RESEARCH PROPOSAL

The effect of orally applied encapsulated lipids in yoghurt on satiety and food intake: a randomized single-blind placebo-controlled study with cross-over design

Protocol title: The effect of orally applied encapsulated lipids in yoghurt on satiety and food intake: a randomized single-blind placebo-controlled study with cross-over design

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

GCP	**Good Clinical Practice**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)**
(S)AE	**(Serious) Adverse Event**
(S)AR	**(Serious) Adverse Reaction**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation 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 subsidising party.**
VAS	**Visual Analogue Scales**
WBP	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: Sensing of lipid fractions in the small intestine can induce negative feedback mechanisms from different parts of the intestine to the proximal gastrointestinal (GI) tract including stomach, gallbladder and pancreas but also to the central nervous system. This negative feedback process is able to inhibit food digestion, appetite sensations and food intake, and is able to increase feelings of satiety and satiation. The ileal brake is considered a potent feedback mechanism not only during short-term intervention with ileal lipid infusion, but also as powerful long-term weight management strategy with orally ingested ileal lipid delivery. However, the major part of dietary lipids will be digested and absorbed in the proximal small intestine and is not likely to reach the distal ileum and induce the strong ileal brake feedback mechanism. To prevent orally applied lipids to be proximal digested and absorbed, we designed a food-grade encapsulation system that releases free fatty acids from safflower oil in the more distal small intestine. The current study will be an explorative study to proof the concept of ileal brake activation: the encapsulated lipid will be added to and mixed with yoghurt (A), and the subsequent satiety and food intake will be compared to an equicaloric mixture of non-encapsulated nutrients (control, yoghurt B) with the same amount of lipids, and the encapsulation components.

Aim: To explore the ability of encapsulation of orally applied lipids in a yoghurt snack to modify *ad libitum* food intake and satiety, without GI symptoms.
Hypothesis: We hypothesize that lipid encapsulates induce satiety and decrease *ad libitum* meal intake compared to control (yoghurt B), due to more distal release in the small intestine.
Primary objective: To investigate whether encapsulation of lipids decreases *ad libitum* food intake compared to non-encapsulated lipids in a formulation with equal nutrients (control).
Secondary objectives:

- To investigate feelings of satiety after orally ingested lipid encapsulates
- To investigate occurrence and severity of GI symptoms after orally ingested lipid encapsulates
- To assess whether differences in food intake, satiety and GI symptoms relate to lipid encapsulation

Study design: Randomized, single-blind, placebo-controlled intervention study with cross-over design.
Study population: 35 human subjects (BMI 25-30 kg/m^2), 18 - 65 years old.
Intervention: Every subject receives two treatments on two different days with at least one week in between, following a randomized cross-over design. On the test day, the subjects will arrive in fasted state and receive a standardized breakfast (small, low in fat, t=0 min). Once major part of the breakfast has been emptied from the stomach (t=90 min), they receive a yoghurt (0.2 L) that contains emulsified safflower oil (6 g) either encapsulated (yoghurt A) or non-encapsulated (control, yoghurt B; same oil droplet size as intervention and in presence of 'empty' encapsulation matrix). Two hours after the intervention yoghurt, once the lipid will be released to the distal small intestine, *ad libitum* meal intake will be measured (t=210 min).

Main study parameters/endpoints: The main study parameter is the intake of *ad libitum* meal (kcal) as measured during pasta lunch, comparing intervention and control treatment.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:

The subjects (in total 35) will have to visit Maastricht University on three occasions: once to sign the consent, fulfil the screening and get an instruction (about half an one hour) and two times to attend the test days (about four hours per test day). The test days will be non-invasive: consumption of a breakfast, yoghurt snack, and lunch; and in between filling questionnaires on satiety feelings (satiety, fullness, hunger, desire to eat, desire to snack) and GI symptoms (bloating, discomfort, pain, nausea). These attributes will be measured using Visual Analogue Scales (VAS, 0 to 100 mm) scores, with the most negative or lowest intensity feelings at the low end and the opposing terms at the high end. The subject will be asked to indicate his feeling at that moment.

1. INTRODUCTION AND RATIONALE

Weight management by ileal brake activation

A key strategy to control the expansion of the worldwide obesity prevalence is by large-scale application of dedicated dietary interventions that help weight maintenance or induce weight loss. A new effective dietary intervention may target to enhance natural gastrointestinal (GI) processes that are involved in food intake regulation, such as gastric distension, and the sensing of nutrients and their digestion products. Gastric distension triggers mechanosensor-mediated signals and suppresses the release of the stomach hormone ghrelin. The presence of macronutrients in the small intestine induces the release of several gut peptides that are known to be associated with food intake, such as cholecystokinin (CCK), peptide YY (PYY), and glucagon-like peptide-1 (GLP-1) [1]. The ileum is believed to give the strongest signal [2], [3].

Ileal brake activation has been proven to acutely reduce food intake with direct intubation of any of the three macronutrients *via* a naso-ileal catheter (overview of literature in [3]). About 30 years ago, ileal lipid infusion was already shown to reduce food intake [4], [5]. Moreover, ileal lipid infusion increased perception of satiety/fullness or decreased hunger in most studies [1], [2], [4]–[7]. Amongst oils with different degree of fatty acid saturation, safflower oil (high in linoleic acid, C18:2) was found to more strongly activate the ileal brake [8]. Therefore, in the current study we will use safflower oil.

The mechanism of action of inhibition of food intake by intraluminal lipids is based on sensing of lipid species at intestinal receptors. When activated more distally in the small intestine, intraluminal lipids are able to induce distal to proximal negative feedback mechanisms [9]. This mechanism is activated more strongly when the *degradation products* of lipid digestion (*i.e.*, fatty acids, monoglycerides) are sensed rather than the intact lipids itself (*i.e.*, triglycerides) (see review [9], paragraph 4.1.1). Hence, for effective ileal brake activation, lipase-mediated hydrolysis (lipolysis) of lipids is required, and in addition, proximal intestinal absorption of lipolysis products has to be prevented.

Ileal brake activation by food

To be an effective and practically applicable weight management strategy, an administration route is required different from direct intestinal intubation, namely orally with the regular route of food intake. When applied orally, however, the major part of dietary lipids will be digested and absorbed in the proximal small intestine and are not likely to induce the ileal brake mechanism. Hence, to activate the ileal brake, lipolysis needs to be controlled. Since lipolysis only occurs at the oil-water interface, the amount and accessibility of this interface are key in controlling it [10], [11].

Many attempts have been made to control lipid digestion, especially through designing a protective interface structure around nano-or-micron-sized lipid droplets. Some of these structures were able to delay lipolysis to a certain extent, including single surfactants that provide steric hindrance [12], [13], more thick interfacial films produced through layer-by-layer adsorption of biopolymers [14]–[18], and specific Pickering-based interface structures [19], [20]. To truly control lipolysis, however, the structuring approach should go beyond interfacial design [21], and may focus on control of enzyme diffusion towards this interface by means of a larger structure. I*n vitro* lipolysis has been controlled by both digestible protein gels [22], [23] and indigestible alginate gels [24], [25]. The latter is the approach taken in the current study.

