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Increased protein intake in healthy males exposed to an appetite modulating, whey-derived peptide hydrolysate †

Erin Stella Sullivan, *[a,b,c,d] Harriët Schellekens,[b,f] Brendan T. Griffin,[b,e] Samantha J. Cushen,[a,b,c] Ken Howick,[b,e] John F. Cryan,[b,f] Darren Dahly,[g] Nessa Noronha[b,h] and Aoife M. Ryan[a,b,c]

The identification of food-grade bioactives with proven orexigenic effects would mark significant progress in the treatment of disease-related malnutrition. To investigate the effects of two milk-derived hydrolysates (UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate)) on appetite and energy intake in healthy humans, a single-blind, placebo-controlled, 3-arm cross-over feeding trial was conducted in 22 fasted, cannulated healthy male volunteers. Participants received 26 mg kg^{-1} of both hydrolysates and placebo and were observed from morning to afternoon with a set breakfast and *ad libitum* lunch. Mean total daily energy and protein intakes when treated with placebo were 2673 kcal (95% CI: 2247–3100 kcal) and 128 g (95% CI: 105–152 g), respectively. Energy intake for either treatment was not significantly different from that for placebo (p = 0.266 for UL-2-141 and p = 0.796 for MF1145). Protein intake significantly increased in the UL-2-141 arm compared with that in placebo (+23 g, p = 0.044), but it did not significantly increase in the MF1145 arm (+13 g, p = 0.189). Appetite, hunger and satiety responses on VAS for either treatment were not significantly different from those obtained for placebo. GLP-1 was significantly higher pre-lunch in the UL-2-141 arm than in placebo (+8 pmol L^{-1}, p = 0.01) and in the MF1145 arm (+7 pmol L^{-1}, p = 0.039). GH was significantly lower pre-lunch only in the UL-2-141 arm than in placebo (–133 pg mL^{-1}, p = 0.027). Protein intake was significantly increased in the UL-2-141 arm, demonstrating appetite modulation, potentially *via* indirect or delayed stimulation of the ghrelin receptor. Since healthy adults are often not in tune with their physiological hunger, repeating the study in subjects with established anorexia may be prudent.

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Introduction

Methods to stimulate appetite in the sick or elderly remain a challenge, with few safe therapeutic options available. In oncology, appetite modulation is of importance as reduced appetite is a strong predictor of malnutrition,[1] and there is strong evidence that malnutrition at diagnosis and deterioration of the nutritional status during treatment are associated with poor outcomes, including increased risk of treatment toxicities and post-operative complications; poor quality of life and overall survival.[2,3]

Corticosteroids, such as dexamethasone, are widely used in oncology to prevent nausea, but short courses of high doses are used to stimulate appetite. However, this is usually limited to those with short life expectancies as a palliative measure[4] as increased appetite is not associated with significant muscle gain and there is a high risk of intolerable or dangerous side effects, such as hyperglycaemia, risk of fracture, GI discomfort and changes in mood including increased aggression.[5] Megestrol acetate is a progestational agent that increases appetite and induces weight gain in patients with cancer cachexia (CC). However, no significant impact on the quality of life (QoL) or survival has been reported to date. Moreover, megestrol acetate is associated with a high risk of serious adverse

[a]*School of Food and Nutritional Sciences, College of Science, Engineering and Food Science, University College Cork, Cork, Ireland.*
E-mail: erin_stella.sullivan@kcl.ac.uk
[b]*Food for Health Ireland, Belfield, Dublin 4, Ireland*
[c]*CancerResearch@UCC, University College Cork, Cork, Ireland*
[d]*Department of Nutritional Sciences, Faculty of Life Sciences & Medicine, King's College London, London, United Kingdom*
[e]*School of Pharmacy, University College Cork, Cork, Ireland*
[f]*Department of Anatomy & Neuroscience, University College Cork, Cork, Ireland*
[g]*School of Public Health, University College Cork, Cork, Ireland*
[h]*School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland*
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events, including thromboembolism and death (84% increased risk of a thromboembolic event).[6] A trial in 2009 found that 11.3% of 97 patients with advanced cancer receiving megestrol acetate with chemotherapy experienced a thrombotic event.[7] These reports demonstrate an increased relative risk and a significant absolute risk of thromboembolic complications of this treatment.[6] A more recent RCT conducted in 190 patients with cancer-related anorexia confirmed this increased risk of potentially serious adverse events while finding no significant improvement in appetite when comparing megestrol acetate, dexamethasone and placebo.[8] Therefore, the likelihood of any potential benefits of megestrol acetate must be carefully considered in light of the risk of potentially life-threatening adverse outcomes.

However, ghrelin, an orexigenic (hunger-inducing) hormone, has received considerable attention as a therapeutic target to stimulate nutritional intake in patients with cancer cachexia and a favourable safety profile.[9–11] In addition, a positive association was demonstrated between baseline circulating levels of ghrelin and body weight gain in individuals with anorexia nervosa.[12] Ghrelin is a peptide hormone that is secreted peripherally, primarily from the stomach and gut, acting on the growth hormone secretagogue receptor, which is expressed throughout the body but concentrated in vagal afferents and pancreatic islets.[13,14] Ghrelin is traditionally considered the 'hunger hormone' unlike other gut hormones with anorexigenic effects, and it induces hunger and provides a signal to initiate feeding. However, it is also involved in other metabolic pathways, including glucose and energy homeostasis. Notably, despite the presence of ghrelin receptors throughout the body, the feeding control mechanisms of ghrelin appear to be primarily mediated *via* the vagus nerve, as ghrelin does not stimulate feeding in cases of vagal ablation or vagotomy.[15] Activation of the ghrelin receptor by peripherally produced ghrelin stimulates the feeding centre in the hypothalamic arcuate nucleus. However, ghrelin can also cross the blood–brain barrier at slow rates.[16]

Given this unique property as a potential appetite stimulant, it has been proposed as a treatment for cachexia; although exogenous ghrelin needs to be administered intravenously or subcutaneously, it may not be the most practical solution for patients needing ongoing appetite support.[9] Intravenous administration of ghrelin causes increased food intake and body weight, decreases catabolism and is well tolerated in cancer patients; however, long-term safety is unclear and the overall level of evidence is low, with a recent Cochrane review unable to make any conclusions on its use, owing to a lack of well-designed studies.[10]

