Probiotic Regulation of Food Intake through Satiety Stimulation and Gut Microbiota Modulation

Probiotische Regulatie van Voedsel Inname door Bevordering van Verzadiging en Verandering van Darm Microbiota

Written by:

Tanja C. Adam, PhD

Department of Nutrition and Movement Sciences

Maastricht University Medical Center +

Maastricht University

P.O. Box 616

6200 MD Maastricht, The Netherlands

Telephone: +31-88-3882123

E-mail: t.adam@maastrichtuniversity.nl

PROTOCOL TITLE

‘**Probiotic Regulation of Food Intake through Satiety Stimulation and Gut Microbiota Modulation’**.

‘Probiotische Regulatie van Voedsel Inname door Bevordering van Verzadiging en Verandering van Darm Microbiota’.

Short title	Probiotics and Satiety
Version	1.0
Date	01-08-2025
Coordinating investigators	Iris Boelen, MSc Department of Nutrition and Movement Sciences Maastricht University Medical Center + Maastricht University P.O. Box 616 6200 MD Maastricht, The Netherlands Telephone: +31-88-3883644 E-mail: Iris.Boelen@maastrichtuniversity.nl T.C. Adam, PhD Department of Nutrition and Movement Sciences Maastricht University Medical Center + Maastricht University P.O. Box 616 6200 MD Maastricht, The Netherlands Telephone: +31-88-3882123 E-mail: t.adam@maastrichtuniversity.nl

Principal investigator	T.C. Adam, PhD Department of Nutrition and Movement Sciences Maastricht University Medical Center + Maastricht University 6200 MD Maastricht, The Netherlands t.adam@maastrichtuniversity.nl
Co-principal investigator	P.J. Joris, PhD Department of Nutrition and Movement Sciences Maastricht University Medical Center + Maastricht University 6200 MD Maastricht, The Netherlands p.joris@maastrichtuniversity.nl
Co-principal investigator	J. Penders, PhD Department of Medical Microbiology, Infectious Diseases and Infection Prevention Maastricht University Medical Center + Maastricht University 6200 MD Maastricht, The Netherlands j.penders@maastrichtuniversity.nl
Study physician	Dr. C.A.J. van Kalkeren Department of Nutrition and Movement Sciences, Maastricht University Medical Center + Maastricht University 6200 MD Maastricht, The Netherlands c.vankalkeren@maastrichtuniversity.nl
Sponsor	Maastricht University P.O. Box 616 6200 MD Maastricht, The Netherlands
Subsidizing party	Stichting LSH-TKI

Independent expert	Prof. Dr. R.P. Mensink, PhD Department of Nutrition and Movement Sciences, Maastricht University Medical Center + Maastricht University P.O. Box 616 6200 MD Maastricht, The Netherlands Telephone: 0883881308 E-mail: r.mensink@maastrichtuniversity.nl

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Principal investigator: T.C. Adam, PhD		1.8.2025

TABLE OF CONTENT

LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS 8

SUMMARY 8

1. INTRODUCTION AND RATIONALE 11

2. OBJECTIVES 13

3. STUDY DESIGN 14

3.1 Screening and Baseline measurements 14
3.2 Test days 14
3.3 Intervention 16

4. STUDY POPULATION 18

4.1 Population 18
4.2 Screening visit 18
4.3 Inclusion criteria 18
4.4 Exclusion criteria 19
4.5 Sample size calculation 19

5. TREATMENT OF SUBJECTS 20

5.1 Randomization, blinding, and treatment allocation 20
5.2 Probiotic intervention 20

6. INVESTIGATIONAL PRODUCT 22

6.1 Name and description of investigation product 22
6.2 Summary of findings from non-clinical studies 23
6.4 Summary of known and potential risks and benefits 24
6.5 Description of justification of route of administration and dosage 24
6.6 Dosages, dosage modifications and method of administration 25
6.7 Preparation and labelling of investigation product 25
6.8 Investigational product accountability 25

7. METHODS 26

7.1 Study procedures 26
7.2 Study parameters/endpoints 27
7.3 Withdrawal of individual subjects 32

8. SAFETY REPORTING 32

8.1 Temporary halt for reasons of subject safety 33
8.2 AEs, SAEs and SUSARs 33
8.3 Follow-up of adverse events 34

9. STATISTICAL ANALYSIS 35

9.1 Descriptive statistics 35
9.2 Statistical analysis 35

10. ETHICAL CONSIDERATIONS 37
10.1 Regulation statement 37
10.2 Recruitment and consent 37
10.3 Benefits and risks assessment, group relatedness 38
10.4 Compensation for injury 39
10.5 Incentives 39
11. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION 40
11.1 Handling and storage of data and documents 40
11.2 Monitoring and Quality Assurance 40
11.3 Amendments 41
11.4 Annual progress report 41
11.5 Temporary halt and (prematurely) end of study report 41
11.6 Public disclosure and publication policy 41
12. STRUCTURED RISK ANALYSIS 42
12.1 Potential issues of concern 42
12.2 Synthesis 42
13. REFERENCES 43

LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

BDI	Beck Depression Inventory
BL	Baseline
BMI	Body Mass Index
BP	Blood pressure
CCMO	Central Committee on Research Involving Human Subjects
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
FFQ	Food-frequency questionnaire
GLP-1	Glucagon-like Peptide 1
HDL	High-density lipoprotein cholesterol
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HR	Heart rate
LDL	Low-density lipoprotein cholesterol
METC	Medical research Ethical Committee
MUMC+	Maastricht University Medical Center +
MRUM	Metabolic Research Unit Maastricht
OGTT	Oral glucose tolerance test
POMS	Profile of Mood Scale
PSS	Perceived Stress Scale
PYY	Peptide YY
QoL	Quality of life
(S)AE	(Serious) adverse event
SBP	Systolic Blood Pressure
Sponsor	The sponsor is the party that commissions the organization or performance of the research, for example a pharmaceutical company, academic hospital, scientific organization, or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidizing party
TAG	Triacylglycerol
TC	Total cholesterol
TFEQ	Three Factor Eating Questionnaire
TNF	Tumor necrosis factor
VAS	Visual Analogue Scale
WMO	Medical Research Involving Human Subjects Act In Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: Satiety hormones, including glucagon-like peptide-1 (GLP-1), are key regulators of food intake. Deficiencies in the release of satiety hormones as well as alterations in the composition and the function of the gut microbiota are hallmarks of overweight and obesity. Probiotics can improve the overall structure of the gut microbiota, and show strain-specific differences with regard to their ability to stimulate satiety hormones. Those animal-based findings, if confirmed in human studies, may pose a gateway for decreased food intake, weight management, and ultimately weight loss. We hypothesize that a newly developed combination of probiotic strains, specifically selected for their potential to *(in vitro)* influence pathways related to weight management, will decrease food intake through increased release of satiety hormones (i.e. GLP-1) and beneficial alteration of the gut microbiota in humans.

Objective: The primary objective of the study is to investigate the effects of a combination of specifically selected probiotic strains in a new probiotic formulation on *ad libitum* food intake, in individuals with overweight and/or obesity.

We will address this objective firstly with an acute study followed by a proof-of-concept 4-months intervention study comparing a total of 98 individuals in a 16-week randomized, placebo-controlled parallel study (49 participants probiotic/ 49 participants placebo).

Study design: This intervention study will have a randomized, controlled parallel design. The total study duration will be 16 weeks.

Study population: A total of 98 men and women between the ages of 20-70 years old, with overweight/obesity (BMI: 26-40 kg/m^2) will be recruited, accounting for a drop-out rate of 20%.

Intervention: Study participants will receive a daily dose of 6 g probiotic supplement (packed in sachets of 3 g) or placebo for 16 weeks.

Main study parameters/endpoints: At baseline and after 16 weeks, participants will visit the research facilities for assessments. The primary endpoint is the difference in satiety measured by *ad libitum* food intake comparing the probiotic and control intervention. Secondary outcome parameters are related to changes in satiety hormones (including GLP-1), fecal microbiota analysis, insulin sensitivity (HOMA-IR), inflammatory markers and lipid metabolism**.** Exploratory outcoms concern perceived satiety, body composition and mental health.