Emulsion-filled Ca-alginate gel particles for ileal brake activation

We have provided evidence that encapsulation of small lipid droplets into millimetre-sized Ca-alginate gel particles is to be considered as a promising approach for ileal brake activation. The benefit of using alginate gel over protein gel, is the degradation of protein and resistance of alginate matrix against gastric proteases [23], so maintenance of the encapsulate structure. In addition, the pH-dependency of volume and porosity of alginate gel particles makes them very suitable for ileal delivery: they are known to shrink under acidic conditions, lowering the pore size, and hence protecting the encapsulated emulsion in the stomach; moreover, they swell at neutral pH, increasing the pore size and favouring lipase diffusion towards the encapsulated oil in the small intestine [24], [26]–[28].

In humans, the transport of alginate beads in the GI system (n=10, after a fatty preload) has been tracked using MRI scanning, and interestingly, the beads were found to end up most in the ileum rather than in duodenum and jejunum [29]. This suggest an extended residence time in the ileum, and therefore stronger ileal brake activation.

Besides, alginate may modify the ileal brake response directly as it is a water-soluble fibre, which can slow transit time and result in spreading of nutrients and slower absorption [30]. The direct effect of alginate on feelings of satiety and hunger have also been evaluated: alginate drinks were found to be able to modulate hunger dependent on its gelling behaviour [31] and viscosity [32]. On the other hand, no effect was found from compressed alginate capsules that were ingested 30 min before meal, on gastric motor functions, satiation, appetite, nor gut hormones [33]. Alginate in the form of Ca-gel particles (not loaded with oil), may affect feelings of satiety (n=10, after a fatty preload), dependent on the gel structure, but not hunger and appetite [29]. Taking all together, the potential direct effect of alginate on hunger modulation may only hold for alginate in solution, but for sure, alginate will not oppose the beneficial effect.

The fate of calcium-alginate gel particles that do contain oil has also been studied in *in vivo* studies. In rat, emulsion-filled Ca-alginate beads improved gastric integrity (in terms of microscopic observation) compared to free emulsion droplets or emulsion micro-clusters, and delayed lipid absorption [34]. These findings were confirmed in a human trial in which oil-core calcium-alginate-shell capsules (0.5 cm) resulted in faster gastric emptying, and delayed lipolysis and absorption as measured with ^{13}C-labelled octanoic acid detected in a breath test [35], though these capsules would be quite large to use as a food additive.

Present encapsulates

We designed encapsulates of safflower oil droplets (S) (d_{32} ~ 25 µm) in Ca-alginate (CA) hydrogel particles (0.5-1.2 mm) with different pore size of the hydrogel (5-10 nm). Lipolysis was controlled simply by gel particle size, and pore size at neutral pH, as shown for studied in a simple static *in vitro* digestion model. As preparation for the current human trial, we added these S-CA particles to yoghurt-meals and studied lipolysis during dynamic *in vitro* digestion in a three-compartment model of the upper GI tract (DIDGI, [36], [37]). Digestive media was sampled and analysed both for total extent of lipolysis and for the fraction solubilised in mixed bile micelles. To distinguish between i) direct inhibition of lipase by alginate [38] and ii) physical barrier to

encapsulated oil [24], meals containing S-CA particles were compared to control meals with non-encapsulated lipid droplets, both in the presence and absence of empty CA particles. Lipolysis of non-encapsulated lipid was fast and would correspond to time scales relevant for duodenal delivery, whearas encapsulated lipid in CA particles showed delayed lipolysis up to four hours after ingestion, corresponding to ileal delivery.

Present study

The present study will be an explorative study to proof the concept of ileal brake activation using yoghurt that contains encapsulated safflower oil droplets in CA gel particles. The effectivity will be tested in a randomized, single-blind, placebo-controlled intervention study with cross-over design. Within each subject we will study the difference in *ad libitum* food intake and satiety after ingestion of the active yoghurt (containing encapsulated lipid) or control (equicaloric mixture of non-encapsulated nutrients: same amount of lipids and empty encapsulates).

2. OBJECTIVES

Primary objective: To investigate whether encapsulation of lipids decreases *ad libitum* food intake compared to non-encapsulated lipids in a formulation with equal nutrients (control).
Secondary objectives:

- To investigate feelings of satiety after orally ingested lipid encapsulates
- To investigate occurrence and severity of GI symptoms after orally ingested lipid encapsulates
- To assess whether differences in food intake, satiety and GI symptoms relate to lipid encapsulation

3. STUDY DESIGN

This intervention study will be a randomized, single-blind, placebo-controlled trial with two treatments in a cross-over design (see Figure 1 for a scheme of the design). The population and intervention are described in relation to the study design here in short, and into more details in the respective sections on population (section 4) and study procedures (section 7.3).