Although exogenous ghrelin has not shown the results hoped for in terms of appetite modulation, anamorelin is a small molecule drug that is an orally active, selective ghrelin receptor agonist and has shown promising results in terms of increased food intake, body weight and lean body mass in cancer populations.[17,18] In the ROMANA 1 and 2 trials, it was shown that anamorelin could increase body mass, specifically lean body mass, in patients with inoperable stage III or IV NSCLC.[19] The safety extension trial (ROMANA 3) found that for up to 24 weeks, anamorelin remained well tolerated, and beneficial responses were maintained.[20] However, these trials failed to show a significant impact on hand-grip strength or QoL. Although licensed and in use in Japan,[21] anamorelin was denied marketing authorisation by the European Medicines Agency (EMA) in 2017, which cited insufficient data on safety outcomes in addition to only a marginal impact on lean body mass and a lack of impact on functional measures, such as quality of life and hand-grip strength.[22] Since then, the results of Japanese anamorelin trials have become available, and these results have confirmed the European results in NSCLC and have shown that improvements in lean body mass and appetite are also transferable to the GI oncology setting and that the drug is well tolerated over 12 weeks of treatment in this cohort.[23,24]

The ghrelin receptor can also be activated by several other ligands and is therefore a target of interest for appetite modulation. Food grade methods for stimulating ghrelin or its receptor are of significant interest because of the difficulties to date with pharmacological options. Interest in food-derived bioactives has developed from the understanding that many nutritive and non-nutritive food components exhibit biological effects, and these components have been employed in functional foods for some time, with the benefit of incorporating these food-grade ingredients into commonly consumed food products.[25] Protein-derived bioactives are a particular area of interest because they serve a dual purpose as a nutritive source of amino acids as well as their constituent peptide sequences have potentially potent effects on various physiological processes, including stimulation of gut hormone release.[26,27] Investigations of food-derived bioactives have often focused on cardiometabolic effects,[25] but the growing interest in the effects of dairy-derived peptides on appetite signalling[28] inspired the Appetite Modulation Work Package of the Food for Health Ireland (FHI-2) programme.

In this FHI-2 study, we investigated the effects on the appetite and energy intake of two bioactive dairy-derived hydrolysates containing a complex mixture of peptides that act as ghrelin mimetics and have been shown to activate ghrelin receptors in murine models.[29] Specifically, we investigated UL-2-141, a whey-derived hydrolysate and MF1145, which is a casein-derived hydrolysate. Initially, MF1145 was shown to increase GHSR-1a-mediated intracellular calcium signalling *in vitro*. Subsequently, *in vivo* studies showed an increase in dietary intake in healthy male and female Sprague–Dawley rats after gavage dosing with the MF1145.[29] An additional ghrelinergic hydrolysate derived from whey was isolated and brought forward in subsequent studies.[30] Owing to enzymatic digestion and acid degradation in the stomach, these orally active hydrolysates require protection from the gastric environment to reach the gut lumen intact; therefore, formulation studies were conducted to optimise the delivery method of the hydrolysates.[30]

In an unpublished work from our laboratory (MSc Thesis of Ms. Fiona Dwyer, UCC), MF1145 was found to be tolerable in healthy males at 26 mg kg^{-1} delivered *via* encapsulated microbeads. Both MF1145 and UL-2-141 were found to be tolerable

in healthy males at 52 mg kg^{-1} in the dose escalation study; however, the high capsule burden was deemed incompatible with the aim of stimulating appetite, so the original 26 mg kg^{-1} was used in the cross-over study. Although these hydrolysates are generally recognised as safe (GRAS), 'safety' studies were conducted as an additional precaution and to assess tolerability at high doses. This study was conducted to assess the effects of these hydrolysates in humans, as the development of food-grade bioactives with proven orexigenic effects would mark significant progress in the treatment of disease and age-related anorexia.

Methods

Aims and objectives

• To examine the effects of the dairy-derived hydrolysate samples (UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate)) *versus* placebo on appetite regulating hormones and biomarkers of glucose metabolism.

• To examine the effect of dairy-derived hydrolysate samples on energy and protein intake *versus* placebo.

• To determine whether dairy-derived hydrolysate samples are efficacious in stimulating appetite on visual analogue scales in their current formulation.

Ethics

Ethical approval was sought from the Clinical Research Ethics Committee of the Cork Teaching Hospitals (CREC). Approval for the Food for Health Ireland (FHI) Appetite Modulation Cross-Over Study was granted on 17/05/2017. All studies were conducted in accordance with the Declaration of Helsinki and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline for Good Clinical Practice E6(R2). Informed consent was obtained for the study and subsequent biobanking. Data will be retained as per the General Data Protection Regulation and identifiable study data held electronically is password-protected spreadsheets created using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). On completion of the study, volunteers received €50 One4All voucher per study day.

Study design

The study was conducted during Autumn/Winter 2017/18 in Mercy University Hospital, Cork, Ireland. Its design was a phase I, single-blind, placebo-controlled, three-way cross-over trial testing the efficacy of 2 dairy-derived hydrolysates (UL-2-141 whey hydrolysate and MF1145 casein hydrolysate) *versus* a placebo in stimulating appetite *via* ghrelin receptor agonism. The study was single blinded to the participants, and the cross-over included different patterns of administration to control for discrepancies based on order of administration.

Study population

The target group for this efficacy study was healthy young males with no significant medical history or dieting behaviours.

Inclusion criteria

• Male

• 18–45 years old

• Body weight 50–100 kg (preferably below 80 kg to lower the burden of per kg doses)

• Body Mass Index (BMI) ≥18.5 kg m^{-2} and ≤30 kg m^{-2}

Exclusion criteria

• Unable or unwilling to consume dairy-derived hydrolysates, *e.g.* veganism and lactose intolerance

• Use of any tobacco or nicotine products (including electronic cigarettes) in the 6 months prior to inclusion

• Unable to swallow capsules

• Weight loss or gain diets, or any other extreme dietary attitudes or behaviour, including disordered eating

• History of obesity

• Weight change ≥5% in the 6 months prior to inclusion

• Any significant medical condition that may interfere with the absorption, metabolism or elimination of the study hydrolysates or that may affect appetite, including but not limited to thyroid disorders, gastrointestinal obstruction, inflammatory disease, malignancies, any acute disease, oedema, ascites, any allergies, liver disease, neurologic disease, any vessel or heart disease, metabolic disorder, diabetes, cardiac, renal or respiratory function impairment and uncontrolled infection

• Use of drugs that may interfere with gastrointestinal motility and visceral sensitivity or absorption, metabolism or elimination of the study hydrolysates, or that may affect appetite, including but not limited to calcium channel antagonists, nitrates, prokinetics, proton pump inhibitors, H_2 receptor antagonists and sedatives

Dose determination (dose escalation and pilot cross-over studies)

The conversion of animal to human doses was performed as per FDA guidelines[31] for the first in-human trials (unpublished work by Fiona Dwyer, MSc). The level at which there were no observed adverse events in animals (NOAEL) can be used to calculate a Human Equivalent Dose (HED) with species-specific conversion factors on a mg kg^{-1} or mg m^{-2} basis. As this hydrolysate is food grade and Generally Recognised As Safe (GRAS), the higher mg kg^{-1} HED was used. Doses found to be safe and effective in *in vivo* studies using Sprague–Dawley rats[29] were converted to HED using a conversion factor of 0.16. Safety factors are available and recommended in pharmaceutical trials, but again, this was not applied, as the hydrolysates were considered relatively safe. Therefore, the HED was rounded up to the nearest 0.5 mg and considered the Maximum Recommended Starting Dose (MRSD).