Nature and extent of the burden and risks associated with participation, benefit, and group relatedness: Subjects will be screened to determine eligibility during one visit of 60 minutes. During the screening visit, anthropometric measurements will be performed and blood pressure will be determined. During the probiotic intervention, participants will receive a daily 6 g of probiotic supplement for 16 weeks. Newly combined strains all have been tested in previous trials, are considered safe, and no expected side effects related to supplement consumption are expected. During the trial at different occasions, tests will be performed and blood will be sampled (a total of 436 ml for the whole trial). During these tests, subjects have

to stay at the university and are allowed to only eat the food they receive from the study team. Some subjects may report pain during venipuncture. Insertion of the cannula can cause some discomfort and possibly a hematoma or bruise. Some adults may also report pain during the insertion of the cannula. Other measurements (i.e. body composition) are routine and are not expected to lead to physical side effects. Participants that do not fully adhere to the study protocol will be excluded from the statistical analyses, because a per protocol analysis will be performed. The total time investment is 17 hours (1000 minutes), excluding travel time. This study will provide insight into the potential beneficial effect of a newly composed probiotic supplement on satiety in overweight/obese adults. Participants will not perceive direct benefits from the study. A benefit for the study population is the acquirement of scientific knowledge concerning underlying mechanisms with regard to satiety and intestinal health.

1. INTRODUCTION AND RATIONALE

Food intake is regulated through an interplay of physiological and psychological parameters conveying either hunger or satiety from gut to brain [1 2]. Satiety hormones, which are released upon nutrient sensing in the intestine, convey satiety perception to the brain through stimulation of receptors in the hypothalamus and in the brainstem. One of the key players responsible for the perception of satiety is glucagon-like peptide 1 (GLP-1), a hormone decreasing food intake, after being released from intestinal L cells in proportion to the size of a meal [1-3]. In individuals living with overweight or obesity however, GLP-1 release appears to be changed, suggesting a role for GLP-1 in the pathophysiology of obesity [4-6]. The gut microbiome, summarizing all microorganisms residing within the human intestine (i.e. bacteria, fungi, and viruses) is continually reshaped by environmental factors such as diet. Several gut microbiota metabolites such as short chain fatty acids and bile acids, have been shown to promote GLP-1 production [7 8], posing a link between gut microbiota and satiety signaling for weight management.

Gut dysbiosis has been previously associated with obesity through dysregulated satiety signaling, altered short chain fatty acid production and low-grade inflammation. The modulation of the gut microbiota is therefore an emerging target for the treatment and the prevention of metabolic disease, including obesity [7].

Probiotics are 'live microorganisms conferring a health benefit to the host' [9], with the ability to improve the overall structure of the host gut microbiota [10 11]. Certain probiotics were shown to decrease food intake in the short term [12] and have encouraged optimism on the strain-specific ability to stimulate satiety hormones, including GLP-1 and insulin [13]. Evidence so far however is predominantly based on animal experiments [14 15] and translation to humans is warranted. A combination of promising bacterial candidate strains, chosen for their specific ability to stimulate GLP-1 and other satiety hormones *in vitro*, may have additive and/or synergistic effects regarding food intake through microbiota mediated effects, and may pose a promising pathway for weight management and overall improvement of metabolic health.

More than one billion people in the world are living with obesity. Although pharmacological incretin agonists are available on prescription now, their availability is limited to a very specific population at a very high cost, side effects can be severe and studies on long-term effects are sparse [16]. Therefore, the urgent need for lifestyle-based interventions that are affordable and available to the general public remains unchanged. While there is some evidence for a role of probiotics in satiety, no studies to date have assessed a combination of bacterial strains specifically selected and geared at the stimulation of satiety hormones, including GLP-1. Well-controlled human studies providing insight in short- as well as long term effects are lacking.

The study has i. the unique and innovative opportunity to provide new hypotheses for the scientific community working to improve metabolic health in humans, ii. delivers novel dietary strategies for consumers, and iii. gives leads for product development for the treatment and prevention of metabolic disease.

The primary objective of the study is to investigate the effects of a new combination of probiotic strains, specifically selected for their potential to modulate satiety hormones such as GLP-1, on satiety, measured by *ad libitum* food intake in individuals with overweight and/ or obesity.
Secondary objective is to assess the effects of the new combination of specific probiotic strains on satiety related hormones, perceived satiety, intestinal microbiota composition, insulin sensitivity, and lipid metabolism.
Our fundamental hypothesis is that a newly developed/formulated combination of probiotic strains, specifically selected for their potential to stimulate satiety hormone release, will decrease food intake and improve satiety through increased GLP-1 release and beneficial modulation of the gut microbiota.

A randomized, controlled parallel study with 98 overweight/obese men and women (aged 20-70) will be performed. Participants will consume 6 g of a probiotic supplement (2 x 3 g) or placebo for 16 weeks on a daily basis. Participants will be randomized into the two intervention arms. Before and after the intervention, the primary outcome food intake will be assessed by means of an *ad libitum* lunch meal 4 hours after a standardized breakfast. The lunch meal will consist of Lasagna together with 300 ml water (8 MJ, protein: 15.2 E%, fat: 30 E%, and carbohydrates: 54.8 E%). Participants who follow a vegetarian diet will receive an energy matched vegetarian version of the lasagna. They will be instructed to drink all water and eat until they feel comfortably satisfied. Afterwards, food intake will be measured, and total energy intake will be calculated. To further determine satiety, blood samples will be drawn to determine hormone concentrations. Furthermore, visual analogue scales (VAS) will be used to register different subjective aspects of appetite, thirst, and well-being during the test day. Stool samples will be collected pre- post- and mid-intervention to determine changes in gut microbiome. Questionnaires will be used to collect information on psychological well-being and physical activity.

2. OBJECTIVES

The following research questions are formulated given the provided background information.

Primary research question:

What is the effect of a 16-week probiotic supplement intervention on satiety - quantified by ad libitum food intake - as compared with a 16-week placebo intervention in overweight/obese study participants?

Major null hypotheses, H_0:

A 16-week probiotic supplement intervention does not affect ad libitum food intake - a marker of satiety - in overweight/ obese men and women compared to a 16-week placebo intervention.

Major alternate hypotheses, H_a:

A 16-week probiotic supplement intervention significantly affects ad libitum food intake - a marker of satiety - in overweight/ obese men and women compared to a 16-week placebo intervention.

Secondary research question:

What are the effects in overweight/obese men and women of a 16-week probiotic supplement intervention on physiological measures of satiety (GLP-1), the microbiome, insulin sensitivity, inflammatory markers and lipid metabolism compared to a 16-week control intervention?

Exploratory research questions:

What are the effects of a 16-week probiotic intervention on:

- other functional outcomes, including perceived hunger and satiety, mental health and overall well-being (focusing on mood, stress, sleep and quality of life);
- other potential mechanisms underlying effects satiety, including changes in body composition?
- gut microbiota-related mechanisms potentially involved in satiety, insulin sensitivity and body weight management.

3. STUDY DESIGN

A longer-term, double-blind, randomized, placebo-controlled parallel study will be conducted.

3.1 Screening and Baseline measurements

Before screening, subjects will be informed about the procedures and informed consent will be obtained. Following screening, study participants that fulfill all inclusion criteria and are willing to participate will come for baseline measurements.

Subjects will be informed about their baseline measurements, including anthropometric measures (weight, length, body mass index), systolic and diastolic blood pressure (SBP and DBP). When treatment with drugs or lifestyle interventions is advised (e.g., fasting glucose ≥ 7.0 mmol/L, fasting serum total cholesterol ≥ 8.0 mmol/L, SBP ≥ 160 mmHg or DBP ≥ 100 mmHg), study subjects will be advised to consult their general practitioner. The results obtained during the intervention trial (i.e. satiety hormone concentrations) will be reported in a descriptive way at a group level, for example: “satiety hormone concentrations are increased/ decreased”, because no accepted normal ranges exist for these measurements.

3.2 Test days

On the test days (baseline, week 8 and week 16) before blood sampling, adults are asked not to perform any strenuous physical exercise or to consume alcoholic beverages. On the morning of blood sampling - after a 12-hour overnight fast (from 8.00 PM) - subjects are only allowed to drink up to two glasses of water together with the study product (probiotic supplement or placebo) and receive a standard breakfast from the study team. Subjects are also asked not to change their habitual diet prior to the test days.

During test days at baseline and at week 16, a total of 8 blood samples will be taken starting 30 minutes before breakfast to assess satiety hormone concentrations. During the test day at week 8 a fasting blood sample will be taken. Stool samples and questionnaires will be taken at all test days (baseline, week 8 and week 16).

3.2.1 Measurements

Subjects will arrive in the morning at the MRUM research facilities. They need to come fasted for all test days. Test rooms are temperature controlled at 22°C.
On all test days, anthropometric measurements (weight, length, waist and hip circumferences) will be performed to characterize study participants and office BP (Omron M7, CEMEX Medische Techniek) will be measured. Thereafter, a fasting blood sample will be collected.