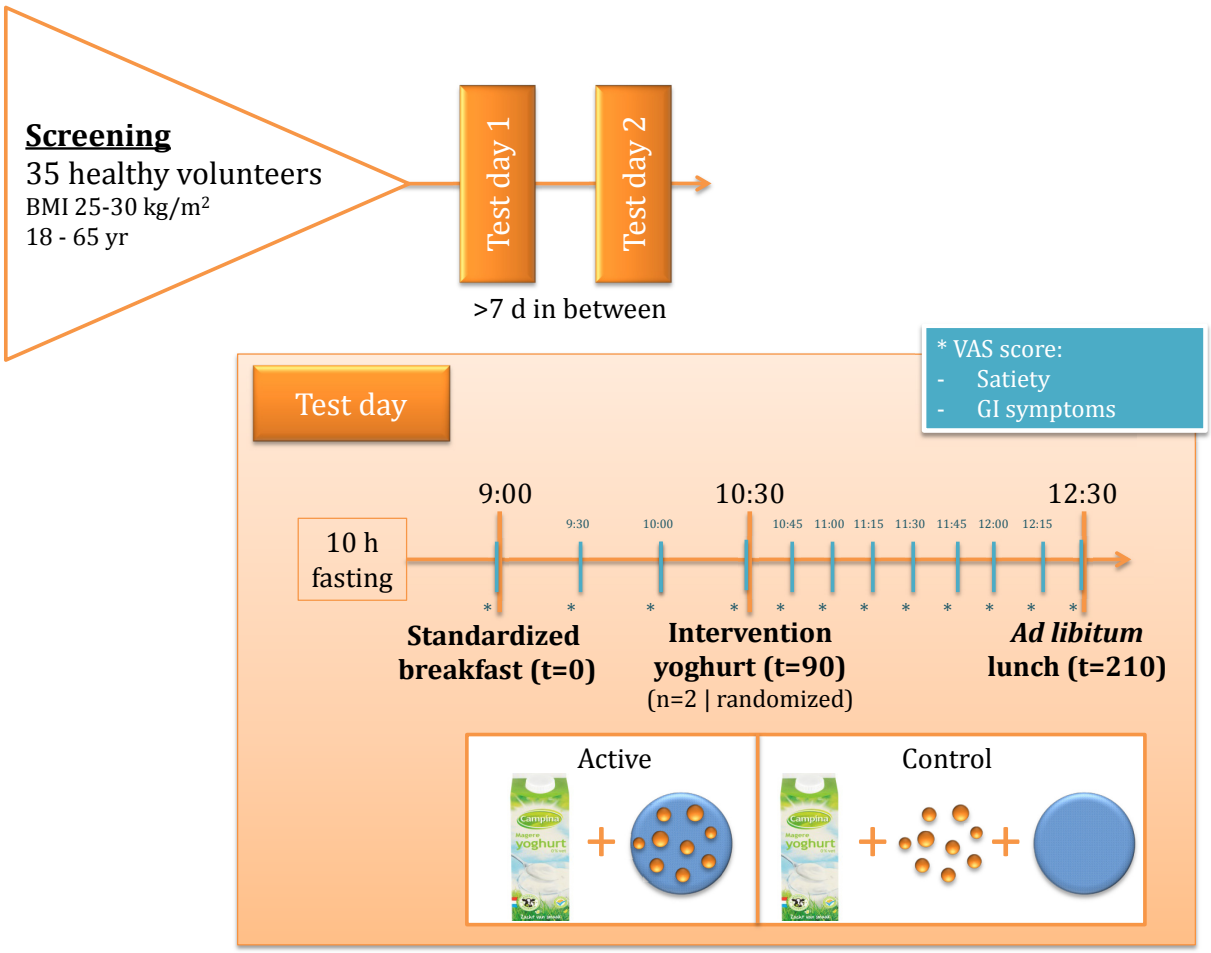

Figure 1. Overview of the study design of this explorative project on lipid encapsulation (in yoghurt) on satiety and food intake, t=0 defined as start of breakfast. The intervention yoghurt differs in the carrier of the lipid (active= encapsulated in gel particle for distal lipid release, control=non-encapsulated for fast-release)
*** VAS= Visual Analogue Scales, will be used for nine attributes per time point.**

Population

Healthy subjects (male and female, any ethnic background, 18-65 years old) with overweight (BMI 25-30 kg/m²) will be studied (inclusion criteria in section 4.2, exclusion criteria in 4.3). Thirty-five subjects will be recruited (section 10.2 for more information).

Intervention

After inclusion, subjects will have to visit Maastricht University on three occasions: once to sign the consent, fulfil the screening and get an instruction (about half an one hour) and two times to attend the test days (about four hours per test day). Both test days will be scheduled in a period of a month, with at least one week in between them.
Every subject receives both treatments on two different test days, following a randomized cross-over design. The treatments differ in intervention yoghurt, which will be ingested in standardized state (relatively empty stomach, ninety minutes after a standardized breakfast that is taken after a 10 hour fast; detailed procedures in section 7.3).

Both intervention products are low-fat yoghurts of 0.2 L that contain 6 g safflower oil (present as droplets with average size of 25 μm), which is encapsulated in gel particles (active, yoghurt A) or a non-encapsulated equicaloric mixture of nutrients (control, yoghurt B).

4. STUDY POPULATION

4.1 Population

Thirty-five healthy subjects (male and female, any ethnic background, 18-65 years old) with overweight (BMI 25-30 kg/m^2) will be recruited from a pool of previously included subjects, and *via* advertisements.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- ✓ Based on medical history and previous examination, no serious gastrointestinal complaints can be defined.
- ✓ Age between 18 and 65 years. A higher age comes with a higher chance of comorbidities. These could influence our study outcomes and therefore this age range was chosen. This study will include healthy adult subjects (male and female). Women must be taking contraceptives (only needed in women with childbearing potential).
- ✓ BMI between 25-30 kg/m^2.
- ✓ Voluntary participation.
- ✓ Able to participate in the study, willing to give informed consent and to comply with the study procedures and restrictions.

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- ✓ Milk (-protein or lactose)- allergy/intolerance.
- ✓ History of severe cardiovascular, respiratory, urogenital, gastrointestinal/ hepatic, hematological/immunologic, HEENT (head, ears, eyes, nose, throat), dermatological/connective tissue, musculoskeletal, metabolic/nutritional, endocrine, neurological/psychiatric diseases, allergy, major surgery and/or laboratory assessments which might limit participation in or completion of the study protocol. The severity of the disease (major interference with the execution of the experiment or potential influence on the study outcomes) will be decided and documented by the principal investigator.
- ✓ Administration of investigational drugs or participation in any scientific intervention study that may interfere with this study, to be decided by the principal investigator prior to the study.

- ✓ Major abdominal surgery interfering with gastrointestinal function (uncomplicated appendectomy, cholecystectomy and hysterectomy allowed, and other surgery) upon judgement of the principal investigator.
- ✓ Dieting (medically prescribed, diabetic and vegetarian).
- ✓ Pregnancy, lactation.
- ✓ Excessive alcohol consumption (>20 alcoholic consumptions per week)
- ✓ Intention to stop smoking.
- ✓ Self-admitted HIV-positive state.
- ✓ Abnormal eating behaviour.
- ✓ Reported unexplained weight loss or gain in the month prior to screening.

4.4 Sample size calculation

The calculations on required sample size should be based on the primary outcome: difference in intake of *ad libitum* meal. To our best knowledge, however, no data is available for this particular encapsulation system, so we need to base our calculation on related studies. First, food intake was significantly reduced with 79 kcal (SEM 41; n=15 so SD of 159 kcal) after ileal intubation (of 6 g lipid; food intake 435 kcal) compared to oral ingestion (of 6 g lipid, combined with saline infusion; food intake 514 kcal) [2]. Second, food intake reduced after active micro-encapsulate intake (655 kcal, SD 216) compared to placebo (699 kcal, SD 238), so a reduction of 44 kcal (P=0.011) [*not yet published randomized placebo-controlled double-blind cross-over study in 60 healthy overweight volunteers*]. Third, intra-ileal infusion of 6 g safflower oil, the food intake (464.3 ± 90.7 kcal) was 122 kcal (21 %) lower compared to placebo (saline infusion, 586.7 ± 70.2 kcal) [1]. On average, these three studies showed a difference of 82 kcal with an average SD of 156 kcal, and we used these values to base our calculation on. For the statistical test (p-value of 5% (α = 0.05), power 80% (B = 0.80)) we used the formula:

$$n = \frac{(t_{n-\frac{1,\alpha}{2}} + t_{n-1,\beta})^2}{d^2}$$

that can be estimated by:

$$n \geq \frac{\sigma^2}{\delta^2} (Z_\alpha + Z_\beta)^2$$

with σ being the standard deviation for paired samples (156 kcal), δ being the minimal detectable difference in food intake (82 kcal), $Z\alpha$ being 1.96 (for α = 0.05), $Z\beta$ being 0.84 (for B= 0.80).

Filling the formula results in a value of n ≥ 28.3, so at least 29 subjects. However, it must be noted that the sample size estimation uses Z-values, derived from a normal distribution. The statistical test, used in this sample size, is based on a t-distribution instead of the normal distribution. Thus, for small samples (n < 100), the sample size n must be estimated iteratively using the appropriate t-values. These t-values depend on 'degrees of freedom' (df), which corresponds to n-1, so in our case for a sample size n of 29, the degree of freedom is 28 (29-1). Therefore, the following formula was used:

$$n \geq \frac{\sigma^2}{\delta^2} \left(t_{n-\frac{1,\alpha}{2}} + t_{n-1,\beta}\right)^2$$

with t-value being 2.048 for a two-sided α of 0.05 with df of 17, and 0.855 for B= 0.80 with df of 17.

