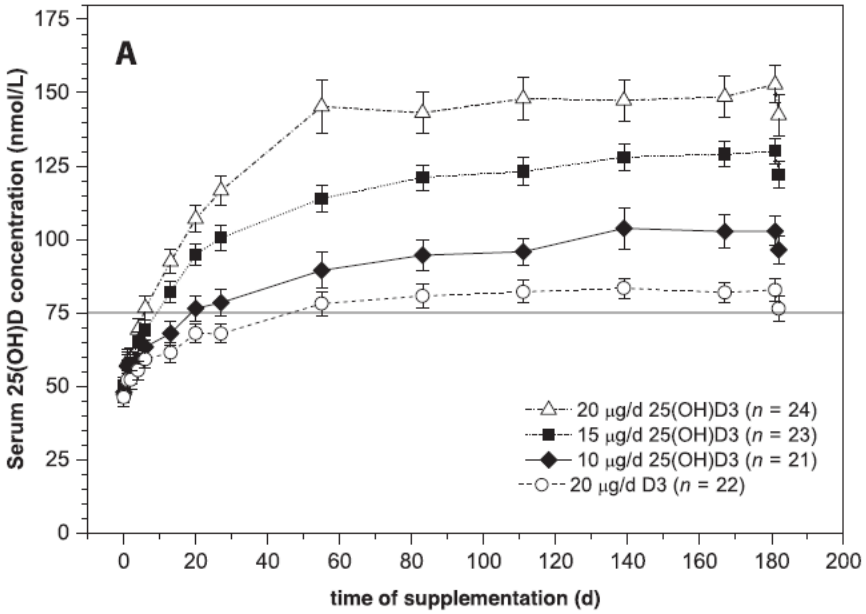
1. Introduction & rationale

The number and proportion of older people is increasing in the UK and many other countries as people are living longer. However, while lifespan is increasing, healthspan is not keeping pace and many older people live with significant illness. Ageing ultimately leads to the loss of functional capacity in many body systems including the cardiovascular, musculoskeletal, osteoarticular and neuroendocrine. A hallmark feature of ageing is immune decline, termed immunosenescence, with the functional decline of the innate and adaptive immune systems resulting in compromised immunity [1-3]. Paradoxically, in parallel there is an elevation in systemic inflammation with ageing, a phenomenon termed inflammageing [4, 5]. Inflammageing and its associated conditions including cardiovascular disease, metabolic diseases, sarcopenia and osteoporosis, some cancers and possibly dementia, make significant contributions to morbidity, poor quality of life, mortality and increased social and health care costs in older people. Furthermore, immunosenescence increases susceptibility to infections contributing to illness and mortality [6]. Importantly, these immune changes also limit responses to public health measures like influenza vaccination [7, 8]. The coronavirus pandemic has highlighted the vulnerability of older people to infections; this vulnerability is likely to be at least in part due to age-related immune changes. Thus, there is an urgent need to understand and overcome the drivers of age-related immune changes that cause ill health.

The intestinal microbiota has an influence on host health by, for example, acting through direct and indirect effects on the host immune system and inflammatory response [9, 10]. The structural complexity and functional capability of the intestinal microbiota decline with ageing [11-14]. How this occurs and the nature of the relationship between changes in the microbiota, the onset of low-grade inflammation, declining gastrointestinal tract function, and immunosenescence in older people are not well described. The loss of intestinal barrier function in older people which can result in translocation of bacterial endotoxins and whole bacteria into the bloodstream is a plausible link between intestinal dysbiosis and inflammaging, and is consistent with the concept that manipulation of the intestinal microbiota may be of therapeutic benefit in older people [15]. Nutritional approaches may be used to modify the intestinal microbiota [16, 17] and to support the immune system including regulating inflammation [18]. Vitamin D is important in this regard and having sufficient vitamin D is likely to be important in maintaining innate and cell-mediated immunity and preventing low-grade inflammation [19-25]. Vitamin D levels are low in the UK population [26] and vitamin D levels among British adults are inversely associated with infection risk [27], suggesting that the influence of low vitamin D status on immune competence is a public health problem. There are a number of reports of low vitamin D status being linked to risk of coronavirus infection and severity of COVID-19 [28-30]. The link between low vitamin D status in older people and immunosenescence and inflammaging requires further exploration. Intriguingly, high dose vitamin D supplements have also been shown to alter the faecal microbiota in animal models [31] and in humans [32, 33]. The intestinal microbiota may also be manipulated through oral intake of probiotics. *Lactobacillus plantarum* is a probiotic organism which has been shown to survive passage through the gastrointestinal tract and colonise in the intestines in humans, with the TIFN101 strain exhibiting the strongest persistence in the gut [33]. *L. plantarum* TIFN101 has been shown to positively affect the immune system by supporting the maintenance of immune cells [34] and stimulating memory responses and antigen presentation [35], supporting intestinal barrier function [36], and protecting against intestinal inflammation by mediating inflammatory signaling molecules in humans [37]. However, the use of this organism is yet to be investigated in older adults in whom