In unpublished work from our laboratory (Ms Fiona Dwyer, UCC), the novel casein-derived ghrelin agonist MF1145 was found to be both safe and tolerable in healthy males at a Total Daily Dose (TDD) of 26 mg kg^{-1} delivered *via* encapsulated microbeads in the initial safety study, and this was used as the MRSD for the dose escalation study. In the dose escalation study, to achieve 26 mg per kg BW, the mean number of capsules administered was 11.8 (11 full capsules and one partially

filled capsule). The standard deviation was 2.06. The minimum capsule prescription was 9 capsules, and the maximum was 14 capsules. To achieve 52 mg per kg BW, the mean number of capsules administered was 23.0. The standard deviation was 4.55. The minimum capsule prescription was 17 capsules, and the maximum was 28 capsules. Both MF1145 and UL-2-141 were found to be safe in healthy males at a Total Daily Dose (TDD) of 52 mg kg^{-1} in the dose escalation study; however, the high capsule burden was deemed incompatible with the aim of stimulating appetite, so the original 26 mg kg^{-1} was used in the pilot cross-over study.

During the pilot cross-over study, the efficacy of the 26 mg kg^{-1} dose of both hydrolysates was assessed *versus* placebo. To achieve 26 mg per kg BW, the mean number of capsules received was 11.7 (11 full capsules and one partially filled capsule). The standard deviation was 1.49. The minimum capsule prescription was 9 capsules, and the maximum was 15 capsules.

Formulation

Whey hydrolysate (UL-2-141). The whey hydrolysate (designated in prior publications as FHI-2571) was prepared by applying a method similar to a previously published method.[30,32] Briefly, bovine milk-derived whey protein (80% w/w protein, Carbery Group, Ballineen, Cork, Ireland) was suspended at 10% protein (w/w) in reverse osmosis-treated water and agitated continuously at 50 °C for 1 h in a jacketed tank. The pH was adjusted using a NaOH 4.0 N solution (VWR, Dublin, Ireland). A bacterial food-grade enzyme preparation was added to the protein solution until a 7–12% degree of hydrolysis was achieved. The enzyme was then inactivated by heat treatment, and the resultant hydrolysate solution was dried in a Niro TFD 20 Tall-Form Dryer (GEA, Düsseldorf, Germany).

Casein hydrolysate (MF1145). Sodium caseinate (NaCas, Kerry Group Plc, Listowel, Ireland) was suspended at 10% (w/w) on a protein basis in water and dispersed under agitation at 50 °C for 1 h using an in-line mixer (total batch size—1000 L). Protein hydrolysis was performed by the addition of a food grade enzyme for 3 h at 50 °C. The pH of hydrolysis was maintained at a constant pH for the duration of hydrolysis by the addition of a hydroxide base (Microbio, Fermoy, Ireland). The enzyme was then inactivated by heat treatment through a plate and frame heat exchanger (Unison Engineering Services Ltd, Limerick, Ireland). Large molecular weight material and aggregates were removed from the hydrolysate through membrane separation or clarification steps. The clarified material was then ultrafiltered at 50 °C using 1 kDa spiral wound organic membranes (Synder Filtration, Vacaville, CA, USA) operating under a transmembrane pressure of 2 bar. A diafiltration step using reverse osmosis was utilised to increase the recovery of small peptides in the permeate. The permeate fraction was dried in a single-stage spray dryer (Anhydro F1 Lab Dryer, Copenhagen, Denmark). This method has also been described in a previous publication.[29]

Capsule delivery. Owing to enzyme-mediated and acid degradation in the stomach, orally active hydrolysates require protection from the gastric environment to reach the gut lumen intact.[30] The formulation used in the dose escalation and cross-over studies was developed as part of the FHI work package.[30] This process began with the conventional extrusion-spheronization of the active ingredient with the bulking agent microcrystalline cellulose (MCC). The microbeads were then sprayed with a dual layer polymer coating solution (PCS) (a methacrylic acid copolymer (MA) subcoat and an aqueous-based ethylcellulose (EC) outer layer) to ensure extended release despite low pH in the stomach. These microbeads were then sealed in standard gelatine capsules.

The hydrolysates and placebo capsules were visually identical, and the subjects were blinded as to which treatment was administered on each day. Pellets of 100 mg were produced containing 33 mg of active ingredient and 67 mg MCC, while the placebo pellets were 100% MCC. The 100 mg pellets were then sprayed with 20 mg PCS so that each 120 mg microbead, of which 27.5% active ingredient, comprised 33 mg hydrolysate, 67 mg MCC, and 20 mg PCS. Each gastro-resistant capsule contained 650 mg microbeads, corresponding to 178.75 mg active ingredient; however, the final capsule of each dose was partially filled to make up the calculated dose.

Following the pilot study, it was decided to proceed with the lower dose of the hydrolysates owing to the capsule burden (ESI1†) and limited availability of hydrolysate. However, the dose was still very large in its current formulation (400 mg of the unprotected hydrolysates would fit into a single capsule, but with the protective formulation, only 179 mg could be contained within a single capsule). Consequently, the average number of capsules required was 12. As shown in ESI2,† the capsules are quite sizeable, so the current formulation is not viable in a clinical population. To counter this, an attempt was made to recruit subjects with body mass at the lower end of the inclusion range. This initial study was to establish if the hydrolysates had bioactivity, and if successfully shown, the plan was to progress the research programme into the development of a food grade matrix to test the bioactives in an appropriate clinical population with suppressed appetite (*e.g.* elderly).