This leads, after a small number of iterations, to a re-estimate of n of at least 31 subjects (n ≥ 30.4). According to this power calculation, 31 subjects will need to complete the study. Because of possible dropouts, 35 subjects will be recruited for this study.

5. TREATMENT OF SUBJECTS

During this study, two treatments will be compared, following a randomized cross-over design. The treatments differ in intervention yoghurt (active or control formulation), which will be ingested in standardized state (relatively empty stomach, ninety minutes after a standardized breakfast that is taken after a 10 hour fast). The yoghurt will be provided with a glass of water or tea (150 mL). Two hours after ingestion of the intervention yoghurt, food intake will be measured in a standardized pasta lunch. Additionally, feelings of satiety and GI symptoms will be determined each 30 minutes before the intervention and each 15 minutes after the intervention. For more details on study procedure, see section 7.3.
On each test day, subjects receive one of the two formulations described in Table 1. Both products are low-fat yoghurts of 0.2 L that contain 6 g safflower oil (present as droplets with average size of 25 μm), which is encapsulated in gel particles (active, yoghurt A) or a non-encapsulated equicaloric mixture of nutrients (control, yoghurt B).

Table 1. Description of the composition of the interventional yoghurts, provided in randomized order.

Formulation	Name	Composition
Yoghurt A	Active	120 g yoghurt + 60 g oil-gel particles (containing 6 g oil) + 24 g water*
Yoghurt B	Control	120 g yoghurt + 54 g empty gel particles + 6 g oil in 24 g water*

** needed to produce the emulsion for the control as 20% oil emulsion.*

5.1 Active investigational product

To activate the ileal brake mechanism, 6 grams of lipid will be ingested of which at least half will be delivered to the ileum following different release profile dependent on encapsulation type. From previous research, we know that ileal infusion with 3 gram of lipid results in the activation of the ileal brake [6]. The lipid encapsulates (60 g total gel particles) are added to 120 g yoghurt, not only to allow easy consumption for the participants, but also to ensure no difference in appearance between control and active formulations. To have the same composition in all formulations, the active product is matched to the control product in terms of total water content.

5.2 Control product

Non-encapsulated lipid will be used as control, to be released into the stomach or proximal duodenum, in contrast to the active that will be delivered into the more distal small intestine. The control yoghurt will be equicaloric to the active yoghurt, with the same amount of lipids (and same droplet size), and the same encapsulation components (empty), only they are present separately rather than forming an oil-gel particle. In addition to being equicaloric, the two types of yoghurt will have the same mouth feel due to the presence of the empty encapsulation components.

6. INTERVENTION PRODUCT

Both intervention products are low-fat yoghurts of 204 mL in total that contain 6 g safflower oil (present as droplets with average size of 25 μm), which is encapsulated in gel particles (active, yoghurt A) or a non-encapsulated equicaloric mixture of nutrients (control, yoghurt B). The used low-fat yoghurt (Campina, Magere yoghurt, 0% fat) itself, to which the lipid formulations are added, is commercially available on the Dutch market and purchased from a local store. Besides, all added ingredients (whey proteins, alginate, calcium, safflower oil) are commercial available food ingredients, and repeatedly used in previous human trials so considered having a history of safe use. The intervention yoghurts will be produced in accordance with safety and quality regulations of the commercial manufacturer, and after preparation properly stored as described in section 6.7.

6.1 Name and description of intervention yoghurts

The active intervention product will be a low-fat yoghurt (120 g, Campina, Magere yoghurt, 0% fat) mixed with 60 gram 'encapsulates' (gel particles that encapsulate safflower oil in such a way to deliver into the ileum). These encapsulates compose for 88 wt% of water (52.9 g per 60 g gel particles), and contain 2 wt% alginate (1.08 g per 60 g gel particles) and 10 wt% safflower oil (6 g per 60 g gel particles, present as droplets with average size of 25 μm). The active intervention yoghurts contain 105 kcal; of which 51 kcal% comes from the lipid (see Table 2).

Control product will be the same low-fat yoghurt as the active product, containing the same components as the 60 gram encapsulates, only with different physical structure: 54 gram empty gel particles (1.08 g alginate) present separately from 6 g safflower oil (same droplet size as in encapsulate). The control yoghurts also contain 105 kcal; of which 51 kcal% comes from the lipid (see Table 2).

Table 2. Overview of the nutrient composition of the studied formulations, and caloric content.

	Yoghurt A	*Yoghurt B*
Name	**Active**	**Control**

Safflower oil (g)	6.0	6.0
Whey protein (g)	0.24	0.24
Alginate (g)	1.08	1.08
Water inter-/intra-gel (g)	76.9	76.9
Yoghurt (g)	120	120
Total (g)	204	204
Total (kcal)	105	105
Lipid contribution (kcal%)	51%	51%

6.2 Summary of findings from non-clinical studies

Lipolysis has be controlled *in vitro* by lipid encapsulation in both digestible protein gels [22], [23] and indigestible alginate gels [24], [25]. The latter is the approach taken in the current study given the resistance of alginate matrix against gastric proteases [23], so ensures maintenance of the encapsulate structure in the stomach. In addition, the pH-dependency of volume and porosity of alginate gel particles makes them very suitable for ileal delivery [24], [26]–[28].
We designed encapsulates of safflower oil droplets (S) ($d_{32} \sim 25$ µm) in Ca-alginate (CA) hydrogel particles (0.5-1.2 mm) with different pore size of the hydrogel (5-10 nm). Lipolysis was controlled simply by gel particle size, and pore size at neutral pH, as shown for studied in a simple static *in vitro* digestion model. As preparation for the current human trial, we added these S-CA particles to yoghurt-meals and studied lipolysis during dynamic *in vitro* digestion in a three-compartment model of the upper GI tract (DIDGI, [36], [37]). Digestive media was sampled and analysed both for total extent of lipolysis and for the fraction solubilised in mixed bile micelles. To distinguish between i) direct inhibition of lipase by alginate [38] and ii) physical barrier to encapsulated oil [24], meals containing S-CA particles were compared to control meals with non-encapsulated lipid droplets, both in the presence and absence of empty CA particles. Lipolysis of non-encapsulated lipid was fast and would correspond to time scales relevant for duodenal delivery, whearas encapsulated lipid in CA particles showed delayed lipolysis up to four hours after ingestion, corresponding to ileal delivery.

6.3 Summary of findings from clinical studies

Ileal brake activation has been proven to be effective with direct intubation of all three macronutrients *via* a naso-ileal catheter (overview of literature in [3]). About 30 years ago, ileal lipid infusion was already shown to reduce food intake [4], [5]. Moreover, ileal lipid infusion increased perception of satiety/fullness or decreased hunger in most studies [1], [2], [4]–[7].

Regarding the current product, all ingredients (whey proteins, alginate, calcium, safflower oil) are commercially available food products with bio-base (so no synthetic additives), and have been repeatedly used in previous human trials (just some examples: alginate [29]–[33], calcium [29], [35], whey proteins even in infants [39], safflower oil [1], [6], [40]). Alginate as such has been used in clinical trials in multiple formulation forms, and it might reduce feelings of hunger (especially when present in solution) or have no effect [29]–[33].