there are changes in the intestinal microbiota [11-14], immune decline [1-3], and elevated inflammation [4, 5].

Robust investigation of diet-microbiota interactions in the context of age-related immune decline and inflammaging requires a cross-disciplinary approach and reliable experimental tools. A consortium of expert researchers from the Universities of Southampton, Birmingham, and East Anglia (The Quadram Institute) have come together to collaborate on this trial, which is funded by the Medical Research Council. The trial is a 2 x 2 factorial trial testing the impact of vitamin D, a probiotic organism, and a combination of the two on immunosenescence, inflammation and the intestinal microbiome in older participants. The interventions will be calcifediol (also known as calcidiol, 25-hydroxycholecalciferol, or 25-hydroxyvitamin D3) to be used at a dose of 10 μg/day vs placebo, *Lactobacillus plantarum* TIFN101 at a daily dose of 5 x 10^9 colony forming units (cfu) vs placebo, and a combination of the two vs placebo over a period of 3 months. Calcifediol has been demonstrated to elevate plasma 25-hydroxyvitamin D3 concentrations more effectively than cholecalciferol [38-40]:



Time course of unadjusted serum 25(OH)D concentration (nmol/L) in healthy older adults.

(Taken from Ref. 39)

Study products will be provided gratis by DSM Nutritional Products; an MTA is in place to cover this.

2. Objective

The objectives of this study are to identify the effect of vitamin D (calcifediol) and *Lactobacillus plantarum* TIFN101 alone and together on the intestinal microbiota, markers of immune function and inflammation and other health-related markers (blood lipids, body composition, muscle strength) in older adults.

Inclusion and exclusion criteria

Inclusion criteria

1. Community dwelling males and females aged 60+ years
2. Body mass index 18.5-35 kg/m^2
3. Willing to adhere to the study protocol
4. Able to provide written informed consent