Procedure

Recruitment. Published studies in this area generally have small sample sizes owing to the participant burden and the high cost associated with the research team and biomarker analysis when conducting hourly blood tests in cannulated subjects. In addition, a recent Cochrane review of ghrelin interventions in cancer reported that studies recruited between 7 and 31 participants.[10] For this cross-over study, which involved three arms, it was decided to recruit 20 subjects, as it was cost prohibitive to include more than this.

Advertisements were placed around the college campus, disseminated *via* email exchanges and placed on online job fora. After an initial telephone conversation to determine eligibility, the participants attended a screening visit in the

Clinical Research Facility-Cork (CRF-C) located in the Mercy University Hospital (MUH). After a successful screening, a participant identification code was assigned, and scheduling of the study visits was agreed upon. Participants were told the commitments required, and it was explained that remuneration of the incentivising vouchers would only be provided on full completion of the study or partial remuneration may be granted in the case of subsequent exclusion for reasons outside the participants' control, at the discretion of the principal investigator (PI). Informed consent was obtained from the research nurse on the morning of the first study visit. Of the 194 people who responded to the advertisements, 66% followed up when provided with the patient information leaflet. Of these 127, 37 were recruited, and after attrition, our final number included in the analyses was 22. See the flowchart in ESI3† for a description of the recruitment and enrolment flow.

Recruitment challenges. One particular issue that came to light was the prevalence of e-cigarette use, which was a *post-hoc* exclusion criterion, as nicotine is known to impact appetite. Subjects who were difficult to cannulate also represented a problem, as this was not consistent from day to day; therefore, one subject who completed day 1 had to be excluded after multiple failed cannulation attempts on day 2. Several subjects disclosed exclusion criteria after starting the trial and were excluded, for example, those who consumed whey protein supplements and alcohol. A major learning point from this study was the difficulty in recruiting and retaining healthy volunteers on a trial of only 3–6 weeks with 4 study visits.

Screening

Participants attended a screening interview with a research dietitian, and a research nurse took blood samples to screen for biochemical abnormalities. Any abnormalities were assessed by the study physician before recruitment was confirmed.

Randomisation

The study was conducted from Autumn 2017 to Winter 2018 in 22 healthy adult males who visited the research unit on 3 occasions with at least 1-week washout between visits. A quasi-randomised cross-over design was used. The subjects recruited were assigned in blocks to the various patterns, as shown in Fig. 1. Each newly enrolled subject was assigned to a predefined block. Each administration pattern was represented by one block, and consecutive blocks were filled before recruiting onto the next block. Each participant was randomised to a pattern of administration whereby they received both hydrolysates and placebos in varying orders. Active treatments and placebos were visually identical. Assignment to each randomisation block was concealed from the participants, and the research nurse administering the treatment was aware of the randomisation. Although not formally concealed, the allocations were not routinely known to the dietitian and laboratory technician during the study days. However, at the time of the analysis, the assignment was unblinded to all members of the study team. Three subjects were unable to complete visit 3 owing to alcohol consumption (n = 2) and personal reasons (n = 1). Thus, there were only 19 subjects who completed all 3 arms and 22 who completed the placebo and MF1145 arms.

Study days

Participants were provided with a standardised meal to consume the evening before each study visit to encourage a consistent level of hunger in the morning. A 420 g serving of cottage pie for reheating was provided, which consisted of minced beef, vegetables and mashed potatoes. In total, it contained 443 kcal, 34 g protein and 5 g fibre. They were instructed to eat this meal at 20:00 and then fast overnight. Subjects were required to abstain from alcohol and avoid vigorous exercise for 24 hours before each study visit. Furthermore, they were asked not to consume any protein supplements or nicotine products for the duration of the study. They were advised to arrive at the unit at 07:45 on each study day. From 08:00, Visual Analogue Scales (VAS) (100 mm) were used hourly to assess hunger, appetite and satiety. A research nurse cannulated the subjects, and they had fasting blood taken along with vitals before receiving a set breakfast. The breakfast was designed to be low in fibre to avoid slowing gastric content emptying, as an empty stomach was preferable at the time of

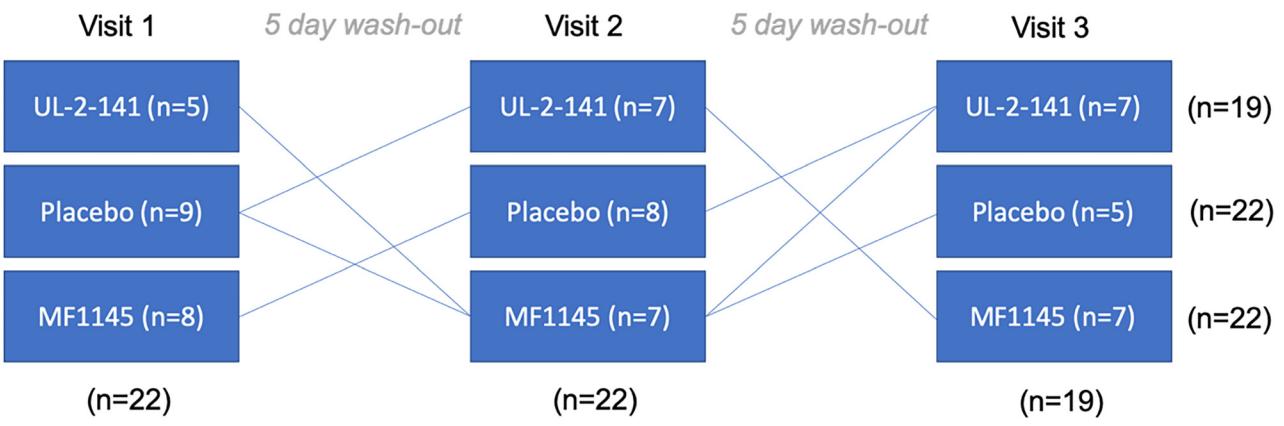

Fig. 1 Schematic representing the block-randomisation pattern of the cross-over trial.