Relatively large Ca-alginate gel particles (3.9-4.4 mm) have been tracked using MRI scanning in the human GI system (n=10) and were more visible in the ileum compared to the jejunum and duodenum [29]. This suggest an extended residence time in the ileum, and therefore favours ileal brake activation.
One human trial has been performed on an encapsulation system containing similar components: oil-core calcium-alginate-shell capsules (0.5 cm), which resulted in faster gastric emptying, and delayed lipolysis and absorption as measured with ^{13}C-labelled octanoic acid detected in a breath test [35], though these capsules would be quite large to use as a food additive.

6.4 Summary of known and potential risks and benefits

In our previous experiments with ileal brake activation *via* nutrient delivery through an ileal catheter, high distal ileal nutrient concentrations have been reached. However, none of the subjects experienced any discomfort during or after direct infusion into the ileum. We do not anticipate on major complaints or side effects for the current study with oral application route and therefore less pronounced ileal nutrient concentrations.
Besides, potential risks are expected to be minimal: First, a commercially available low-fat yoghurt (Campina, Magere yoghurt, 0% fat) will be used. Second, all of the added components are commercially available food ingredients with bio-base (so no synthetic additives)that have been repeatedly used in previous human trials. These include: alginate[29]–[33], calcium [29], [35], whey proteins [39], safflower oil [1], [6], [40]). Thus, all ingredients have a documented history of safe use. As mentioned previously, only for alginate we foresee a possibility for GI symptoms, for example abdominal bloating (3 out of 48 participants); but no significant difference (in nausea, fullness, bloating and pain scores) were detected compared to placebo [33]. Although alginate is used for multiple human applications (*e.g.,* acid burn), it may cause discomfort and/or GI symptoms due to viscosity-induced delay of the GI transit. To minimize possible discomfort and GI symptoms, the study will be halted when pain (as burning/cramp/colic) is measured to be extremely unpleasant (VAS scores > 95 out of 100) in three or more subjects.

6.5 Description and justification of route of administration

The lipids will not be administered *via* an ileal catheter, as the effectivity of this administration route has been proven already in previous studies (for an overview of studies, we refer to the review [3]). For an effective weight management strategy, administration should be performed *via* the normal route of food intake: oral administration. The current study will focus on the ability of lipid to induce the ileal brake when given orally. The lipids will be mixed with yoghurt, not only to allow easy consumption for the participants, but also to ensure no difference in appearance between control and active formulation. The lipids from the active intervention yoghurt will be delivered into the more distal small intestine within the two hours between ingestion and meal intake, and might consequently activate the ileal brake, whereas the control intervention yoghurt will deliver nutrients more proximally (to the stomach and duodenum). This allows investigating the effects of distal small intestinal delivery of undigested nutrients on food intake.

6.6 Dosages, dosage modifications and method of administration

To determine the dosage, we combined knowledge on effective dosage upon direct ileal fat perfusion with knowledge on the release profile of the current encapsulation system. Previous ileal brake studies have shown no dose dependency of satiety between 3 g and 9 g [6]. The current encapsulation system is expected to deliver at least 50% of the encapsulated lipid into the ileum, based on *in vitro* release data from static and dynamic digestion models (as summarized in section 1 and 6.2). We chose the investigational products to contain as little lipid as possible to still be effective, because lipid is a highly caloric nutrient and difficult to be compensated in the subsequent lunch. Therefore, the intervention yoghurts contain 6 grams of encapsulated safflower oil to release ≥ 3 g into the ileum (≥ 50%).
The method of administration of the active is based on an encapsulation method, using a matrix of alginate that is gelled by calcium divalent ions. The release of encapsulated lipid from these gel particles is based on pH-induced swelling at neutral pH as in the small intestine. This swelling increases the porosity of the gel matrix, which favours diffusion *via* increasing its coefficient. We found this diffusion coefficient under *in vitro* intestinal conditions to determine the release rate. By reverse engineering, we designed encapsulation systems that encapsulate safflower oil, and tuned the release profile based on gel matrix.

6.7 Preparation and labelling of Investigational Product

Handling and labelling will be done in the food facility of the Metabolic Research Unit Maastricht (MRUM, Maastricht University), following relevant HACCP guidelines. All ingredients (whey proteins, alginate, calcium, safflower oil) are commercial available food products, so no special precautions have to be taken in handling of the substances (see section 12 for a structured risk analysis). The alginate gel particles, both empty and filled with lipid, will be produced in accordance with safety and quality regulations of the commercial manufacturer. After preparation of these particles, they will be stored not longer than four days (96 hours) in 0.5 M $CaCl_2$ solution under refrigerated conditions (temperature of 4 °C) to ensure microbial safety. The ingredients (*i.e.*, yoghurt, oil-gel particles, or gel particles and emulsion) will be mixed freshly (1 to 20 hours before consumption) and stored under refrigerated conditions as suggested by the yoghurt supplier (Campina, Magere yoghurt, 0% fat). A detailed description of the procedures can be found in a separate standard operating procedure for the active and control yoghurt.
Labelling of the products will be according to the following code: study title (Lipid encapsulates, LE), subject number (01-35), yoghurt (A, B), and date. For example: LE07/A 13-02-17 means subject 7 of lipid encapsulates study-yoghurt A (active) produced on February 13th 2017.

6.8 Drug accountability

Not applicable as all used substances are food grade.

7. METHODS

7.1 Study parameters/endpoints

7.1.1 Main study parameter/endpoint

The main study parameter is the intake of *ad libitum* meal (kcal), as measured during pasta lunch (based on weight reduction of the plate assuming a homogenous meal). The reduction in amount of intake is supposed to reflect the activation of the ileal brake. The food intake will be compared between encapsulated lipids (active, yoghurt A) and non-encapsulated lipids in a formulation with equal nutrients (control, yoghurt B) within a subject.

7.1.2 Secondary study parameters/endpoints

Feelings of satiety (VAS scores)
The secondary study parameter is the effectivity to activate the ileal brake and, hence, induce feelings of satiety and reduce feelings of hunger, as measured on VAS scores (satiety, fullness, hunger, desire to eat, desire to snack). The VAS scores will be compared in time between intervention and control within a subject.

GI symptoms (VAS scores)
Another secondary study parameter is the occurrence and severity of gastrointestinal (GI) symptoms that orally ingested lipid encapsulates might give, as measured on VAS scores of GI symptoms (bloating, discomfort, pain, nausea). The VAS scores will be compared in time between intervention and control within a subject. Besides, the number of AEs and SAEs will be recorded. Moreover, we will assess whether differences in GI symptoms relate to encapsulate type.

7.2 Randomisation, blinding and treatment allocation

All 35 participants receive two treatments of one day each, following a cross-over design. The order in which they receive the treatments will be determined through randomisation. Randomisation will minimize the risk of bias and enhance the validity of statistical comparisons. As subjects qualify for the study, they will be assigned a subject number (01 - 35, *via* lottery) that corresponds with a predefined randomization schedule.