Exclusion criteria

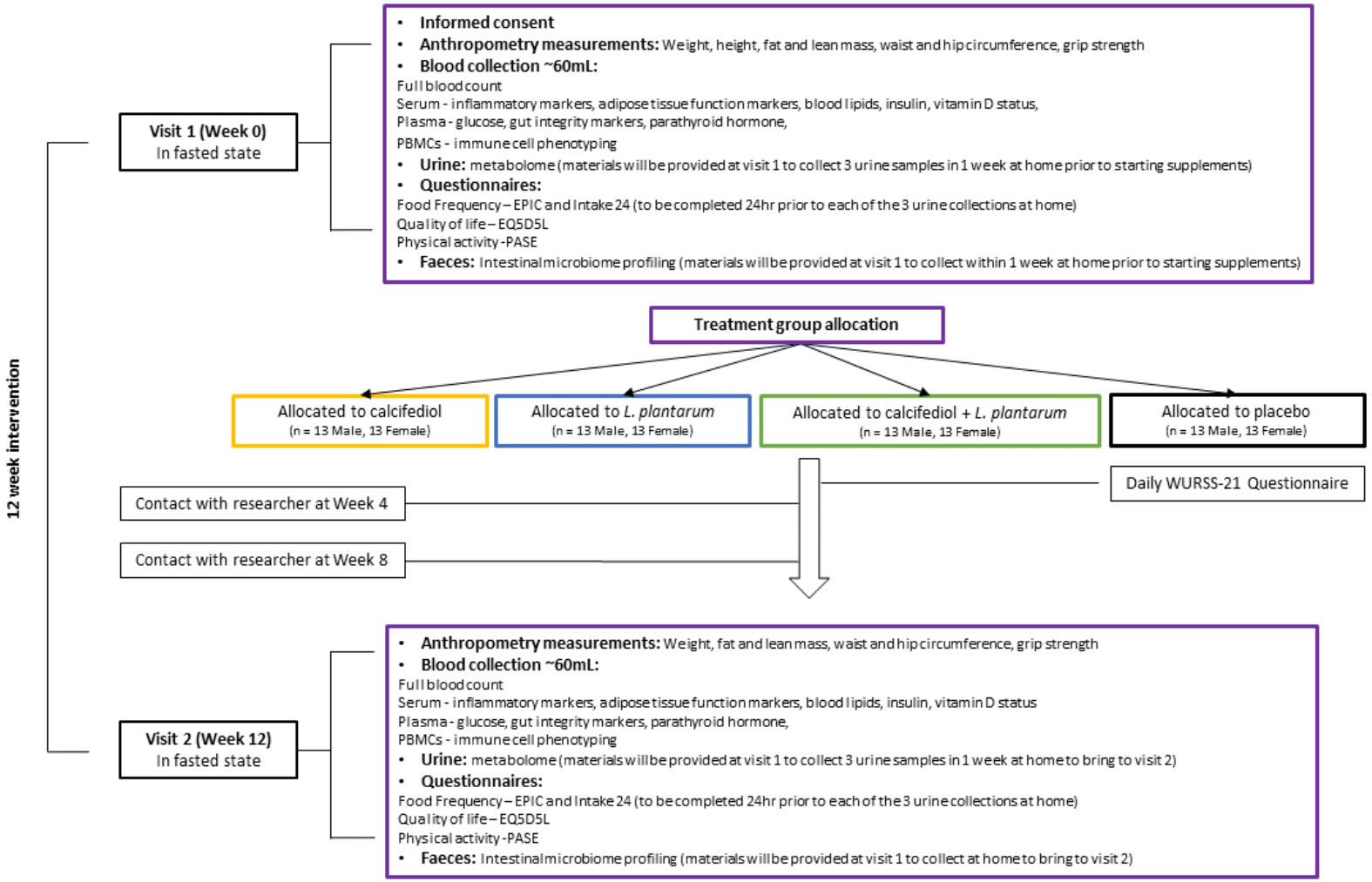
1. Living in a care or nursing home
2. Diagnosed with diabetes or other metabolic and endocrine disorders
3. Presence of active gastrointestinal disease (coeliac disease, Crohn's disease, diagnosed IBD etc.), autoimmune disease, or inflammatory disease (lupus, rheumatoid arthritis, multiple sclerosis), COPD, active cancer or current cancer treatment or having had cancer within the last year, having had a heart attack or other cardiac event within the last year
4. Use of steroid inhalers, or use of prescribed medicine to control inflammation (e.g. non-steroidal anti-inflammatory drugs; NSAIDs) or prescribed vitamin D or calcium+vitamin D or regular use of over-the-counter NSAIDs
5. Use of dietary supplements (will allow a 4-week washout period)
6. Use of probiotic drinks or yoghurts (will allow a 4-week washout period)
7. Blood donation in the previous 3 months
8. Participation in any other clinical trial in the previous 3 months

3. Study design and participant schedule

All procedures involving human participants will be approved by a relevant NHS Research Ethics Committee. This study will be conducted according to the guidelines established in the Declaration of Helsinki. The study will be registered at www-controlled-trials.com.

The study will be a 2 x 2 factorial double-blind randomised, placebo-controlled trial carried out with clinically healthy older adults (aged 60 years and above). Participants will be assessed at baseline, take daily supplements for 3 months, then return for further assessment and conclude the study (see Figure 1 and Figure 2).