Ad libitum food intake (Mealtest): On the day of the meal test (baseline and week 16), participants arrive at the Metabolic Research Unit Maastricht (MRUM) at about 8.00 and a fasted blood sample will be taken. A standardized breakfast meal will be served. Participants will have a maximum of 15 minutes to eat the breakfast. Hereafter, blood samples will be drawn at time: 15, 30, 45, 60, 90, 120 and 240 minutes. After the 240-minute blood sample, participants will be served an *ad libitum* lunch meal consisting of Lasagna together with 300 ml water (8 MJ, protein: 15.2 E%, fat: 30 E%, and carbohydrates: 54.8 E%). The participants will be instructed to drink all water and eat until they feel comfortably satisfied. Afterwards, food intake will be measured, and the energy intake will be calculated.

Blood samples/ Satiety hormones: To determine satiety hormones, blood samples will be drawn at time: -30,15, 30, 45, 60, 90, 120 and 240 minutes. For analyses of satiety hormones, EDTA-aprotinin tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) will be used. Syringes and EDTA-aprotinin tubes will be ice-chilled before blood collection and samples will be snap frozen in liquid nitrogen immediately. Total GLP-1, PYY3-36 and ghrelin concentrations will be determined using a radioimmunoassay method. A total of 436 ml blood will be drawn throughout the study. A 4ml fasted blood sample will be taken at screening to exclude type 2 diabetes.

Subjective appetite sensation Visual Analogue Scales (VAS) will be used to register different aspects of appetite, thirst, and well-being during the test day. VAS will be conducted showing a 100 mm horizontal line with a question above the line and will be anchored with two extremes of answers at each end. The questions concern hunger, satiety, fullness, thirst, prospective food consumption, and desire to eat. Participants will be asked to mark their responses to the questions with a vertical line (18). Appetite sensation will be assessed for a total of 8 times throughout the testday. Assessments will take place at -30 minutes before the standard breakfast and +30: +60; +90; +120; +180; +240 and +270 minutes after breakfast.

Fecal microbial composition Stool samples will be collected at home and immediately stored in the freezer at -20°C. Participants will bring the stool sample to the laboratory, while preserving the cold chain, where samples will be kept in a -80°C freezer until further analysis.

Diet and physical activity: Participants will be instructed to continue their usual physical activity and dietary regimen throughout the whole intervention period to study the isolated

effect of probiotic supplementation. At baseline, at week 8 and in week 16 participants' diet and physical activity pattern will be assessed. Participants will complete a 4-day dietary record, which includes week as well as weekend days. In addition, they will fill out the SQUASH questionnaire to assess 24-hour daily activity patterns for 1 week.

Questionnaires include the assessment of eating behavior with the three-factor eating questionnaire (TFEQ), stress perception with the perceived stress scale (PSS), assessment on depression with the Beck Depression Inventory (BDI), and mood with the Profile of Mood Scale (POMS). All assessments are validated and reliable instruments of mental health assessments.

3.3 Intervention

Participants will receive daily 6 g of probiotic supplement or placebo for 16 weeks. Beneficial results from individual strains of the this newly composed supplement have been reported previously [17-20]. During the intervention, adults will visit our research facilities every eight weeks to receive new supplies and/or talk with the coordinating investigator(s) in case there will be questions. Investigators will also be available for participants over the phone or via email throughout the whole intervention. Two sachets for daily consumption containing 3 g of probiotic supplement each, or placebo, will be provided in 8-week allotments to the adults during the intervention at the time of their monthly consult with the investigator(s). Throughout the present study, adults will be instructed to dissolve one sachet (3 g) in the morning before breakfast, and one sachet (3 g) in the evening before they go to bed. On the test days both sachets will be taken at the university. All participants will be requested and instructed to keep their habitual diet. Finally, food intake between test days will be measured using validated food-frequency questionnaires (FFQs), which will be checked in the presence of the coordinating investigator(s) immediately.

Participants are not allowed to use other supplements, in their background diet. Also, participation within another trial is not allowed. Except for these restrictions, they will maintain their habitual lifestyle throughout the total study period. Finally, participants will be requested to record daily in study diaries any signs or symptoms of illnesses, the use of medication, consumption of alcohol, any deviations of the study protocol and any other complaints. All adverse events reported spontaneously by the participants or observed by the investigators will be recorded.

Empty bags and daily portions that are not used must be returned at each visit to calculate the compliance of the study participants. Subjects will be considered compliant when they take at least 80% of the study product.

4. STUDY POPULATION

4.1 Population

Subjects will include 98 men and women (aged 20-70 years) with overweight or obesity (BMI: ≥ 25 kg/m^2). Because we do not want to encumber these adults unnecessarily, we have decided to set this upper limit for age. Subjects should be free of clinical diseases as assessed by medical history and blood chemistry and weight stable for at least three months before the start of the study.

4.2 Screening visit

The subjects will be invited for a screening visit, this visit will include recording of:

- Body weight
- Height
- Waist circumference
- Blood pressure: systolic (SBP) and diastolic (DBP)
- Use of medication and/ or supplements
- Current and history of diseases

Men and women with overweight/ obesity will attend the research facilities for a screening visit. During this visit, anthropometric measurements (weight, length, waist circumference) will be performed and BP (SBP and DBP) will be determined in four-fold (the first measurement will be discarded, and the last three measurements will be averaged). A fasted blood sample (4ml) will be taken to determine fasted glucose concentration and to exclude type 1 and 2 diabetes. In case the study participants are blood donors, they will be told that they cannot donate blood from 8 weeks before the start of the study, during the study and for 4 weeks after completion of the study. When participants fulfill all inclusion criteria as formulated in **paragraph 4.3**, they may enter the study.

4.3 Inclusion criteria

The inclusion criteria are:

- Men and women, aged between 20-70 years
- BMI ≥ 25 kg/m^2
- Stable body weight (weight gain or loss < 3 kg in the past three months)
- Willingness to give up being a blood donor from 8 weeks before the start of the study, during the study and for 4 weeks after completion of the study

- No difficult venipuncture as evidenced during the screening visit

4.4 Exclusion criteria

The exclusion criteria are:

- Diabetic patients (type 1 and type 2)
- Abuse of drugs
- More than 2 units alcoholic consumptions per day for women and 3 for men
- Use of products or dietary supplements known to interfere with the main outcomes 3 months prior to the start of the study as judged by the principal investigators
- Gastrointestinal disease (including IBD and IBS)
- Use of an investigational product within another biomedical intervention trial within the previous 1-month
- Severe medical conditions that might interfere with the study, such as epilepsy, asthma, kidney failure or renal insufficiency, chronic obstructive pulmonary disease, autoimmune disease and rheumatoid arthritis
- Active cardiovascular disease like congestive heart failure or cardiovascular event, such as an acute myocardial infarction or cerebrovascular accident
- use of lipid- and glucose lowering medication, corticosteroid use
- Psychiatric diagnoses including clinical depression
- Intolerances or dietary habits that might interfere with the study (i.e. lactose intolerance or veganism)
- pregnancy
- smoking, vaping
- use of antibiotics up to 3 months prior to the study

4.5 Sample size calculation

Based on data derived from Bjerg et al. [12] reporting a 15% difference in *ad libitum* food intake after probiotic supplementation, and Gregersen et al. [21], who have assessed the reproducibility and power of ad libitum energy intake assessed by repeated single meals. Thirty-nine participants per group (N = 39) are needed to detect a change in our primary outcome parameter *ad libitum* food intake with a power of at least 80%, an α value of .05 (two-sided) and an effect size of 15% difference. The inclusion of 98 participants accounts for a 20% drop-out rate. In our previous study involving a total of 32 overweight/obese subjects, weight loss reduced postprandial GLP-1 concentrations [4], indicating that we also have enough power for the analysis of the secondary outcome parameters listed in this proposal.

5. TREATMENT OF SUBJECTS

After screening participants have to attend the research facilities to perform the baseline measurements. Participants will receive their first two sachets of supplement (or placebo) to be taken on the morning of the baseline measurements. After baseline measurements adults will receive daily 6 g of probiotic supplement or placebo for 16 weeks. Anthropometric data, blood and stool samples will be collected at baseline and after 8 and 16 weeks.

5.1 Randomization, blinding, and treatment allocation

Participants will receive daily 6 g of probiotic supplement or placebo for 16 weeks. A computerized randomization schedule will be generated (a priori) and safeguarded by an independent researcher, who is neither involved in data analysis, not has any contact with participants. In the schedule, blocks of 4 study participants will be made. Within each block, participants will be randomly allocated to one out of two interventions (probiotic supplement or placebo) in a 1:1 ratio. Adults will be assigned to a participant number, which corresponds with the numbers of the randomization schedule, in the order in which they sign the informed consent. Outcome assessors are thus masked to the allocation process. The coordinating and investigating researchers as well as the research participants will be blinded to the intervention. Both, the placebo and probiotic formulation will be made available in 3g dry powder sachets and will be indistinguishable in looks, taste, and consistency.