Blinding of packing and treatment will be assured for subjects. We will check at the end of the last test day whether the subjects were expecting to consume a control on one or more of the test days, and in case yes, on which day(s) they received it. Besides, on this day we will ask how they liked the yoghurts.

The VAS questionnaires will be labelled by the researcher with study title (Lipid encapsulates, LE), subject number (01-35), yoghurt (A, B) and time point (t= 0, 30, 60, 90, 105, 120, 135, 150, 165, 180, 195, 210 min). For example: LE07/ A / 120 - Subject 07, active yoghurt, VAS scores at 120 minutes.

Besides, labelling of the products will be according to the following code: study title (Lipid encapsulates, LE), subject number (01-35), yoghurt (A, B), and date. For example: LE07/A 13-02-17 means subject 7 of lipid encapsulates study-yoghurt A (active) produced on February 13th 2017.

7.3 Study procedures

Study procedure of the total study period

Thirty-five healthy subjects (male and female, any ethnic background, 18-65 years old) with overweight (BMI 25-30 kg/m^2) will be recruited (for more information on recruitment and consent see section 10.2; inclusion criteria in section 4.2, exclusion criteria in 4.3). If the volunteer is interested, an appointment will be made to sign the consent and complete the medical screening (discuss questionnaire (medical history, health status and medicine, and physical activity level (low, moderate or active)). If the volunteer is suitable, we will measure height and body weight as described below). After the subject is included, (s)he will receive an introduction lecture (of about half an one hour) and two test days (that consist of about four hours) will be scheduled per participant (with at least seven days in between the test days). Both test days will be scheduled in a period of a month.

Height measurement for BMI calculation- Height will be measured using a standard stadiometer. The participant has to remove any footwear. All participants should have their height measured while in bare or stocking feet. The participant will be instructed to stand on the base of the stadiometer, and the investigator will check if the participant is standing straight with shoulders back and resting flat on his/her feet. The height will be recorded in centimetres (cm) to the nearest 0.1 cm.

Body weight measurement- Body weight will be measured using an electronic or digital scale, which is regularly calibrated. Subjects are asked to remove their footwear, outer garments (*e.g.*, jackets, hats, heavy sweaters, etc.), belt, heavy jewellery, and they are asked to empty their pockets. The participant will be asked to remain on the scale, and the investigator will read the screen once stabilized and record the value in kg to the nearest 0.1 kg.

Procedure of the intervention study days

The procedure of the intervention study days is summarized in Table 3. On each test day, the subjects will arrive at 8:50 am at Maastricht University, after a ten hour fast, so no eating and drinking is allowed other than *ad libitum* consumption of water and tea (without milk or sugar) after 22:00 the night before each test day.

Upon arrival at Maastricht University (8:50 am), compliance to the previously mentioned rules will be checked and baseline measurements will be done ("t=0" min), including nine VAS scores for satiety and GI symptoms. Then, the experiment starts with the ingestion of a standardized breakfast (9:00 am, t=0 min). During all study days, this breakfast will be relatively light (±120 kcal) and low in fat (≤0.5 g total lipid): 1 sandwich (tijgerbrood tarwe 1 g lipid/100g, 1 boterham: 35g (±84 kcal)) with marmalade (0 g lipid/100g, 1 portion: 15g (±36 kcal)) and one glass of water or tea (150 mL).

Ninety minutes after breakfast (10:30 am, t=90 min), once major part of the breakfast has been emptied from the stomach, subjects receive a low-fat yoghurt-like product of 0.20 L that contains one of the two formulations of emulsified safflower oil (6 g lipid as active or control formulation, for product details see section 5), following a randomized cross-over design. Each 30 minutes before the intervention and each 15 minutes after the intervention, the nine VAS scores for satiety and GI symptoms will be measured. Two hours after the intervention yoghurt (12:30, t=210 min), *ad libitum* meal intake will be measured as amount eaten from a large pasta meal (>1 kg, obtained from a local supermarket). After collection of these results, the test day is finished and the subject returns home (around 13:00 am). For an overview of the complete study design, see Figure 1.

VAS scores for satiety and GI symptoms- During a test day, satiety and GI symptoms will be measured at 12 time points (t= 0, 30, 60, 90, 105, 120, 135, 150, 165, 180, 195, 210 min). Questionnaires will be used to measure satiety feelings (satiety, fullness, hunger, desire to eat, desire to snack) and GI symptoms (bloating, discomfort, pain, nausea). These nine attributes will be measured using Visual Analogue Scales (VAS, 0 to 100 mm) scores, with the most negative or lowest intensity feelings at the low end and the opposing terms at the high end. The subject will be asked to indicate his feeling at that moment. The investigator can easily quantify the rating as shown in previous studies (for example: METC 13-2-025; 11-3-034; 14-3-062). The scoring forms will be collected immediately so that they cannot be used as a reference for later scorings.

Table 3. Schedule of the participant prior and during the test days.

When?	Action/Intervention	Measurement
Interview	Sign consent, complete screening (inclusion); and information lecture	Screening questionnaire, height, body weight
Test days (2x)		
8:50 am	Arrival* and baseline	VAS (S & GI, t=0 min)**
9:00 am	Standardized breakfast(t=0 min)	
9:30 am		VAS (S & GI, t=30 min)
10:00 am		VAS (S & GI, t=60 min)
10:30 am	Intervention yoghurt(t=90 min)	VAS (S & GI, t=90 min)**
10:45 am		VAS (S & GI, t=105 min)
11:00 am		VAS (S & GI, t=120 min)
11:15 am		VAS (S & GI, t=135 min)
11:30 am		VAS (S & GI, t=150 min)
11:45 am		VAS (S & GI, t=165 min)
12:00 am		VAS (S & GI, t=180 min)
12:15 am		VAS (S & GI, t=195 min)
12:30 am	*Ad libitum* lunch(t=210 min)	VAS (S & GI, t=210 min)**
13:00 am	Return home	

*VAS= Visual Analogue Scales for satiety (S) and GI symptoms (GI). * after 10 h fasting. ** VAS score measured just before the intervention.*

7.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason (also without reason) if they wish to do so without any consequences. The responsible investigator can also withdraw a subject if continuing participation is in his opinion deleterious for the subject's wellbeing. Subjects can also be withdrawn in case of protocol violations and non-compliance. When a subject withdraws from the study, a medical examination can be performed if considered necessary by the nurse practitioner/physician. In case of withdrawal because of AEs or SAEs haematological, blood chemistry and urine laboratory test or other special examinations may be performed.

7.4.1 Specific criteria for withdrawal

Not applicable.

7.5 Replacement of individual subjects after withdrawal

Thirty-five subjects will be included at the start of this explorative study. Subjects will not be replaced after withdrawal.

7.6 Follow-up of subjects withdrawn from treatment

No formal follow-up visits are planned in case a subject withdraws from the study after administration of a study investigational product.

7.7 Premature termination of the study

The study will be discontinued if the treatments are intolerable for the majority of the subjects.

8. SAFETY REPORTING

8.1 Temporary halt for reasons of subject safety

In accordance to 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 a further positive decision 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)

AEs are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the investigational product, trial procedure, or the experimental intervention. All AEs reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

8.2.2 Serious adverse events (SAEs)

A SAE 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 SAE.