Figure 1. Overview of the study plan and of participant flow through the study



Recruitment

Participants will be sought through poster advertisements; articles in the media (newsletters, newspapers, radio, university project specific social media pages, targeted social media click advertisements); posters and email within the University of Southampton and University Hospital Southampton NHS Foundation Trust; contacting those on a GDPR compliant database held by the University Hospital Southampton; through contact with Age UK and other organisations particularly relevant to older people; and via local GP surgeries who will act as Participant Identification Centres (these will be identified with the help of the Wessex clinical research network (CRN)). Individuals who are interested will contact the research team by telephone. They will then be sent the Participant Information Sheet. They will be able to contact the researcher to confirm their interest. If they do not contact the researcher within 7 days they will be contacted by the researcher to see if they remain interested in the study. Those individuals who indicate interest in the study will answer a small number of questions to ascertain whether they are likely to meet the inclusion/exclusion criteria (screening questionnaire). If so, an appointment will be made for them to attend visit 1 (V1) at the NIHR Wellcome Trust Clinical Research Facility at Southampton General Hospital.

Visit 1

Participants will attend the NIHR Clinical Research Facility at Southampton General Hospital in the morning (between 8 and 10:30 am) in the fasted state (no food or drink apart from water from 9 pm the night before). Participants will be given the opportunity to discuss the study and have any questions answered. If they are happy to be enrolled, they will be asked to sign an Informed Consent Form.

At this visit, participant's height, weight, mid-arm upper circumference, waist and hip circumferences and blood pressure will be measured. In addition, their body composition will be measured using a Tanita bioelectric impedance apparatus and grip strength will be measured using a hand grip dynamometer taking the maximum of three readings with each hand. ~60 mL blood will be collected to provide whole blood, serum and plasma.

After blood collection, participants will be given breakfast (orange juice, toast and jam, tea or coffee). Following consumption of breakfast, participant's diet will be assessed using the widely used EPIC Food Frequency Questionnaire, their quality of life will be assessed using EQ5D5L and their physical activity will be assessed using PASE. Participants will then be randomised to one of four groups (vitamin D (calcifediol, 10 µg/day), probiotic (*Lactobacillus plantarum* TIFN101 (5 x 10^9 cfu/day)), vitamin D + probiotic, or placebo) and will receive 3 months supply along with a diary to record that they have taken their supplement each day. Blinding, randomisation, and supplement packaging will be completed by individuals independent of the researchers involved in the study. Participants will be randomised stratified by sex to ensure equal numbers of males and females in each treatment group.

Participants will also take away materials for collection of faecal and urine samples. They will be provided with a pack and instructions for collecting a faecal sample and 3 urine samples at home within one week of V1. Two urine samples will be collected on weekdays (one per day) and one urine sample will be collected at a weekend or first thing on a Monday morning. Participants will be asked to complete a 24 hr food diary using the online tool 'intake 24' or by completing a

paper diary 24 hr prior to collecting each urine sample. Participants will be asked to keep the urine samples in their home fridge until all 3 samples have been collected. Participants will be asked to collect a faecal sample within 24 hours of the last urine collection. Participants will be asked to either return their samples to the Clinical Research Facility in person or to contact the researcher once samples are available who will travel to their home to collect the samples or will arrange for them to be collected by courier.

At the end of V1 participants will be provided with their supplements and instructed to start taking these once they have produced their faecal sample and three urine samples. Participants will receive instructions on taking their supplements and will record this daily use in a paper diary.

Participants will be contacted every 4 weeks by the researcher (at week 4 and week 8) and will be requested to bring unused supplements to their end of study clinic visit (visit 2; week 12). These will be used to assess compliance.

Between clinic visits 1 and 2, participants will complete the daily WURSS-21 questionnaire to assess respiratory symptoms. If they have no symptoms, they will not need to complete this questionnaire.

Blood will be collected for the following measurements:

Sample	**Measurement**	**Analysed by**	**Reason**
Blood	Full blood count	University Hospital Southampton Chemical Pathology	Reports blood immune cell numbers (plus red cells and platelets)
Blood – Serum	TNFα IL-1β IL-1RA IL-4 IL-6 IL-8 IL-10 IL-17 IFN-γ GM-CSF sCD14 IFN α/β	University of Birmingham	Inflammatory markers
Blood - Serum	Insulin Total cholesterol HDL- cholesterol LDL- cholesterol Triglycerides 25-hydroxyvitamin D3 Calcium	University Hospital Southampton Chemical Pathology	Glucose homeostasis Blood lipids Vitamin D status
Blood - Serum	Leptin Adiponectin Visfatin Resistin CRP LPS binding protein	University of Southampton	Adipose tissue function markers Inflammatory markers