5.2 Probiotic intervention

Participants will receive daily 6 g of probiotic supplement or placebo for 16 weeks. Supplement and placebo are matched for energy and macronutrient content. Nutrition facts (per 100g) are shown in **Table 1**.

Table 1: Nutrition facts of the product (per 100g)

	Probiotic supplement	**Placebo**
Energy (kcal)	310	357
Protein (g)	3.1	2.2
Total fat (g)	0.4	0.5
Saturated fat (g)	0.1	0.1
Carbohydrates (g)	64.8	87.1
Dietary fiber (g)	21.7	0.5

During the intervention, adults will visit our research facilities every eight weeks to receive new supplies and/or talk with the coordinating investigator(s). Sachets for daily consumption containing 3 g of probiotic supplement or placebo will be provided as 8-week allotments to the participant during the intervention at the time of their consult with the investigator(s). Throughout the study, participants will be instructed to consume the probiotic supplement as well as the placebo dissolved in 300 ml of water 30 min before the first meal in the morning or at least 30 min after the last meal before they go to bed. On the days of blood sampling, the supplement will be consumed 30 min before the first blood draw. Participants will be requested and instructed to adhere to their habitual diet, meaning that intervention effects will be evaluated as part of a habitual diet. Instructions from the investigator(s) prior to and during the intervention will help the participants to follow the instructions. Additionally, subjects will receive written information. In fact, we already have extensive experience with this research approach that has deliberately been chosen, since it closely resembles the real-life setting, maximizing the applicability of the study results.

Participants are not allowed to use other supplements, in their background diet. Also, participation within another trial is not allowed. Except for these restrictions, they will maintain their habitual lifestyle throughout the total study period. Finally, participants will be requested to record daily in study diaries any signs or symptoms of illnesses, the use of medication, consumption of alcohol, any deviations of the study protocol and any other complaints. All adverse events reported spontaneously by the participants or observed by the investigators will be recorded.

Empty bags and daily portions that are not used must be returned at each visit to calculate the compliance of the study participants. Subjects will be considered compliant when they take at least 80% of the study product.

6. INVESTIGATIONAL PRODUCT

6.1 Name and description of investigation product

The multispecies probiotic formulation Winclove 750 and the placebo will be produced and provided by Winclove Probiotics (Amsterdam, the Netherlands) for the participants of this study. Participants will receive two sachets per day for a duration of 16 weeks. The probiotic formulation consists of 9 probiotic strains, selected to improve satiety hormone signaling, lipid accumulation, bile acid metabolism, immune and inflammatory properties, as well as oxidative stress: *Bifidobacterium breve* W25, *Bifidobacterium lactis* W51, *Lactobacillus acidophilus*

W22, *Lactobacillus brevis* W63, *Lactobacillus gasseri* W137, *Lactobacillus plantarum* W21, *Lactobacillus reuteri* W192, *Lactobacillus rhamnosus* W5, *Lactococcus lactis* W58. The product is provided as a white to ivory colored powder. Both, the placebo and probiotic formulation will be made available in 3g dry powder sachets and will be indistinguishable in looks, taste, and consistency.

Ingredients of the probiotic formulation sachet: Maize starch, Acacia fibre P16, Maltodextrin Inulin P2, Hydrolysed rice protein, Enzymes (amylase), Probiotic strains (*Bifidobacterium breve* W25, *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W22, *Lactobacillus brevis* W63, *Lactobacillus gasseri* W137, *Lactobacillus plantarum* W21, *Lactobacillus reuteri* W192, *Lactobacillus rhamnosus* W5, *Lactococcus lactis* W58).

Ingredients of the placebo sachet: Maize starch, Maltodextrin Inulin P2, Hydrolysed rice protein, Enzymes (amylase).

The technical data sheets with detailed product and placebo information can be found in **C1_Appendix_1A** and **C1_Appendix_1B**, respectively.

Each sachet will be dissolved in approximately 300 ml of water prior to consumption. All sachets will be manufactured according to generally accepted procedures and Good Manufacturing Practices. Winclove Probiotics will take care of the costs for the production, packaging, and transport of the sachets, as well as quality control. The sachets will be produced in several batches after each of which Winclove Probiotics will check the quality and composition. Hereafter, the sachets will be sent to the NUTRIM institute at the Maastricht University. Sachets will be stored at the department of Nutrition and Movement Sciences at room temperature (15-25°C).

Table 2: Winclove 750 product specification

Analysis	Limits
Appearance	White to ivory coloured powder with good flow
Solubility	Waterdispersable, partly soluble
Density	0.4-0.7 g/ml
Salmonella spp.	Absent in 25 grams
Coagulase positive staphylococcus	Absent in 1 gram
Escherichia coli	Absent in 1 gram

Bacillus cereus	< 500 cfu/g
Moulds and Yeasts	< 500 cfu/g
Moisture content	< 5%

6.2 Summary of findings from non-clinical studies

The strains of this new product were selected based on in-vitro assay for satiety hormone stimulation, leptin and lipid accumulation, bile acid metabolism, oxidative stress, and immune modulatory effects. GLP-1 was significantly increased (≥150%) by *L. rhamnosus (W5) and L. brevis (W63) whereas* CCK was increased by *L. rhamnosus (W5) and L. brevis (W63).* PYY was increased by: *L. rhamnosus (W5), L. gasseri (W137), L. brevis (W63), B. breve (*W25), and GIP was increased by *B. breve (W25).*
Regarding the screening for leptin and lipid accumulation, *B. animalis (W51)* showed the most promising dual benefit by decreasing lipid accumulation and increasing leptin secretion. By analyzing the activity of bile salt hydrolase (Bsh) the metabolism of secondary bile acids was analyzed. Bile salts may contribute to GLP-1 secretion (via TGR5 activation) and cholesterol metabolism, both of which are relevant to weight control. *B. lactis (W58)* showed activity in this assay *with L. reuteri (W192) showing the highest Bsh activity.*

By analyzing a big dataset on the transcriptome profile of co-culture of PBMCs and probiotic strains, the effects of our strains on immune modulation, inflammatory responses and oxidative stress were determined. All strains showed potential benefit based on this screening, with the best performing strain from this scoring system being *Lactobacillus gasseri* (W137) for immune and inflammatory responses.

6.3 Summary of findings from clinical studies

No clinical interventions have been performed with this exact combination of strains, as the formulation was specifically developed for the purpose of the study. Three of the 9 strains of the formulation of the product developed for this study (W51, W63, and W58) are also part of the probiotic formulation Ecologic® Barrier, that has been on the market for over 20 years. Several clinical studies have been performed, showing improvements across several areas of metabolic health (**see C1_Appendix_2_BARRIER_studyoverview**).

6.4 Summary of known and potential risks and benefits

The probiotic formulation developed for this study contains only qualified presumption of safety (QPS) strains. Additional safety documentation is attached to this document under D6 (product information).

As food supplement, probiotic formulations are generally considered safe. Most previous studies with probiotics have not resulted in adverse events, besides incidental sensations of bloating and change in stool consistency (Allen et al., 2010; Huurre et al., 2008; Kuitunen et al., 2009; Kukkonen et al., 2008; Luoto et al., 2010; Nierset al., 2009).

However, Besselink et al. (2008) terminated their RCT treating acute pancreatitis with probiotics because of increased mortality in the experimental group. Of course, this could have been a coincidence, but it does highlight the potential risk of probiotics in critically ill people. The present study excludes people with critical conditions of the pancreas, liver and bowel.

A main general benefit of probiotics is supporting a healthy digestive tract which was reinforced by evidence gathered on a large number of different probiotic strains representing commonly studied species (Hill et al., 2014).In a meta-analysis performed by Ritchie and Romanuk (2012) which included a broad range of probiotic strains; containing 74 studies, 84 trials and 10,351 patients, the authors concluded that, in general, probiotics are beneficial in the treatment and prevention of gastrointestinal diseases. Probiotics have also shown to have immune modulatory effects as well as effects on the epithelial barrier in healthy adults (Ohland & Macnaughton, 2010; Klaenhammer et al., 2012).