administration of the hydrolysate. It consisted of orange juice, toast, cornflakes, full-fat milk, jam, butter and sugar. This contained 832 kcal, 19 g protein and 4 g fibre. After breakfast, a 24-hour dietary recall and activity history were taken by the research dietitian to confirm adherence to behavioural restrictions during the study period (no consumption of dietary supplements for the duration of study involvement, no alcohol or nicotine during 24 hours prior to each visit and abstaining from vigorous exercise in the 24 hours preceding each visit). Blood was taken 2 hours after breakfast, followed immediately by the administration of hydrolysate or placebo, depending on the randomisation pattern (Fig. 1), and blood was taken hourly thereafter. Participants received 26 mg kg^{-1} of both the bioactives and placebo, as described above (see study timeline in ESI4†). Two hours after dosing, participants were offered an *ad libitum* lunch consisting of a mixed platter, with each item being provided at 1.5 times the 3rd quartile of intake for Irish adult males.[33,34] The full lunch served included 2770 kcal, 144 g protein and 28 g fibre. A full description of the *ad libitum* meal is shown in ESI5–SI6.† The meal is in ESI7.† They were instructed to eat until comfortably full. Leftovers were taken away after 30 minutes, and the items were weighed individually. The food was weighed on a laboratory scale (Fisher Scientific Portable 2alance, 2000 g capacity, accurate to 0.1 g). Subjects were observed for a further 3 hours after consuming lunch. On leaving the facility, subjects were asked to complete food diaries (including all food and fluids consumed, typically dinner and a snack), specifying quantities using household measures until the end of the day. Nutritional analyses were conducted using Nutritics Research Edition (version 5, Nutritics Ltd, Dublin, Ireland).

Laboratory analyses

At each of 7 time points, 3 × 2 ml samples of blood were taken from a peripherally inserted cannula. Owing to ethics committee concerns, the volume of blood collected each day was limited, so only 800–1000 μl plasma was yielded at each time point from the 4 ml samples when centrifuged. This limited the replicates and reruns of the ELISAs. Biobanked bloods were analysed using commercially available manual enzyme-linked immunosorbent assays (ELISA) (R&D Systems, MN, USA & Merck Millipore, MA, USA) for Total Ghrelin, Active Ghrelin, Growth Hormone (GH), Glucagon-Like Peptide-1 (GLP-1), Insulin-Like Growth Factor-1 (IGF-1) and Insulin. The relative absorbance of each well on the plates was read using spectrophotometry at a wavelength of 450 nm on a Dynex D-2 workstation (Dynex Technologies Inc., VA, USA).

Statistical analyses

Statistical analyses were performed using R (version 3, R Foundation, Vienna, Austria) and SPSS (version 24.0, SPSS Inc., Chicago, IL, USA). Mixed effects models were fit using the R package lme4.[35] Certain figures were also created in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Differences in total daily energy/nutrient intakes were estimated using mixed effects models, accounting for period and treatment effects and adjusting for age and body weight. Differences in the levels of blood biomarkers (*e.g.* ghrelin) measured at serial time points on each of the three treatments were estimated by plotting curves for each biomarker of interest and calculating the area under the curve (AUC) for each treatment. AUCs were compared according to treatment condition using Kruskal–Wallis non-parametric tests. AUC provides information about the extent of biomarker peaking for each treatment arm. Treatment effects were assessed by mixed effects models, accounting for period and treatment effects and adjusting for age and body weight as well as baseline measures. Biomarker AUCs were the primary outcome, and all other analyses should be considered exploratory and should not be adjusted for multiple testing.

Results

Baseline characteristics

Overall, 22 male participants (mean age 27.4 years (6.1 SD), range 20.5 to 40.9 years) were included, with an average BMI of 24.6 kg m^{-2} (SD 2.9; range 19.7 to 30.2 kg m^{-2}). Table 1 describes the baseline anthropometrics and fasting characteristics of the study cohort.

Dietary intake

The majority of the breakfast provided was consumed by each participant. The set breakfast contained 823 kcal and 19 g protein, while the averages consumed were 629–657 kcal and 17–18 g protein, respectively (Table 2). The breakfast was a low fibre meal intended to prevent prolonged satiety and avoid delayed transit of gastric contents, which needed to be cleared before the administration of the IMP/placebo.

Lunch was the meal in which the greatest amounts of food were consumed. The average amounts consumed were approximately half of the platter served, irrespective of the treatment. Although 2770 kcal and 144 g protein were served, the averages consumed were 1337–1356 kcal and 74–78 g protein, respectively (Table 3).

Table 1 Characteristics of the study population. Mean (standard deviation)

	Male (n = 22)
Age (years)	27.4 (6.1)
Weight (kg)	76.8 (10.4)
Height (m)	1.76 (0.07)
Body Mass Index (kg m^{-2})	24.6 (2.9)
Systolic Blood Pressure (mm Hg)	120.1 (10.0)
Diastolic Blood Pressure (mm Hg)	78.7 (8.9)
Heart Rate (bpm)	74.3 (14.0)
Insulin-Like Growth Factor-1 (ng mL^{-1})	158.3 (72.4)
Growth Hormone (pg mL^{-1})	243.8 (373.4)
Glucagon-Like Peptide-1 (pmol L^{-1})	47.2 (23.6)
Insulin (pmol L^{-1})	46.1 (30.1)
Glucose (mmol L^{-1})	4.6 (0.5)
Total Ghrelin (pg mL^{-1})	844.1 (355.2)
Active Ghrelin (pg mL^{-1})	531.3 (336.3)

Table 2 Nutritional intake at breakfast on days of treatment with placebo, UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate). Mean (standard deviation)

Breakfast		Placebo (n = 22)	UL-2-141 (n = 19)	MF1145 (n = 22)
Energy	kJ	2677.2 (586.2)	2798.2 (555.4)	2720.9 (451.0)
	kcal	628.8 (137.1)	656.8 (130.2)	638.1 (106.7)
Fat	g	13.0 (3.7)	13.2 (3.4)	13.0 (3.2)
of which Saturates	g	7.4 (2.3)	7.5 (2.1)	7.4 (2.0)
Carbohydrate	g	111.9 (27.9)	117.8 (25.4)	114.2 (20.8)
of which Sugars	g	48.7 (14.5)	50.4 (13.7)	46.1 (13.9)
Protein	g	17.1 (3.6)	17.9 (3.0)	17.1 (3.0)
Fibre	g	3.0 (0.7)	3.1 (0.6)	3.2 (0.4)
Sodium	mg	689.7 (150.5)	720.4 (134.3)	714.1 (97.4)

Table 3 Nutritional intake at lunch on days of treatment with placebo, UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate). Mean (standard deviation)

Lunch		Placebo (n = 22)	UL-2-141 (n = 19)	MF1145 (n = 22)
Energy	kJ	5678.6 (1334.2)	5731.4 (1011.2)	5647.6 (1438.9)
	kcal	1343.3 (319.8)	1356.0 (241.9)	1336.9 (344.3)
Fat	g	74.8 (28.2)	77.3 (26.3)	76.4 (30.0)
of which Saturates	g	17.3 (7.4)	16.7 (6.3)	17.0 (8.0)
Carbohydrate	g	154.3 (30.2)	152.9 (30.1)	151.6 (35.0)
of which Sugars	g	54.2 (20.2)	55.2 (16.1)	53.7 (19.0)
Protein	g	73.6 (18.6)	77.9 (17)	73.8 (18.0)
Fibre	g	12.7 (2.4)	12.6 (2.5)	12.3 (3.6)
Sodium	mg	2148.7 (596.3)	2230.5 (424.7)	2123.7 (701.7)

As dinner was self-reported by the subjects, some data were missing. Excluding these, dinner (and any other snacks until midnight) consumed was marginally smaller than the lunch consumed in the unit. Self-selected intake was 1025–1201 kcal and 48–62 g protein (Table 4). Inter-individual variation in energy and protein intake throughout the day across arms is shown in ESI8 and ESI9,† with the main differences between arms observed after leaving the research facility.