The investigator will report all SAEs without undue delay after obtaining knowledge of the events, through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within seven days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of eight 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 SAE.

8.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable.

8.3 Annual safety report

Not applicable.

8.4 Follow-up of AEs

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.

8.5 Data Safety Monitoring Board (DSMB) / Safety Committee

Not applicable.

9. STATISTICAL ANALYSIS

The SPSS statistical software package Version 23.0.0.2 (IBM© SPSS Statistics, Chicago, IL, USA) will be used for statistical analysis. The data will be organized in this software, containing descriptive independent variables (ID, age, gender, BMI), fixed variables (test day (1, 2), treatment (A, B), time) and dependent variables (food intake and VAS scores). A descriptive analysis of the study population will be performed first, about the independent variables age, gender and BMI, including mean ± S.D. The dependent variables will be checked to meet the requirements of the statistical models described, including and normality, and homogeneity of variance. Continuous data will be reported as mean ± S.D and/or S.E.M. and statistical significance will be set at $p< 0.05$, two sided.

9.1 Primary study parameter(s)

The main study parameter is the intake of *ad libitum* meal (kcal). The difference in intake of *ad libitum* meal (kcal) will be compared within-subjects, between ingestion of the active or control yoghurt.
H_0: There is no significant difference in *ad libitum* meal intake after control and active yoghurt.
H_1: A*d libitum* meal intake differs between control and active yoghurt.
If the food intake data (2 values per subject) is normally distributed and homogeneity of variance holds, a paired-samples T-test will be performed to evaluate the null hypothesis that there is no difference in *ad libitum* food intake after control and active yoghurt ($n=35$, $p \leq 0.05$).
Missing values of the primary study parameter food intake will be left out of the analysis, because the paired-samples T-test compares food intake within-subjects.

9.2 Secondary study parameter- VAS scores for satiety and GI symptoms

Secondary parameters include differences between treatments in feelings of satiety (as measured by VAS) and GI symptoms (as measured by VAS). During each of the test days, participants (n=35) fill in nine VAS scores (VAS1-VAS9) per time point, at 12 time points (t= 0, 30, 60, 90, 105, 120, 135, 150, 165, 180, 195, 210 min). If the VAS data is normally distributed and homogeneity of variance holds, we will use a repeated measures analysis of variance (ANOVA) model that includes the fixed factors treatment, time, and the interaction *treatment * time* to evaluate the null hypothesis that there is no difference in VAS scores after control and active yoghurt ($n=35$, $p \leq 0.05$). If the results of the ANOVA indicate a significant effect of intervention yoghurt (Wilks' Lambda test, $p \leq 0.05$), the treatments will be compared based on pairwise comparison with a Tukey post hoc test.
Missing values of the secondary study parameter will cause the intervention to be left out, due to the use of ANOVA.

10. ETHICAL CONSIDERATIONS

10.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki (as amended in Tokyo, Venice, Hong Kong, Somerset West, Edinburgh, Seoul and Fortaleza. Note of clarification added in Washington and Tokio) and in accordance with the Medical Research Involving Human Subjects Act (WMO). Last updated during WMA General Assembly meeting in Fortaleza, Brazil, October 2013.

10.2 Recruitment and consent

Subjects will be recruited from a pool of previously included subjects that participated in previous studies, and besides, using posters at the local libraries and student frequented areas, advertising in local newspapers, and if needed using www.onderzoeksmachine.nl. Initial information will be provided verbally or *via* email. The written information brochure will be send by regular mail or email. If a volunteer is interested, an appointment will be made to sing an informed consent and complete the medical screening (for the procedure see section 7.3). A minimum of one week is provided to decide whether the volunteer would like to participate. Volunteers will not be contacted if they do not respond to the sent information brochure. Upon arrival for the first visit, the participants will have to sign an informed consent before screening can start. If participants withdraw from the study after the screening, these results will be destroyed. Participants can make at any time an appeal to the independent doctor that has been appointed to this study. Participants will be informed that their decision to participate is voluntary and they can withdraw at any time without giving a reason. At the end of the study, participants will have the opportunity to be informed about their individual results and the group results.

10.3 Objection by minors or incapacitated subjects

Not applicable.

10.4 Benefits and risks assessment, group relatedness

The benefits of this study can be great: it will provide knowledge about the principle of intestinal brake activation through dietary yoghurt that contains encapsulated lipid (effectivity compared to control). If found effective in this study, such a yoghurt snack could be directly implemented in a diet, to prevent overeating, and in that way provide a long-term weight management strategy. However, besides that there is no personal benefit for the subjects.
This study is non-invasive, so we do not expect any great risks for the subjects. The oral consumption of a breakfast, yoghurt snack, and lunch clearly reduces the discomfort in subjects compared to our previous intestinal brake studies with naso-ileal catheter. Moreover, all consumed products are food-grade and could appear in a regular diet. To minimize discomfort further, the study will be halted when pain (as burning/cramp/colic) is measured to be extremely unpleasant (VAS scores > 95 out of 100) in three or more subjects.

10.5 Compensation for injury

Insurance for participants will be provided in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). Maastricht University has a standard WMO insurance a liability insurance for research participants.

The sponsor/investigator has a liability insurance that is in accordance with article 7 of the WMO. The sponsor (also) has an insurance that 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.

1. € 650.000,-- (*i.e.,* six hundred and fifty thousand Euro) for each subject who participates in the Research;
2. € 5.000.000,-- (*i.e.,* five million Euro) for all subjects who participate in the Research;
3. € 7.500.000,-- (*i.e.,* seven million five hundred thousand Euro) for the total damage incurred by the organisation 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 four years after the end of the study.

10.6 Incentives

Once completed the full study, the subjects will receive 75 euro (including travel expenses). In case a subject stops participation during the study, he or she will be reimbursed *pro rato*.

11. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

11.1 Handling and storage of data and documents

Data are handled confidentially and anonymously in accordance to WBP (in Dutch: Wet Bescherming Persoonsgevens; Personal Data Protection Act). To guarantee the privacy of the participants, subject numbers will be used in such a way that no personal information about the participants will be available (allocated *via* lottery). All questionnaires and data will be coded (using study title (Lipid encapsulates, LE), and subject number (01-35), yoghurt (A, B) and time point (t= 0, 30, 60, 90, 105, 120, 135, 150, 165, 180, 195, 210 min). For example: LE07/ A / 120- Subject 07, active yoghurt, VAS scores at 120 minutes. The researcher keeps the key of the code in a locked cabinet. Next to the research team, the data is assessable for the METC, IGZ and monitors. No human material will be taken during this study. All primary documents and data shall be kept for 15 years after the end of the experimental phase of the study for possible inspection. All future studies will always be sent to the METC for approval.

11.2 Monitoring and Quality Assurance

The study will be monitored by the Clinical Trial Centre Maastricht (CTCM).

11.3 Amendments

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

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

11.4 Annual progress report

The current study is expected to take substantially shorter than a year. In case of considerable delay, 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, SAE/ SARs, 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 eight 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.
The investigator/sponsor will send the publication on the results of the study to the accredited METC after peer-reviewed by a scientific journal; if not accepted within one year after the end of the study, the manuscript will be send as submitted to the journal within one year after the end of the study to the accredited METC.