6.5 Description of justification of route of administration and dosage

The probiotic formulation and placebo will be provided as a powder that should be dissolved in water, which makes it easy to consume. The daily dose of the probiotic was determined based on clinical efficiency of other probiotic products, technological and commercial considerations, and is considered safe. The probiotics will be delivered in two sachets per day of 3 g each, that should be dissolved in water, and administered twice daily (eg. one sachet before breakfast, the second one after dinner). The placebo will also be provided by Winclove Probiotics. It contains all components of the probiotic formulation except the bacteria and acacia fiber, including maize starch and maltodextrin. The placebo and probiotic had indistinguishable appearance, shape, smell, consistency, and taste.

6.6 Dosages, dosage modifications and method of administration

The daily dose will be 6 grams a day with the daily dose of 1,3 x 10^{10} cfu/day, portioned in sachets of 3 grams. Participants will be instructed to add the powder to a glass of water (approx. 300ml) and to stir well before drinking it in the morning and evening.

6.7 Preparation and labelling of investigation product

The probiotic will be provided in single dose foil sachets. For the duration of 16 weeks, the participants will receive 2 boxes per 8-week period, containing 56 sachets each. Boxes will be separately labeled in accordance to GMP guidelines, with the name, address and telephone number of the sponsor, dosage form, route of administration, quantity of dosage units; the batch and/or code number to identify the contents and packaging operation; the trial reference code allowing identification of the trial; the trial subject identification number/treatment number; the name of the investigator; directions for use; “For clinical trial use only” or similar wording; the storage conditions; period of use (use-by date, expiry date or re-test date as applicable) and “keep out of reach of children”.

6.8 Investigational product accountability

Study product will be stored in a secure area with restricted access. The investigator agrees that the study product will be dispensed only to study subjects who have provided written consent. Compliance will be monitored by return of all product containers and sachets (used and unused). Any deviations will be noted in the Source documents and the subjects CRF’s. Participants will be instructed to store the sachets at room temperature away from moisture, direct heat and sunlight. Subjects will be asked to return any non-used sachets, which will be destructed as appropriate after the number of returned sachets has been counted.

7. Methods

7.1 Study procedures

Overweight or obese men and women will attend the research facilities for a screening visit. During this visit, anthropometric measurements (weight, length, body mass index) will be performed and BP (SBP and DBP) will be determined in four-fold (the first measurement will be discarded, and the last three measurements will be averaged). In case the study participants are blood donors, they will be told that they cannot donate blood from 8 weeks before the start of the study, during the study and for 4 weeks after completion of the study. When subjects fulfill all inclusion criteria, they may enter the study.

On the days test days after screening (baseline, week 8 and week 16), adults are asked not to perform any strenuous physical exercise or to consume alcoholic beverages. On the morning of the baseline measurement - after a 12-hour overnight fast (from 8.00 PM) - subjects are only allowed to drink up to two glasses of water. Subjects are also asked not to change their habitual diet prior to the test days.

For baseline measurements, participants have to attend the research facilities for blood draws, before and after a standardized breakfast and an *ad libitum* lunch.
Measurements at baseline will be repeated at follow up week 16. During these tests, participants have to stay at the university and are not allowed to eat anything other than the food provided by the study. Adults will receive daily 6 g of probiotic supplement or placebo for a total of 16 weeks. On week 8 measurements will be limited. Only anthropometric data, another blood sample and a stool sample will be collected, but no extensive meal test with multiple blood draws will be conducted.
At 16 weeks study participants have to attend the research facilities to perform the follow-up measurements which are equal to the baseline measurements. During the intervention, adults will visit our research facilities every eight weeks to receive new supplies and/or talk with the coordinating investigator(s).

All venipunctures and measurements will be performed as much as possible by the same investigator, at the same location, and at the same time of the day. Serum or plasma will be obtained by centrifugation and stored as appropriate. Samples will be stored coded for 15 years for possible further analyses. Only the study investigators have access to the code. When possible, samples from one subject will be analyzed within one run at the end of the study under strict quality control. The blood-sampling scheme at each study day is given in **Table 3**.

The amount of blood drawn will be 436ml (4ml at screening; 200 ml during the baseline and week 16 visit and 32 ml at week 8) during the whole study period.

Table 3: Blood sampling scheme at each day

	screening	Baseline-measurements	Blood sample 8 weeks	Blood sample 16 weeks
Action	1 blood sample	8 blood samples	1 blood sample	8 blood samples
Blood volume (ml)	4	200	32	200

7.2 Study parameters/endpoints

The primary objective is to investigate the effect of a 16-week supplementation with a probiotic in overweight or obese men and women compared to placebo on satiety, while we will also focus on on satiety related hormones, perceived satiety, intestinal microbiota composition, insulin sensitivity, and lipid metabolism (secondary objectives). Satiety will be assessed by means of an *ad libitum* food intake challenge.
Based on new insights that are related to the original research question, it may be decided to analyze the samples for other parameters related to food intake regulation. Consequently, adults will explicitly be asked in the informed consent form to agree with this approach.

7.2.1 Ad libitum food intake

On the day of the meal test, participants arrive at the Metabolic Research Unit Maastricht (MRUM) at about 8.00 and a fasted blood sample will be taken. A standardized breakfast meal will be served. Participants will have a maximum of 15 minutes to eat the breakfast. Hereafter, blood samples will be drawn at time: 15, 30, 45, 60, 90, 120 and 240 minutes. After the last blood sample (240 minutes) participants will be served an *ad libitum* lunch meal consisting of Lasagna together with 300 ml water (8 MJ, protein: 15.2 E%, fat: 30 E%, and carbohydrates: 54.8 E%). The participants will be instructed to drink all water and eat until they feel comfortably satisfied. Afterwards, food intake will be measured, and the energy intake will be calculated.

7.2.2 Satiety hormones

To determine satiety hormones, blood samples will be drawn at time: 0, 15, 30, 45, 60, 90, 120 and 240 minutes. For analyses of satiety hormones, EDTA (Becton, Dickinson and Company, Franklin Lakes, NY, USA) will be used. Syringes and EDTA-aprotinin tubes will be

ice-chilled before blood collection and samples will be snap frozen in liquid nitrogen immediately. Total GLP-1, PYY3-36 and ghrelin concentrations will be determined using a radioimmunoassay method.

7.2.3 Subjective Appetite Sensation

Visual Analogue Scales (VAS) will be used to register different aspects of appetite, thirst, and well-being during the test day. VAS will be carried out using 100 mm horizontal lines with a question above the line and will be anchored with two extremes of answers at each end. The questions concern hunger, satiety, fullness, thirst, prospective food consumption, and desire to eat. Participants will be asked to mark their responses to the questions with a vertical line (18). Appetite sensation will be assessed at 30-minute intervals starting at time -30 until after lunch for a total of 270 minutes.

7.2.4 Fecal microbial composition

Stool samples will be collected at home and immediately stored in the freezer at -20°C. Participants will bring the stool sample to the laboratory, while preserving the cold chain, where samples will be kept in a -80°C freezer until further analysis.
We will profile the gut microbiota composition at baseline and after treatment using 16S rRNA gene amplicon sequencing techniques. Sequencing data will be processed using our validated in-house Bio-IT pipeline and microbial richness, diversity, community structure and taxonomic composition will be linked to the probiotic intervention as well as treatment outcomes.
In addition, we will collect and use biobank samples to be able to measure microbial metabolites (e.g., SCFA and bile salts) in the future. This would enable us to gain more functional insights into the microbiota in association to probiotic intervention and treatment outcomes. Together these data will elucidate whether i. the baseline microbiota impacts probiotic colonization and treatment outcomes (responders vs non-responders), ii. probiotic supplementation influences the microbiota composition, and iii. probiotic-induced shifts in microbiota composition impact treatment outcomes. These findings will provide important leads to potentially further improve the efficacy of the probiotic product.

7.2.5 Diet and physical activity

Participants will be instructed to continue their usual physical activity and dietary regimen throughout the whole intervention period to study the isolated effect of probiotic supplementation. Before, at week 8 and in the last week of the intervention period, participants' diet and physical activity pattern will be assessed. Participants will complete a 4-

day dietary record, which includes week as well as weekend days. In addition, they will fill out the SQUASH questionnaire [22] to assess 24-hour daily activity patterns for 1 week.

7.2.6 Insulin resistance

Fasted blood samples for glucose and insulin will be taken to determine the homeostatic model assessment of insulin resistance (HOMA-IR) and beta-cell function [23]. By means of the fasted glucose and insulin concentrations HOMA-IR can be calculated according to the following equation:

$$\text{HOMA-IR: } \frac{\text{Glucose x Insulin}}{22.5}$$

7.2.7 Blood pressure and heart rate

Office BP (SBP and DBP) and the heart rate (HR) will be determined in four-fold (the first measurement will be discarded, and the last three measurements will be averaged) using a semi continuous blood pressure monitoring device (Omron M7, CEMEX Medische Techniek, Nieuwegein, The Netherlands). BP will also be measured during the measurements.