The average consumption throughout the entire study day varied in the ranges of 3090–3233 kcal and 140–157 g protein depending on the treatment administered (Table 5). During placebo treatment, daily protein intake accounted for 17.6% of total calories, fat calories accounted for 38.3% and 44.1% of energy came from carbohydrates. Treatment with UL-2-141 was associated with 18.5%, 39.0% and 42.5% intake of protein, fat and carbohydrate calories. Treatment with MF1145 was associated with 18.0%, 36.5% and 45.6% intake of protein, fat and carbohydrate calories, respectively. Although simple ANOVA comparisons of total daily nutritional intake according to treatment did not reveal significant differences, mixed effects models, which controlled for period, weight and breakfast baseline, demonstrated a significantly increased total daily intake of protein among those who received the UL-2-141 treatment.

Table 4 Nutritional intake following the return home (dinner and snacks) on days of treatment with placebo, UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate). Mean (standard deviation)

Dinner		Placebo (n = 18)	UL-2-141 (n = 15)	MF1145 (n = 16)
Energy	kJ	4694.9 (2522.8)	5031.9 (2177.4)	4300.6 (2067.7)
	kcal	1120.4 (603.0)	1201.0 (521.0)	1024.7 (493.5)
Fat	g	47.7 (28.5)	57.1 (32.4)	41.9 (23.1)
of which Saturates	g	19.6 (13.5)	24.1 (15.5)	17.0 (10.5)
Carbohydrate	g	108.2 (71.5)	107.2 (55.4)	105.5 (50.5)
of which Sugars	g	32.6 (22.6)	37.2 (28.3)	35.2 (23.3)
Protein	g	47.6 (23.1)	62.0 (41.7)	51.1 (27.6)
Fibre	g	9.9 (5.0)	9.1 (6.4)	10.3 (5.5)
Sodium	mg	1052 (678.4)	1192.8 (559.2)	1306.7 (982.0)

Table 5 Total daily nutritional intake on days of treatment with placebo, UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate). Mean (standard deviation)

Total Daily Intake		Placebo (n = 18)	UL-2-141 (n = 15)	MF1145 (n = 16)
Energy	kJ	13037.7 (3474.6)	13640.6 (2873.8)	12880.2 (2757.4)
	kcal	3090.1 (830.7)	3233.0 (684.8)	3049.5 (657.7)
Fat	g	135.4 (42.6)	146.8 (42.9)	128.3 (39.4)
of which Saturates	g	44.6 (17.8)	47.7 (19.0)	41.6 (13.8)
Carbohydrate	g	372.6 (95.9)	384.8 (83.7)	384.2 (75.7)
of which Sugars	g	138.4 (40.4)	148.2 (39.8)	143.3 (33.0)
Protein	g	139.7 (33.9)	156.9 (51.1)	142.2 (32.6)
Fibre	g	25.5 (6.1)	25.0 (7.6)	26.5 (6.7)
Sodium	mg	3889.1 (1011.7)	4115.5 (878.6)	4167.0 (1313.3)

Objective changes in dietary intake

Energy. Mean energy intake at lunch when treated with placebo was 1353 kcal (95% CI: 1236–1469 kcal). Energy intake was not significantly different from the placebo for either treatment (p = 0.729 and p = 0.776 for UL-2-141 and MF1145, respectively). Total daily intake (including self-reported dinner after leaving the study site) was 2673 kcal (95% CI: 2247–3100) in the placebo group and was not significantly different across groups (p = 0.266 and p = 0.796 for UL-2-141 and MF1145, respectively).

Protein. Mean protein intake at lunch when treated with placebo was 73.7 g (95% CI: 66.6–80.9 g). Protein intake was not significantly different from the placebo for either treatment (p = 0.353 and p = 0.954 for UL-2-141 and MF1145, respectively). However, total daily intake significantly differed in those receiving UL-2-141 when controlling for period effect, body weight and age (Table 6). During placebo treatment, the total daily protein intake was 128.4 g (95% CI: 104.9–151.9). During treatment with UL-2-141, the total daily intake was 151.4 g (95% CI: 105.5–197.3), p = 0.044. During treatment with MF1145, total daily intake was 141.5 g (95% CI: 98.5–184.6), p = 0.189.

Subjective appetite ratings

Throughout the study day, appetite, fullness and hunger ratings were dynamic, as expected, responding to both breakfast and lunch. However, no significant differences were observed in the

Table 6 Total daily protein intake – mixed effects model. UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate)

	Simple			+ Covariates		
Predictors	Estimates	CI	*p*	Estimates	CI	*p*
Intercept	120.03	99.75–140.31	<0.001	128.41	104.93–151.88	<0.001
UL-2-141 (*vs.* placebo)	23.99	0.60–47.38	0.044	22.99	0.59–45.39	0.044
MF1145 (*vs.* placebo)	11.57	−8.86–32.00	0.267	13.1	−6.46–32.67	0.189
Weight (kg)	1.74	0.10–3.39	0.037	1.85	−0.06–3.75	0.058
Day 2 (*vs.* 1)				−9.32	−29.14–10.50	0.357
Day 3 (*vs.* 1)				−21.55	−43.49–0.38	0.054
Age (years)				0.54	−2.73–3.80	0.747
Random effects						
σ^2	1195.2			1086.5		
τ_{00}	1160.13_{id}			1451.08_{id}		
ICC	0.49			0.57		
N	22_{id}			22_{id}		
Observations	59			59		

VAS between the placebo and either treatment group at any individual point or in the area under the curve (AUC).