11.6 Public disclosure and publication policy

All trial results, both positive and negative, will be disclosed in agreement with the CCMO statement on publication policy. Based on the results of this study, at least one publication will be submitted for publication to a peer-reviewed scientific journal. The authorship of the article shall be determined in appropriate consultations based on a considerable contribution to the set-up and execution of the study and an active participation in publication. None of the parties concerned has the right of veto considering publication.

12. STRUCTURED RISK ANALYSIS

12.1 Potential issues of concern

a. Level of knowledge about mechanism of action

Ileal brake activation has been proven to be effective with direct intubation of all three macronutrients *via* a naso-ileal catheter (overview of literature in [3]). About 30 years ago, ileal lipid infusion was already shown to reduce food intake [4], [5]. Moreover, ileal lipid infusion increased perception of satiety/fullness or decreased hunger in most studies [1], [2], [4]–[7]. The mechanism of action is based on sensed lipid species at intestinal receptors, which induce distal to proximal negative feedback mechanisms [9]. This mechanism is induced more strongly when the degradation products of lipid digestion (fatty acids, monoglycerides) are sensed rather than by the intact lipids itself (triglyceride molecules) (clear overview of literature in [9], paragraph 4.1.1). Hence, for efficient ileal brake activation, lipase-mediated hydrolysis of lipids is required, and in addition, proximal absorption of degradation products has to be prevented.

To be an effective weight management strategy, a different administration route is required than direct intubation, namely orally with the normal route of food intake. When applied orally, however, the major part of dietary lipids will be digested and absorbed in the proximal small intestine and are not likely to induce the strong ileal brake mechanism. Hence, to activate the ileal brake, the digestion of lipid needs to be controlled. To truly control lipid digestion, the structuring approach should go beyond interfacial design [21], and may focus on control of enzyme diffusion towards its substrate by means of a larger structure (more details on this approach can be found in section 1).

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

Humans have not been exposed to the current test products. However, humans have been exposed regularly to the separate components present in the current products: low-fat yoghurt, safflower oil, whey protein, calcium, alginate. First of all, the low-fat yoghurt (Campina, Magere yoghurt, 0% fat) is a product from a local supermarket that is included in the diet of major part of the Dutch population. Safflower oil is a vegetable oil that can be part of a normal diet. Moreover, the exact same safflower oil has been intubated at different locations in the small intestine in previous ileal brake studies by the current principal investigator without GI symptoms. The biopolymers whey protein and alginate can be used in food preparations, and are safe to use *in vivo*. No GI symptoms were observed in the previous study by the current principal investigator, where whey protein, carbohydrates and alginate were present (but no lipid), even at higher concentrations [Alleleyn and *co-workers*, in press].

c. Can the primary or secondary mechanism be induced in animals and/or in ex-vivo human cell material?

The primary mechanism, the ileal brake, has been induced multiple times *in vivo* in clinical trials, as described in 12.1a). Moreover, the transposition of the ileum to the jejunum in rat resulted in higher GLP-1 and PYY levels, and weight loss [41]. In an e*x vivo* pig study increased GLP-1 and PYY release was found after ileal exposure to nutrients [42]. These results all contribute to the suggestion that satiety hormones are involved in the ileal brake mechanisms.

d. Selectivity of the mechanism to target tissue in animals and/or human beings

The satiety hormone receptors (GLP-1r, PYYr) are expressed in the distal gut regions [43]. And after stimulation of *ex vivo* ileal tissue, satiety hormone release was the highest in the most distal part of the small intestine [42]. In older animal studies it has been shown that signals arising in the lower duodenum and upper jejunum have no effect on the short-term control of food intake whereas signals from the more distal jejunum and ileum significantly inhibited total daily food intake [44]. Furthermore a study of Maljaars et al. (2011) showed that ileal fat infusions had the most pronounced effect on food intake and satiety compared to oral, duodenal and jejunal fat ingestion [2]. The data all contribute to the selectivity of the mechanism to the target tissue: the ileum.

e. Analysis of potential effect

To our best knowledge, no data is available for this particular encapsulation system about food intake and satiety. Our encapsulation system, however, had been extensively studied in simulated digestive conditions, including both static and dynamic *in vitro* models. Based on these models, the behaviour of the encapsulation system and rates of lipolysis were being estimated. Based on previous studies using the same oil, it is known that already at 3 g intra-ileal infusion, satiety can be induced compared to control (orally taken lipid) [6]. Intra-ileal infusion of 6 g safflower oil, the food intake (464.3±90.7 kcal) was 21 % lower compared to placebo (saline infusion, 586.7 ±70.2 kcal) [1]. Besides, food intake was significantly reduced with 79 kcal (SEM 41; n=15 so SD of 159 kcal) after ileal intubation (of 6 g lipid; food intake 435 kcal) compared to oral ingestion (of 6 g lipid, combined with saline infusion; food intake 514 kcal) [2]. In another study on encapsulation, food intake reduced after active micro-encapsulate intake (655 kcal, SD 216) compared to placebo (699 kcal, SD 238), so a reduction of 44 kcal (P=0.011) [*not yet published randomized placebo-controlled double-blind cross-over study in 60 healthy overweight volunteers*]. On average, these three studies showed a difference of 82 kcal with an average SD of 156 kcal.

f. Pharmacokinetic considerations

Not applicable.

g. Study population

The study population includes healthy overweighed subjects (BMI 25-30 kg/m^2), in the age of 18-65 years.

h. Interaction with other products

Not applicable.

i. Predictability of effect

This study is non-invasive, with the primary outcome variables being food intake and the secondary study parameters measured using a questionnaire, so no biomarkers will be used. The questionnaires will include VAS scores for satiety (satiety, fullness, hunger, desire to eat, desire to snack) and GI symptoms (bloating, discomfort, pain, nausea). To minimize possible discomfort, the study will be halted when pain (as burning/cramp/colic) is measured to be extremely unpleasant (VAS scores > 95 out of 100) in three or more subjects.

j. Can effects be managed?

Not applicable.

12.2 **Synthesis**

The overweight of the subjects (BMI 25-30 kg/m^2) might bring a higher risk compared to persons with normal weight. To reduce this risk, potential subjects are medically screened and only included when relatively healthy.

The meals and yoghurts are bought from a local supermarket and only used before the expiry date. In addition, all added ingredients are food-grade according to EU regulation: safflower oil, calcium, alginate and whey protein. New batches will be ordered for this study and opened only in rooms that are suitable for human consumption. Handling and labelling will be done in the food facility of the Metabolic Research Unit Maastricht (MRUM, Maastricht University), following relevant HACCP guidelines.

This study is non-invasive, so we do not expect any great risks for the subjects. The oral consumption of a breakfast, yoghurt snack, and lunch clearly reduces the discomfort in subjects compared to our previous intestinal brake studies where naso-ileal feeding catheters were introduced. To minimize discomfort further, the study will be halted when pain (as burning/cramp/colic) is measured to be extremely unpleasant (VAS scores > 95 out of 100) in three or more subjects.

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