7.2.8 Metabolic risk markers

Satiety hormones (see **Table 4**):
- Fasted and postprandial GLP-1 concentrations will be measured.
- Fasted and postprandial PYY concentrations will be measured.
- Fasted and postprandial ghrelin concentrations will be measured.

Lipids (see **Table 4**):
- Fasting serum triacylglycerol (TAG) concentrations will be measured.
- Fasting serum total cholesterol (TC) and HDL-C will be measured.

Glucose and insulin (see **Table 4**):
- Fasting and postprandial plasma glucose concentrations will be measured.
- Fasting and postprandial serum insulin concentrations will be measured.

Short chain fatty acids (see Table 4):
- Fasting and postprandial plasma short chain fatty acid concentrations will be measured.

Table 4: Time points at which blood samples will be drawn during test days.

	Blood samples **Baseline (fasting and postprandial)**	**Blood samples** **8 weeks (fasting only)**	**Blood samples** **16 weeks** **(fasting and postprandial)**
TAG, TC, HDL-C (4ml)	X (fasting only)	X	X (fasting only)
Insulin (4ml)	X	X	X
GLP-1(4ml)	X	X	X
PYY (4ml)	X	X	X
Ghrelin (4ml)	X	X	X
Inflammatory markers (4ml)	X (fasting only)	X	X (fasting only)
Glucose (4ml)	X	X	X
Short chain fatty acids (4ml)	X	X	X
ml	**200**	**32.0**	**200**

TAG: triacylglycerol; TC: total cholesterol; HDL: high-density lipoprotein cholesterol;

7.2.9 Inflammatory markers

Fasting hemostatic factors and plasma markers related to systemic inflammation (e.g. interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)α, and C-reactive protein (hsCRP) will be measured to assess low-grade systemic inflammatory responses.

7.2.10 Mental health

Mental health assessed using the validated Beck Depression (BDI; a self-reported questionnaire comprised of 21 items measuring behavioral manifestations and the severity of key symptoms of depression) [24] and State-Trait Anxiety Inventories (STAI; a 40-item self-report questionnaire for measuring state anxiety and trait anxiety) [25].

7.2.11 Mood

Mood will be tested using the widely-applied Profile of Mood States (POMS), which is a standard validated psychological test for the assessment of an individual's mood [26].

7.2.12 Stress

Stress will be assessed using the Perceived Stress Scale (PSS) [27].

7.2.13 Sleep characteristics

Sleep characteristics (sleep duration, quality, latency and efficiency) will be assessed at the end of the interventions using the 10-item Pittsburgh Sleep Quality Index [28] [28].

7.2.14 Three factor Eating questionnaire

Cognitive restrained, disinhibition and hunger will be assessed using the Dutch three factor eating questionnaire [29].

7.2.15 Body Composition

Measurement of body composition is done by BodPod®. The BodPod is one of the newest and least invasive forms of body composition testing involving state of the art technology, developed by Life Measurements Instruments, Concord, CA. The use of Bod Pod correlates nicely with the concept of hydrostatic weighing and deuterium dilution measurements. Instead of using water to measure body volume, the Bod Pod uses air displacement to measure body volume [30]. Measurement time takes roughly 10 minutes per individual and allows to determine the amount and percentage of fat mass and fat free mass, taking physical activity level into account. The measurements are done according to the manufacturer's instructions, and when the participant is in the fasted state. The same device and software will be used throughout the entire study and will be calibrated according to manufacturer's instructions.

7.2.16 Quality of Life

The quality of life will be assessed at the end of the interventions using a 32-item questionnaire [31]. This measure will quantify quality of life on different dimensions: social, spiritual, emotional, cognitive, physical, activities of daily living, and integrated quality of life.

7.2.17 Anthropometric measurements

At all test days, anthropometric measurements (body weight, length, waist and hip circumferences as indirect measures of fat distribution, and skinfold measurements) will be measured before the measurements without shoes and heavy clothing.

7.2.18 Compliance

Empty sachets must be returned at each visit to calculate the compliance of the study participants. Subjects will be considered compliant when they take at least 80% of the study product.

7.3 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons or non-compliance.

7.3.1 Specific criteria for withdrawal

Study subjects will also be removed from the study if the study team becomes aware of any of the following conditions:

- Reported (major) non-compliance with the described interventions or other significant protocol violations.
- Reported illness and/or use of medication by the subject (principal investigators and study physician to decide).

7.3.2 Replacement of individual subjects after withdrawal

Withdrawn subjects will not be replaced.

7.3.3 Follow-up of subjects withdrawn from treatment

After withdrawal, no follow-up of the study subjects will take place. In case of withdrawal due to medical complications, study participants will be referred to a general practitioner

7.3.4 Premature termination of the study

In case this study is ended prematurely, the investigator will notify the accredited METC, including the reason for the premature termination. Premature termination is only possible:

- if the judgement of the competent medical research ethics committee that has assessed the study is irrevocably revoked;
- if a reasonable case can be made for terminating the study in the interests of the health of the research subjects;

- if it transpires that continuation of the study cannot serve any scientific purpose, and this is confirmed by the medical research ethics committee that has issued a positive decision on the study;
- if one of the parties or the funder has been declared insolvent or a bankruptcy/winding-up petition has been filed in respect of one of the parties or the financier, or one of the parties or the financier is dissolved as a legal entity;
- if the principal investigator is no longer capable of performing the tasks of the principal investigator, and no replacement agreeable to both parties can be found;
- if one of the two parties fails to comply with the obligations arising from the agreement and, provided compliance is not permanently impossible, this compliance has not taken place within thirty days of the defaulting party receiving a written request to comply, unless failure to comply is not in reasonable proportion to the premature termination of the study;
- if circumstances beyond the control of the sponsor, investigator or funder make it unreasonable to require the study's continuation.

8. SAFETY REPORTING

8.1 Temporary halt for reasons of subject safety

In accordance with section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending further review by the accredited METC. The investigator will take care that all subjects are kept informed.

8.2 AEs, SAEs and SUSARs

8.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the investigational product. All adverse events reported spontaneously by the study participants or observed by the investigator, or his staff will be recorded.

8.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;

- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

The investigator will report all serious adverse events (SAEs) to the sponsor without undue delay after obtaining knowledge of the events. The sponsor will report the SAEs through the web portal Research Portal to the review committee that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the SAEs.

8.3 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.
SAEs need to be reported until end of study within the Netherlands, as defined in the protocol.

9. STATISTICAL ANALYSIS

9.1 Descriptive statistics

For the normal distributed parameters, data will be presented as mean values and standard deviations. Non-normal distributed parameters will be presented as medians (ranges).

9.2 Statistical analysis

The primary study outcome parameter is satiety. The major null and alternate hypotheses are:
H_0: A 16-week probiotic supplement intervention does not affect ad libitum food intake - a marker of satiety - in overweight/ obese men and women; and a 16-week probiotic supplement intervention does not affect GLP-1 concentrations - a marker of satiety - as compared with a 16-week placebo intervention.
H_a: A 16-week probiotic supplement intervention significantly affects ad libitum food intake - a marker of satiety - in overweight/ obese men and women; and a 16-week probiotic supplement intervention does affect GLP-1 concentrations - a marker of satiety - as compared with a 16-week placebo intervention.

Differences in baseline values between intervention groups will be tested with an unpaired Student's t test. A repeated measures ANCOVA (with time as within and intervention as between subjects-factor) will be conducted to evaluate differences in ad libitum food intake comparing probiotic and placebo intervention.
A repeated measures ANCOVA will be conducted to assess the effect of intervention on hormone release (i.e. GLP-1) at baseline and at week 16, with intervention as a between subjects' factor and hormone release as a within subjects' factor. Baseline values will be considered as a covariate.
To further evaluate potential mechanisms underlying observed beneficial effects of long-term probiotic supplementation, Pearson's correlation coefficients will also be calculated. Differences are considered to be statistically significant when $P < 0.05$. Statistical analyses will be performed using SPSS (SPSS Inc., Chicago, IL, USA). Participants that not fully adhere to the study protocol will be excluded from the statistical analyses. A per protocol analysis will be performed to assess the true efficacy and missing data will not be replaced by estimates. The per protocol analysis will allow us to evaluate the physiological effects of intervention as intended. Participants that dropped-out will be excluded from the statistical analyses, but results from the first testing day will be compared with those who completed the study. An additional Intention to Treat (ITT) analysis (with Multiple Imputation) will be performed with all participants included in the study to assess effectiveness of the intervention [32]. All collected

data from the participants will be presented in tables for the whole group and separated for the intervention groups.