Blood - plasma	Parathyroid hormone Glucose	University Hospital Southampton Chemical Pathology	Related to vitamin D status Glucose homeostasis
Blood - plasma	Zonulin-1 Occludin iFABP Citrulline sCD14	University of Southampton	Gut integrity markers
Blood mononuclear cells	Differentiated CD57+ve NK cells T cells PTK7+ve recent thymic emigrant cells Naïve memory T cells (CD3+ve but differential expression of CD45RA, CD28, CD57 and KLRG1) Regulatory T cells (FOXP3 and CD25+ve) B cells (CD24hi, CD38hi)	University of Birmingham	Immune cell phenotypes
Urine	Metabolome	University of Southampton	Microbiota and diet-related metabolome
Faeces	Whole genome shotgun metagenomics, and metabolomic analysis Wet/dry weight	Quadram Institute	Quantitative intestinal microbiome profiling

Volumes of blood to be collected will be as follows:

Matrix	**Collection tube**	**Volume**	**Analytes**
Serum	SST	~ 3.5 ml	Insulin
Serum	SST	~ 5 ml	CRP, cholesterol, HDL, LDL, triglycerides, vitamin D, calcium
Serum	SST	~ 3.5 ml	Leptin, adiponectin, visfatin, resistin
Serum	Red top serum	~ 6 ml	TNFα, IL-1β, IL-1RA, IL-4, IL-6, IL-8, IL-10, IL-17, IFN-γ, GM-CSF, IFNα/β
Blood mononuclear cells	Lithium heparin	~ 18 ml (3 x 6 ml)	Immune cell phenotypes
Plasma	Fluoride oxalate	~ 5 ml	Glucose
Plasma	EDTA	~ 6 ml	Zonulin-1, Occludin, iFABP, Citrulline, sCD14
Blood	EDTA	~ 3.5 ml	Full blood count
Plasma	Lithium heparin	~ 6 ml	Parathyroid hormone

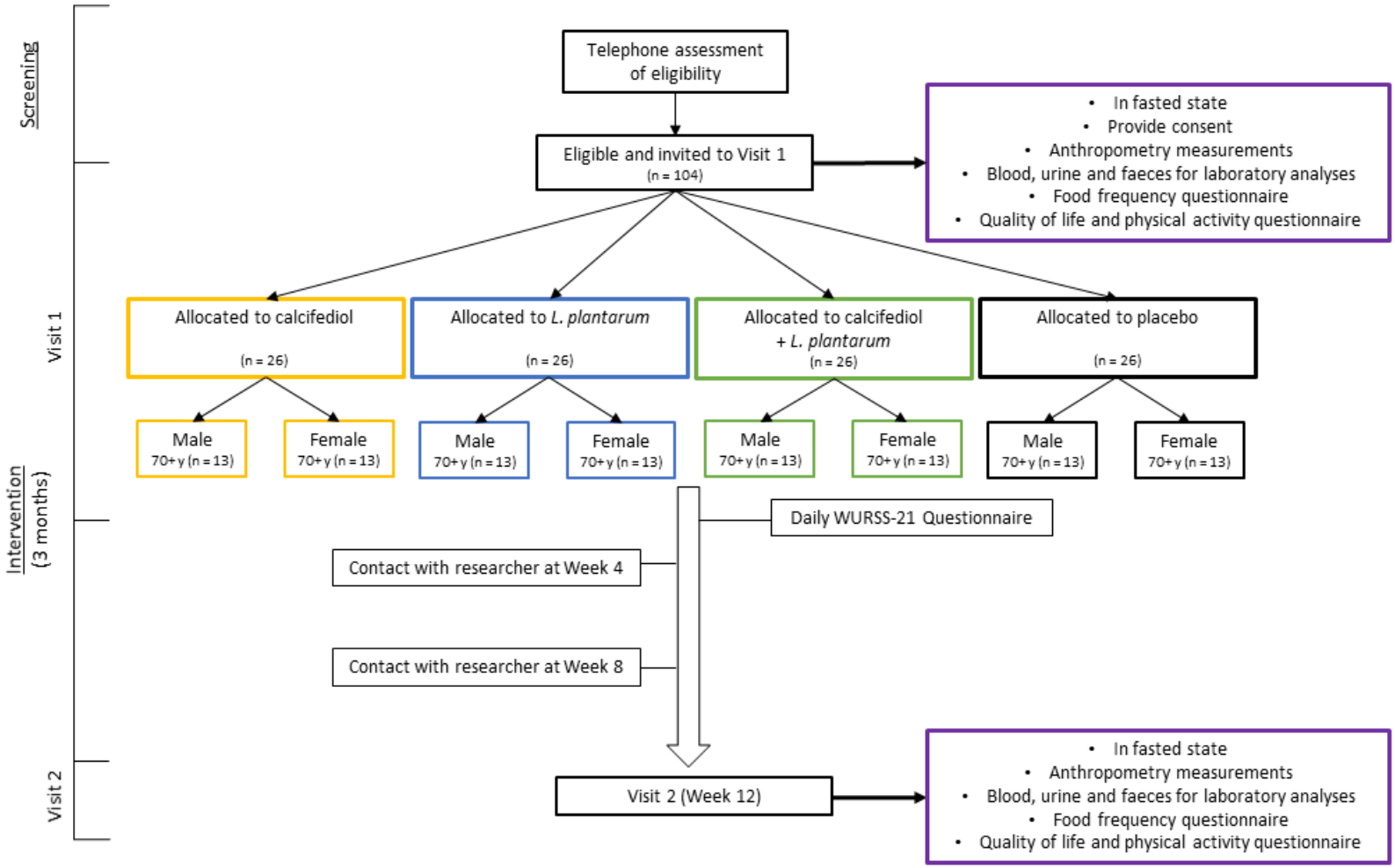
A summary of the questionnaires to be used at each time point is as follows:

Questionnaire	**V1 (baseline)**	**V2 (3 months)**
Food frequency (EPIC and 3 x intake 24)	YES	YES
Quality of life (EQ5D5L)	YES	YES
Physical activity (PASE)	YES	YES
Respiratory symptoms (WURSS-21)	Daily between V1 and V2 (Only if participant has symptoms)	

Visit 2

Three months (12 ± 1 weeks) after Visit 1, participants will return to the NIHR Clinical Research Facility at University Hospital Southampton for visit 2 (V2). Participants will be asked to bring a recent faecal sample and three recent urine samples with them; these will have been collected and stored in the same way as for post-V1 and again a 3 day food diary will be completed. All measurements, blood samples and questionnaires conducted at visit 1 will be repeated at visit 2.

Figure 2. Further overview of the study plan.



4. Variables and analyses

The primary outcomes measured will be vitamin D status (measured as 25-hydroxyvitamin D3 in serum), colonisation of the probiotic organism (measured as numbers of *L. plantarum* in faeces), and serum CRP concentration (measured with a high sensitivity kit).

In addition, the following will be measured as secondary outcomes:

1. Immune cell phenotypes
2. Inflammatory markers
3. Faecal microbiome taxonomy
4. Weight, body mass index, body fat mass, body lean mass, hip circumference, waist:hip ratio
5. Grip strength
6. Dietary intake (from food frequency questionnaire)
7. Quality of life (questionnaire)
8. Physical activity (questionnaire)
9. Respiratory symptoms (questionnaire)
10. Blood glucose, insulin, HOMA-IR
11. Blood lipids (total, LDL and HDL cholesterol, triglycerides)
12. Blood adipokines (leptin, adiponectin, leptin/adiponectin ratio, visfatin and resistin)
13. Blood markers of intestinal barrier integrity
14. Blood PTH and calcium, as markers of vitamin D homeostasis
15. Faecal metabolome
16. Urinary metabolome

Serum calcium and plasma parathyroid hormone will be measured as these relate to vitamin D status and action.

Full blood count will be conducted to obtain absolute numbers of different types of immune cells.