Objective changes in biomarkers

The AUCs for glucose, insulin, AG, and TG were not significantly different in the UL-2-141 arm or the MF1145 arm compared to the placebo. The AUC for IGF-1 was significantly reduced in the UL-2-141 arm ($p < 0.001$) and MF1145 arm ($p < 0.001$) compared to placebo; however, on adjustment for baseline IGF-1 level, there was no difference ($p = 0.622$ for UL-2-141 and $p = 0.287$ for MF1145).

The AUC for GLP-1 was not significantly different in the UL-2-141 arm ($p = 0.09954$) or the MF1145 arm ($p = 0.3017$) compared to the placebo. Despite the lack of difference in the AUC, in the adjusted models (which consider baseline measures), GLP-1 was significantly increased pre-lunch in the UL-2-141 arm compared to placebo (+8 pmol L^{-1}, $p = 0.01$) and in the MF1145 arm (+7 pmol L^{-1}, $p = 0.039$). Fasting baseline and weight were both significant covariates in the adjusted model, as shown in Table 7.

The AUC for GH was not significantly different in the UL-2-141 arm ($p = 0.1362$) or the MF1145 arm ($p = 0.2803$) compared to the placebo. The only significant difference in GH is in the pre-lunch period in the UL-2-141 arm. GH is lower in the UL-2-141 arm compared to the placebo in a clinically significant magnitude (−133 pg mL^{-1}, $p = 0.027$). The normal physiological range of GH reported in the literature is 0–99 pg mL^{-1}; therefore, a change of 133 pg mL^{-1} is a significant magnitude of change. GH was similar in the MF1145 arm initially but appeared to decrease compared to the UL-2-141 arm later in the day. The change in trajectory toward the end of the day may be related to a delayed effect if the hydrolysates required more time for digestion and absorption than allowed for in the follow-up period; therefore, the true effect may not have been observed owing to limited follow-up.

The temporal evolution of the biomarkers measured according to arm, at both the group and individual levels (representing inter-individual variation), is represented in ESI10–ESI23.†

Post hoc analyses

Although intra-assay coefficients of variation were within the expected range of <10% for all assays except total and active ghrelin, inter-assay CVs were not within the limit of 15% for

Table 7 Pre-lunch GLP-1–mixed effects model. UL-2-141 (whey hydrolysate) and MF1145 (casein hydrolysate)

	Simple			+ Covariates		
Predictors	Estimates	CI	*p*	Estimates	CI	*p*
Intercept	53.37	48.44–58.29	<0.001	54.09	48.37–59.81	<0.001
UL-2-141 (*vs.* placebo)	7.83	1.59–14.06	0.014	8.38	2.00–14.76	0.01
MF1145 (*vs.* placebo)	5.66	−0.40–11.72	0.067	6.53	0.32–12.73	0.039
Fasting baseline	1.21	1.02–1.40	<0.001	1.3	1.10–1.50	<0.001
Day 2 (*vs.* 1)				−0.3	−6.38–5.77	0.922
Day 3 (*vs.* 1)				−3.63	−10.06–2.79	0.268
Age (years)				−0.23	−0.83–0.37	0.458
Weight (kg)				−0.42	−0.78–−0.06	0.024
Random effects						
σ^2	101.2			104.52		
τ_{00}	37.23_{id}			25.02_{id}		
ICC	0.27			0.19		
N	22_{id}			22_{id}		
Observations	63			63		
Marginal R^2/Conditional R^2	0.729/0.802			0.746/0.795		

any assay. Owing to the limits on blood samples allowed by ethics, we were unable to run repeat analyses.

Based on the estimates of outcome variances from this study, we conducted power calculations for a 2-arm RCT and found that to reliably detect a statistically significant difference of 200 kcal in a 2-arm RCT with 0.8 power, we would need 250–1000 participants (125–500 per arm). To detect a 400 kcal difference, we would need 50–300 participants (25–150 per arm). To detect a statistically significant difference of 10 g protein in a 2-arm RCT with 0.8 power, we would need 200–1375 participants (100–690 per arm). To detect a 20 g protein difference, we would need 50–375 participants (25–188 per arm).

Discussion

Herein, we report the results of a three-arm placebo-controlled cross-over study involving 2 food-grade bioactive hydrolysates derived from dairy protein, whey hydrolysate UL-2-141 and casein hydrolysate MF1145, which were previously studied in cell lines[30] and murine models.[29] In this study, we found that UL-2-141 was capable of eliciting an increased intake of protein after a single dose in healthy adult males. Several significant differences between the placebo and both treatment arms were identified with respect to GH and GLP-1 response, indicating involvement of these hormonally driven appetite regulating pathways, despite no difference in ghrelin being detected at the timepoints measured. However, in people with sarcopenic obesity (common in cancer), or in older age, the typical response to ghrelin exposure may be attenuated, and fasting levels may be altered;[36] therefore, the effective activation of the ghrelin receptor and observation of downstream effects, rather than the stimulation of the ghrelin production, may be a more important outcome.

No differences in intake were observed during the supervised *ad libitum* lunch meal. However, food diaries for the evening after leaving the research facility demonstrated a trend toward increased self-selected energy and protein intakes in the range of 5–13.5 hours post-capsule administration. A statistically significant increase in protein intake was observed (+ 23 g, $p = 0.044$), which is promising in terms of application in an anorectic population with higher protein requirements. This is a clinically significant response that is significantly higher than the proposed leucine threshold for stimulating muscle protein synthesis.[37–39] Moreover, the increased protein intake represented a 12.3% increase over the placebo, which compared favourably with phase I trials of the pharmaceutical ghrelin receptor agonist anamorelin, where healthy young males consumed 18.4% more energy in an *ad libitum* meal 4 hours post-capsule administration.[18] In the same study, VAS scores for hunger were also observed to be increased,[18] which was not observed during lunch for the present study. However, VAS measures were not available at the timepoint during which our study found a difference in protein intake later in the day. Therefore, the lack of difference in the VAS scores in this study may be attributable to insufficient follow-up time. However, healthy adults often eat beyond their physiological satiety cues and thus may not accurately self-report hunger or satiety, even if the physiological processes underlying such cues are present. Moreover, a recent *meta*-analysis of anamorelin trials found that although body weight, muscle mass and QoL were improved with administration of anamorelin, there was no significant difference in appetite,[40] which confirms that appetite self-report may not adequately predict observed changes in dietary intake and should be paired with objective dietary intake data. Although energy intake did not statistically significantly increase, the mean energy intake in the UL-2-141 arm was 81 kcal higher than placebo in the evening, and our *post hoc* power calculations suggest that owing to the large variances in energy intake, a much larger magnitude of change is required to be detected at the current sample size. Nonetheless, an increase in protein and maintenance of energy intake is clinically important.