Secondary outcome parameters that are assessed on baseline as well as at the 8-week and 16-week assessment will be analysed with linear mixed model analysis. This includes hormone measurements, questionnaires and microbiota. Treatment and time will be used as fixed factors, participants and baseline values will be used as random factors. Time x treatment interactions will provide information if the treatment effect -if any- is comparable at all timepoints.

10. ETHICAL CONSIDERATIONS

10.1 Regulation statement

The study has to be approved by the Medical Ethical Committee (METC) of Maastricht University (UM). The present human intervention study will be conducted according to the Declaration of Helsinki (Finland, October 2024) and the Principles of GCP and in accordance with the Medical Research Involving Human Subjects Act (WMO).

10.2 Recruitment and consent

Study participants will be recruited by advertisements in local newspapers, the internet and posters. Participation will be on voluntary basis. Adults who are interested to participate will be contacted via telephone or email about the study. These persons will be asked questions to check for several basic inclusion and exclusion criteria (e.g. sex, age, BMI and general health) on the phone. In case these persons are interested to participate and the basic inclusion criteria follow our study protocol, subjects will receive the information brochure via email and will be informed about the study in detail during an in-person meeting. Volunteers can always contact the researchers if any questions arise by phone and by email. If desired, study subjects can contact an independent expert, who will not be involved in the study. Subjects will be instructed to read the information folder carefully and to ask questions when things are not clear. If they are still interested in participating at least one week after having received the information brochure and after carefully reading the subject information, study subjects can contact the researcher by phone or email and a screening visit will be planned. Before signing the informed consent form, participants will again be asked whether they have any remaining questions and whether the procedures are clear. The informed consent form will then be signed by both the study subject and the researcher. Only when the informed consent form is signed, subjects will be insured and the screening measurements can be performed.
Participation will be on a voluntary basis and study subjects are free to withdraw at any stage of the study without further explanation.

Besides recruitment via the above-mentioned methods, subjects who have participated in earlier studies and who agreed to be approached for future studies will be contacted by email to ask if they are interested to participate. If these subjects show interest, the same procedure as described above will be followed.

10.3 Benefits and risks assessment, group relatedness

The present study will provide insight into the potential beneficial effect of probiotics on satiety in overweight or obese men and women. Participants will not perceive direct benefits from the study. A benefit for the study population is the acquirement of scientific knowledge concerning underlying mechanisms of healthy foods, more specifically longer-term effects of a newly composed probiotic supplement on satiety.

Study participants will record in diaries any signs of illness, medication used, deviations from the study protocol and any experienced side effects such as headache, stomach complaints, nausea bloated feeling, flatulence, diarrhea, constipation, itching, eruptions/rashes, fatigue, and dizziness. During the intervention, adults will receive daily 6 g of probiotic supplement for 16 weeks. Of note, long-term probiotic regimens have already been provided in previous trials and were well-tolerated, and there are no expected side effects related to the probiotic intervention [17-20]. During the trial on different occasions, tests will be performed and blood will be sampled (a total of 436 ml during the whole trial). During these tests, study subjects have to stay at the university and are not allowed to eat.

Venipuncture can occasionally cause a local hematoma or bruise to occur. Some subjects may report pain during venipuncture. Insertion of the cannula can cause some discomfort and possible a hematoma or bruise. Some study subjects may also report pain during the insertion of the cannula. Other measurements are routine and are not expected to lead to physical side effects.

Time investment, as indicated in **Table 5**, is 17 hours (1000 minutes), excluding travel time, and blood sampling volume will be 436 ml during the whole study (see **Table 3**).

In the case of any incidental findings, the participant and their general practitioner will be informed. Subjects who do not agree with this procedure are not allowed to participate.

Table 5: Time investment scheme

	Screening	Baseline measurements	Blood sample 8 weeks	Measurements at 16 weeks	Diaries
Time cost (min)	60	1x 360	1 X 90	1 X 360	100

10.4 Compensation for injury

The sponsor/investigator has liability insurance, which is in accordance with article 7 of the WMO. Maastricht University has an insurance, which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.

€ 650.000, -- (i.e., six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
€ 5.000.000, -- (i.e., five million Euro) for death or injury for all subjects who participate in the Research;
€ 7.500.000, -- (i.e., seven million five hundred thousand Euro) for the total damage incurred by the organization for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the current human intervention study.

10.5 Incentives

Study participants will receive a compensation for the time invested in participating. This compensation will be € 300 after complete participation or pro rata after withdrawal from the study. No financial reward will be given for the screening visit, except for traveling costs. These traveling costs will also be compensated during the study, either on the basis of public transport (economy class), or with a maximum of € 0.23 per kilometer.

11. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

11.1 Handling and storage of data and documents

All data will be handled confidentially. Study subjects will receive a code (two-digit number). This code will (except for the list mentioned below) not be traceable to the natural person and not contain a birth date or initials of the study subject. A subject identification list will be prepared that links this code to the personal identifiable information of the study participants. The key to the code will be safeguarded by the principal investigator. Only the principal and coordinating investigators, the Health and Youth Care Inspectorate, and people who monitor the study will have access to the source data. Source data will be stored at the University for 15 years in accordance with GCP standards to re-use them or to validate results. Subjects who do not agree with this cannot participate in the study. The handling of personal data will comply with the EU General Data Protection Regulation Act and the Dutch Act on Implementation of the General Data Protection Regulation.

Only members of the research team, the Health and Youth Care Inspectorate, and people who monitor the study will have access to samples. All collected samples from the study subjects will be coded (subject ID, time of sampling, and type of sample [serum or plasma]) and stored for a period of maximum 15 years after which they will be destroyed. Future research in line with the current study -namely effects in the area of nutrition and satiety- will only be conducted with study subject's approval on the informed consent form for re-use of the data and approval of the new study by the accredited METC that gave a favorable opinion. Data will also be stored at the University for 15 years in accordance with GCP standards to re-use them or to validate results. Coded samples may be sent outside of the university for specific sample analyses. If this is the case, an amendment will be submitted to the METC. In this amendment, it will be specified which country the analyses will take place, what will happen to data and body material after the analyses, and which measures have been taken to protect personal data.

11.2 Monitoring and Quality Assurance

Trained and qualified monitors will perform monitoring of this trial. Monitoring visits will take place at several time points by the Clinical Trial Center Maastricht (CTCM). Before the start of the study, a site initiation visit will take place. During the study, one or multiple visits will be scheduled as agreed upon by the researcher and the monitor. When the study is completed, the close out visit will take place.

11.3 Amendments

Amendments are defined as changes made to the research proposal after a favourable opinion by the accredited METC has been provided. All the amendments will be notified to the accredited METC that gave the favourable opinion.

11.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

11.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit. The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action. In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the trial, including any publications/abstracts of the study, to the accredited METC.

11.6 Public disclosure and publication policy

Publication policy is in line with the CCMO publication statement. The results of the current study will be published in peer-reviewed scientific journals. Both positive and negative results of the study will be disclosed. The principal investigators will always try to publish and/or present results to the general public.

12. STRUCTURED RISK ANALYSIS

12.1 Potential issues of concern

The total study duration will be 16 weeks. The amount of blood that will be taken in this study period is less than 500 ml. Blood sampling itself can cause bruises or local hematoma. All measurements are routine and are not expected to lead to physical side effects. In the case of any unexpected medical findings, the participant and their general practitioner will be informed. Participants who do not agree with this procedure are not allowed to participate.

Overweight/ obese men and women will receive daily 6 g of probiotics supplement or placebo for 16 weeks. This regimen was well-tolerated in previous trials [17-20], is considered safe, and no expected side effects related to the supplement intake are expected. Newly combined microbial strains all have been tested in previous trials, are considered safe, and no expected side effects related to supplement consumption are expected.
Other measurements (i.e. body composition) are routine and are not expected to lead to physical side effects. Participants that do not fully adhere to the study protocol will be excluded from the per protocol analyses. The total time investment is 17 hours (1000 minutes), excluding travel time.
Participants will not perceive direct benefits from the study. A benefit for the study population is the acquirement of scientific knowledge concerning underlying mechanisms with regard to satiety and intestinal health.

12.2 Synthesis

Probiotic supplement regimens were well-tolerated in previous trials and are safe, and there are no expected side effects related to the intervention.