5. Sample size and statistical analysis

The primary outcomes of the study are serum 25-hydroxyvitamin D3 concentration, colonisation with the probiotic organism (as detected in faeces), and serum CRP concentration. The study sample size has been calculated based upon blood 25-hydroxyvitamin D3 concentration. Assuming a study entry concentration of 45 nmol/l and an increase to 70 nmol/l after receiving 10 μg/day calcifediol for 3 months (both with a standard deviation of 25 nmol/l), a sample size of 72 (n = 36 per group i.e. + or – vitamin D) will give 95% power to detect the difference in concentrations as significant at $p < 0.01$. To allow for a drop-out rate of 30% a total of 104 participants will be enrolled. This sample size will be sufficient to detect colonisation with the probiotic, as such colonisation (with other organisms) has been shown with smaller sample sizes. Using our data for serum CRP concentrations in older people resident in care homes (mean 6.3 $\pm$ 2.2 mg/dl) we estimate that a sample size of study completers of 36 per group will give 70% power to detect a 20% reduction as significant.

Changes between V1 and V2 in all outcomes will be compared between groups by ANOVA for 2 x 2 factorial design; subsequent pairwise comparisons between groups will be performed. Statistical analysis will be conducted using the current version of SPSS.

Study materials (i.e. the supplements) will be provided prepackaged by DSM. DSM will allocate a study ID to each participant's package of supplements in order that both participants and researchers are blind to allocation. DSM will provide to the researchers a sealed envelope (one per participant) containing the treatment allocation for each participant. These envelopes will be kept in a locked filing cabinet and will be accessed a) if there is a SAE and b) once the database is complete and locked and prior to statistical analysis being performed.

6. Adverse events

6.1 What is an adverse event?

An adverse event (AE) is defined as any untoward medical occurrence in a patient or clinical study subject administered an investigational product and which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational product, whether or not related to the investigational product.

An adverse reaction is defined as all untoward and unintended responses to an investigational product related to any dose administered, i.e. where a causal relationship between the investigational product and an adverse event is at least a reasonable possibility.

An unexpected adverse reaction is an adverse reaction, the nature or severity of which is not consistent with the information about the investigational product or intervention in question set out in the Summary of Product Characteristics or Investigator's Brochure.

An adverse event, adverse reaction, or unexpected adverse reaction, is defined as serious if it:

a) results in death;

b) is life-threatening;

Life threatening in the definition of a serious adverse event (SAE)/serious adverse reaction (SAR) refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event that hypothetically might have caused death if it were more severe.

c) requires hospitalisation or prolongation of existing hospitalisation;

In general, hospitalisation signifies that the participant has been detained (usually involving an overnight stay) at the hospital or emergency ward for observation and/or treatment which would not have been appropriate at the investigator site. When in doubt as to whether hospitalisation occurred or was necessary, the adverse event should be considered as serious. Hospitalisation for elective surgery or routine clinical procedures, which are not the result of an AE, need not be considered AE and should be recorded on a Clinical Assessment form and added to the study file. If something untoward is reported during the procedure, this must be reported as an AE and either 'serious' or 'non-serious' attributed according to the usual criteria.

d) results in persistent or significant disability or incapacity;

e) consists of a congenital anomaly or birth defect.

Medical judgement should be exercised in deciding whether an SAE/SAR is serious in other situations. Important SAE/SARs that are not immediately life-threatening or do not result in death or prolonged hospitalisation but may jeopardise the subject or may require intervention to prevent one or the other outcomes listed in the definition above, should also be considered serious.

A suspected serious adverse reaction (SSAR), is any serious adverse reaction that is suspected (possibly or probably) to be related to the investigational product.

A suspected unexpected serious adverse reaction (SUSAR) is an SSAR which is also "unexpected", meaning that its nature and severity are not consistent with the information about the investigational product in question set out in the IB.

6.2 Intensity

The assessment of intensity will be based on the investigator's clinical judgement using the following definitions:

- Mild: An event that is easily tolerated by the participant, causing minimal discomfort and not interfering with everyday activities.
- Moderate: An event that is sufficiently discomforting to interfere with normal everyday activities.
- Severe: An event that prevents normal everyday activities.

The term severity is often used to describe the intensity (severity) of a specific event. This is not the same as 'seriousness', which is based on participant/event outcome or action criteria.

6.3 Causality

The relationship between the investigational product/procedure and the occurrence of each AE will be assessed and categorised as below by the investigator. The investigator will use clinical judgement to determine the relationship. Alternative causes, such as natural history of the underlying diseases, concomitant therapy, other risk factors etc. will be considered. The Investigator will also consult the IB or other product information.