13. REFERENCES

1. Adam TC, Drummen M, Macdonald I, et al. Association of Psychobehavioral Variables With HOMA-IR and BMI Differs for Men and Women With Prediabetes in the PREVIEW Lifestyle Intervention. *Diabetes care* 2021 doi: 10.2337/dc21-0059 [published Online First: 2021/06/06]
2. Field BC, Chaudhri OB, Bloom SR. Obesity treatment: novel peripheral targets. *Br J Clin Pharmacol* 2009;68(6):830-43. doi: 10.1111/j.1365-2125.2009.03522.x
3. Tschop MH, Friedman JM. Seeking satiety: From signals to solutions. *Sci Transl Med* 2023;15(723):eadh4453. doi: 10.1126/scitranslmed.adh4453 [published Online First: 20231122]
4. Adam TC, Jocken J, Westerterp-Plantenga MS. Decreased glucagon-like peptide 1 release after weight loss in overweight/obese subjects. *Obes Res* 2005;13(4):710-6. doi: 13/4/710 [pii]
10.1038/oby.2005.80 [published Online First: 2005/05/18]
5. Adam TC, Westerterp-Plantenga MS. Glucagon-like peptide-1 release and satiety after a nutrient challenge in normal-weight and obese subjects. *The British journal of nutrition* 2005;93(6):845-51. doi: S0007114505001297 [pii] [published Online First: 2005/07/19]
6. Popoviciu MS, Paduraru L, Yahya G, et al. Emerging Role of GLP-1 Agonists in Obesity: A Comprehensive Review of Randomised Controlled Trials. *Int J Mol Sci* 2023;24(13) doi: 10.3390/ijms241310449 [published Online First: 20230621]
7. Bastings J, Venema K, Blaak EE, et al. Influence of the gut microbiota on satiety signaling. *Trends Endocrinol Metab* 2023;34(4):243-55. doi: 10.1016/j.tem.2023.02.003 [published Online First: 20230303]
8. Zeng Y, Wu Y, Zhang Q, et al. Crosstalk between glucagon-like peptide 1 and gut microbiota in metabolic diseases. *mBio* 2024;15(1):e0203223. doi: 10.1128/mbio.02032-23 [published Online First: 20231206]
9. FAO W. Probiotics in food: Health and nutritional properties and guidelines for evaluation. FAO Food and Nutrition paper Food and Agriculture Organization of the United Nations and World Health Organization, 2006.
10. van Best N, Rolle-Kampczyk U, Schaap FG, et al. Bile acids drive the newborn's gut microbiota maturation. *Nat Commun* 2020;11(1):3692. doi: 10.1038/s41467-020-17183-8 [published Online First: 20200723]
11. Vliex LMM, Penders J, Nauta A, et al. The individual response to antibiotics and diet - insights into gut microbial resilience and host metabolism. *Nat Rev Endocrinol* 2024;20(7):387-98. doi: 10.1038/s41574-024-00966-0 [published Online First: 20240314]
12. Bjerg AT, Kristensen M, Ritz C, et al. Lactobacillus paracasei subsp paracasei L. casei W8 suppresses energy intake acutely. *Appetite* 2014;82:111-8. doi: 10.1016/j.appet.2014.07.016 [published Online First: 20140715]
13. Simon MC, Strassburger K, Nowotny B, et al. Intake of Lactobacillus reuteri improves incretin and insulin secretion in glucose-tolerant humans: a proof of concept. *Diabetes care* 2015;38(10):1827-34. doi: 10.2337/dc14-2690 [published Online First: 20150617]
14. Wang Y, Dilidaxi D, Wu Y, et al. Composite probiotics alleviate type 2 diabetes by regulating intestinal microbiota and inducing GLP-1 secretion in db/db mice. *Biomed Pharmacother* 2020;125:109914. doi: 10.1016/j.biopha.2020.109914 [published Online First: 20200205]
15. Wang Y, Wu Y, Sailike J, et al. Fourteen composite probiotics alleviate type 2 diabetes through modulating gut microbiota and modifying M1/M2 phenotype macrophage in db/db mice. *Pharmacol Res* 2020;161:105150. doi: 10.1016/j.phrs.2020.105150 [published Online First: 20200817]
16. Dankner R, Murad H, Agay N, et al. Glucagon-Like Peptide-1 Receptor Agonists and Pancreatic Cancer Risk in Patients With Type 2 Diabetes. *JAMA Netw Open*

2024;7(1):e2350408. doi: 10.1001/jamanetworkopen.2023.50408 [published Online First: 20240102]
17. Horvath A, Leber B, Feldbacher N, et al. Effects of a multispecies synbiotic on glucose metabolism, lipid marker, gut microbiome composition, gut permeability, and quality of life in diabesity: a randomized, double-blind, placebo-controlled pilot study. *Eur J Nutr* 2020;59(7):2969-83. doi: 10.1007/s00394-019-02135-w [published Online First: 2019/11/16]
18. Majewska K, Kregielska-Narozna M, Jakubowski H, et al. The Multispecies Probiotic Effectively Reduces Homocysteine Concentration in Obese Women: A Randomized Double-Blind Placebo-Controlled Study. *J Clin Med* 2020;9(4) doi: 10.3390/jcm9040998 [published Online First: 20200402]
19. Smarkusz-Zarzecka J, Ostrowska L, Leszczynska J, et al. Effect of a Multi-Strain Probiotic Supplement on Gastrointestinal Symptoms and Serum Biochemical Parameters of Long-Distance Runners: A Randomized Controlled Trial. *Int J Environ Res Public Health* 2022;19(15) doi: 10.3390/ijerph19159363 [published Online First: 20220730]
20. Smith-Ryan AE, Mock MG, Trexler ET, et al. Influence of a multistrain probiotic on body composition and mood in female occupational shift workers. *Appl Physiol Nutr Metab* 2019;44(7):765-73. doi: 10.1139/apnm-2018-0645 [published Online First: 20181219]
21. Gregersen NT, Flint A, Bitz C, et al. Reproducibility and power of ad libitum energy intake assessed by repeated single meals. *The American journal of clinical nutrition* 2008;87(5):1277-81. doi: 10.1093/ajcn/87.5.1277
22. Terpstra SES, Hoogervorst LA, van der Velde J, et al. Validation of the SQUASH physical activity questionnaire using accelerometry: The NEO study. *Osteoarthr Cartil Open* 2024;6(2):100462. doi: 10.1016/j.ocarto.2024.100462 [published Online First: 20240318]
23. Sasaki N, Ueno Y, Higashi Y. Indicators of insulin resistance in clinical practice. *Hypertens Res* 2024;47(4):978-80. doi: 10.1038/s41440-023-01566-7 [published Online First: 20240104]
24. Dixon JB, Dixon ME, O'Brien PE. Depression in association with severe obesity: changes with weight loss. *Archives of internal medicine* 2003;163(17):2058-65.
25. Tenenbaum G, Furst D, Weingarten G. A statistical reevaluation of the STAI anxiety questionnaire. *J Clin Psychol* 1985;41(2):239-44. [published Online First: 1985/03/01]
26. Norcross JC, Guadagnoli E, Prochaska JO. Factor structure of the Profile of Mood States (POMS): two partial replications. *J Clin Psychol* 1984;40(5):1270-7. doi: 10.1002/1097-4679(198409)40:5<1270::aid-jclp2270400526>3.0.co;2-7 [published Online First: 1984/09/01]
27. Harris KM, Gaffey AE, Schwartz JE, et al. The Perceived Stress Scale as a Measure of Stress: Decomposing Score Variance in Longitudinal Behavioral Medicine Studies. *Ann Behav Med* 2023;57(10):846-54. doi: 10.1093/abm/kaad015
28. Buysse DJ, Reynolds CF, 3rd, Monk TH, et al. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989;28(2):193-213. doi: 10.1016/0165-1781(89)90047-4 [published Online First: 1989/05/01]
29. Stunkard AJ, Messick S. The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. *J Psychosom Res* 1985;29(1):71-83.
30. Tseh W, Caputo JL, Keefer DJ. Validity and reliability of the BOD POD(R) S/T tracking system. *Int J Sports Med* 2010;31(10):704-8. doi: 10.1055/s-0030-1255111 [published Online First: 20100708]
31. Nijssen KMR, Mensink RP, Joris PJ. Effects of Intranasal Insulin Administration on Cerebral Blood Flow and Cognitive Performance in Adults: A Systematic Review of Randomized, Placebo-Controlled Intervention Studies. *Neuroendocrinology* 2023;113(1):1-13. doi: 10.1159/000526717 [published Online First: 2022/10/12]
32. Singal AG, Higgins PDR, Waljee AK. A primer on effectiveness and efficacy trials. *Clin Transl Gastroenterol* 2014;5(1):e45. doi: 10.1038/ctg.2013.13