- Not related: Temporal relationship of the onset of the event, relative to administration of the product, is not reasonable or another cause can by itself explain the occurrence of the event.
- Unlikely: Temporal relationship of the onset of the event, relative to administration of the product, is likely to have another cause which can by itself explain the occurrence of the event.
- Possibly related: Temporal relationship of the onset of the event, relative to administration of the product, is reasonable but the event could have been due to another, equally likely cause.
- Probably related: Temporal relationship of the onset of the event, relative to administration of the product, is reasonable and the event is more likely explained by the product than any other cause.
- Definitely related: Temporal relationship of the onset of the event, relative to administration of the product, is reasonable and there is no other cause to explain the event, or a re-challenge (if feasible) is positive.
- Where an event is assessed as possibly related, probably related, definitely related the event is an adverse reaction.

6.4 Expectedness

Adverse reactions must be considered as unexpected if they add significant information on the specificity or severity of an expected adverse reaction. The expectedness of an adverse reaction shall be determined according to the reference documents.

- Expected: Reaction previously identified and described in protocol and/or reference documents.
- Unexpected: Reaction not previously described in the protocol or reference documents.

All AEs occurring during the period from screening visit to the trial completion will be registered and reported if applicable.

For all adverse event/reactions the investigator will make an assessment of intensity, causality, expectedness and seriousness.

The PI will keep the Sponsor and the REC informed of any significant findings.

At the end of the study all adverse events recorded during the study will be subject to statistical analysis and analysis and subsequent conclusions will be included in the final study report. All AEs experienced by study subjects will be registered. After trial completion these study subjects will be unblinded.

6.5 Expedited reporting of serious adverse events

All patient safety related incidents will be reported according to University Hospital Southampton NHS Foundation Trust (UHS) Incident Reporting and Management Policy. In addition to the Trust Incident reporting, SAEs are expedited to the people and departments identified below. The PI (or delegated person) will make an initial report, orally or in writing. The initial report will include as much information as is available at the time.

The PI (or delegated person) will report the following:
SUSAR
Immediately report to:
- the PI
- the sponsor
- UHS R&D department
- UHS patient safety team (using Trust incident Reporting form)
- the University of Southampton

UHS will be responsible to further expedite the Reporting of SUSAR to the REC that gave approval as soon as possible but within 7 days. The investigator (or delegated person) will make an initial report, orally or in writing. The initial report will include as much information as is available at the time. Oral reports will be followed up in writing within a further 24 hours of the initial report.

After the initial report the investigator will actively follow up the subject. The
Investigator (or delegated person) will provide information missing from the initial report within five working days of the initial report.

Written reports will be made by completing an SAE/SUSAR reporting form provided by University Hospital Southampton R&D.

UHS incident report template available from UHS Staffnet or departmental log books

SAE
Within 24 hours report to:
- the PI
- the Sponsor
- UHS R&D Department
- the University of Southampton

As above; but no expedited reporting to the REC.

Urgent Safety Measures/ Temporary Halt of the Trial
Implement and report immediately as a substantial amendment to:
- the PI
- the Sponsor

The PI
must inform as soon as possible but within 3 days:
- the REC that granted approval
- the University of Southampton

The Sponsor and the PI must be notified of any urgent safety measures/temporary halt of a trial that have had to be taken that are not part of the protocol.

The report must include the reasons for the urgent safety measure and the plan for further action.

7. Ethical and governance considerations

The study will be approved by an NHS Ethics Committee; such approval will be sought as soon as the protocol is finalised.

The study will be approved by the University of Southampton Research Governance Office.

The study sponsor will be University of Southampton.

The study will be registered at a relevant clinical trial registration site.

The study will be conducted in accordance with the recommendations for physicians involved in research on human participants adopted by the 18th World Medical Assembly, Helsinki 1964 as revised and recognized by governing laws and EU Directives; and the principles of GCP and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework and the Medicines for Human Use (Clinical Trial) Regulations 2004, as amended in 2006 and any subsequent amendments.

The PI will submit a final report at conclusion of the trial to the REC within the timelines defined in the Regulations.

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