Increased GLP-1 was observed pre-lunch in both the UL-2-141 and MF1145 arms, independent of baseline values. The differences observed were of clinically significant magnitude; given that the normal physiological range reported in the literature for GLP-1 is 2–25 pmol L^{-1} (90% CI),[41] a change of 7–8 pmol L^{-1} would represent a 28–400% increase from baseline at either extreme of normal.[41] This increase in pre-prandial GLP-1 was unexpected, as this would typically be associated with an anorectic effect; however, as GLP-1 is known to delay gastric emptying,[42,43] this may partially explain the delayed observation of increased protein intake. If delayed gastric emptying was present, this may have reduced the rate of digestion and subsequent peptide release, resulting in delayed stimulation of orexigenic pathways. Because we did not have GLP-1 levels at the time preceding the meal where an increased intake was observed, it is possible that GLP-1 was normal or reduced in the lead up to the increased intake in the evening. However, pre-prandial peaks in GLP-1 have been previously described in rats as part of the anticipatory response to expecting a meal, and it has been shown that blocking this pre-prandial peak was associated with reduced dietary intake.[44] Thus, the results in the present study showing a GLP-1 pre-prandial peak before lunch may also represent a normal physiological stage of the anticipatory response in humans.

No significant differences were observed in ghrelin response; despite this, protein intake was significantly increased in the UL-2-141 arm, which shows that the bioactive has the potential to modulate appetite, potentially *via* indirect stimulation of the ghrelin receptor *via* GH. GH is intrinsically stimulated by ghrelin and could show an impact on the ghrelin/growth hormone secretagogue receptor (GHS-receptor), which is not directly modulated *via* ghrelin, as the receptor is capable of being activated by many ligands.[14] Pre-lunch, we observed decreased GH in the UL-2-141 arm compared to placebo, in a clinically significant magnitude. Later in the day, GH in the MF1145 arm appears to increase to meet the UL-2-141 arm. We propose that while this is expected to reduce appetite, it does not rule out the expected biological effect on the GHS-receptor occurring later in the day, closer to the time of the observed increase in dietary protein. It is also possible

that later in the day, as UL-2-141 continued to be digested into its constituent peptides distally in the gut, there may have been synergistic effects on intestinal L-cell receptors known to mediate anorexigenic pathways involving PPI and GLP-1.[45] However, a longer follow-up time in subsequent studies is required to confirm either of these potential mechanisms.

There are several possible mechanisms that might explain the superior performance of UL-2-141 in this human trial, in contrast to the superior performance of MF1145 in prior rat models.[29] As the UL-2-141 sample was considerably less hydrolysed (7–12%)[30] than the MF1145 sample (>80%),[29] it may be that the breakdown of UL-2-141 occurs at a more optimal location in the human gastrointestinal system than in the rat models, where MF1145 performed superiorly.[29,30] As gut transit time in humans is many times longer than that of a rat,[46,47] whose gastrointestinal system is physiologically adapted for a cellulose-rich diet[48] and hydrolysis of protein in the gut is time-dependent, our hypothesis is that UL-2-141 is sufficiently hydrolysed more proximally than in corresponding animal models, which improves efficacy given that the ghrelin-receptor expressing cells occur proximally in the gut, in the vagal afferents and the pancreas.[14] Although there is limited evidence directly comparing the digestion of the same dairy-derived bioactive peptides in humans and rat models,[48] studies examining the rate of peptide release from dairy-derived protein in human gastrointestinal systems have also shown that whey-derived peptides are fully released more rapidly than casein-derived peptides,[49] which supports the hypothesis that earlier release of UL-2-141 in humans may explain the results discordant in our *in vivo* findings. Given that the bioactive effect identified *in vivo* was mediated by the GHS-receptor,[29] and our GH findings are consistent with this pathway being affected by UL-2-141 in humans, this may be a rationale to re-evaluate the need for enteric coatings, as earlier digestion may be a favourable outcome, facilitating more direct stimulation of the GHS-receptors. Moreover, it may be that future mechanistic studies should use an animal model better situated to model human digestion, such as pigs, whose digestive physiology is closer to that of humans.[48] The current study has many strengths, including the tight regulation of subjects' behaviour and strict monitoring of dietary intake during the study visit, with frequent biomarker assessments throughout the day. However, there are several limitations that must be acknowledged. This study was conducted in a homogeneous population of young, healthy males; future studies should examine the impacts of these hydrolysates in females, and in subjects who are older and experiencing either age-related or disease-related anorexia, as their physiological responses may differ from those observed in this study. This study was designed as a pilot, and *a priori* power calculations were not possible owing to a lack of reference data. However, using these results, we conducted *post hoc* power calculations for a 2-arm RCT and have reported the required sample size for future trials. Notably, these calculations suggest that we were powered adequately to detect a true change in protein in the range observed in this study; however, the current study was not powered to detect a significant change at the level we observed in energy intake. Therefore, it is unsurprising that statistical significance was not reached for this marker despite a trend toward a clinically significant difference. Additionally, appetite regulation is complex and associated with diverse factors, such as genetic polymorphisms, gut microbiome, and even factors, such as weather or personal stressors. Although it was beyond the scope of this study to comprehensively assess these confounders, it may be advisable in large definitive trials of appetite modulators to consider assessing a broader array of known confounders that may mediate or explain variance in appetite responses. Finally, although we found that these hydrolysates are safe and tolerable in healthy males, the quantity of capsules required in their current formulation is not viable commercially or clinically, which is of utmost importance in designing natural product trials.[50] This has highlighted the importance of considering incorporation in a food matrix, especially considering the impact of acid degradation in the stomach and the uncertainty surrounding the need for gastro-protection in the formulation. Furthermore, refining pharmacological delivery systems and determining the smallest bioactive components of the compounds facilitate a more tolerable dose volume. Finally, it is suggested that clinical trials utilising natural products should be designed with 'de-risking' in mind to avoid significant investment in large-scale trials, which are unlikely to provide robust data;[50] similarly, the feasibility of scale-up should be evaluated at the time of mechanistic studies.[51] The non-significant results of this pilot trial are very useful in this context because they confirm the need for prolonged observation periods in future trials and also prompt further elucidation of the mechanisms of action for these hydrolysates in the human system to inform optimal formulation and dosage and to improve our understanding of the expected time of onset of any observed physiological effects.

Conclusion

Although dairy-derived peptide-containing hydrolysates have previously demonstrated ghrelinergic effects in rats, no such effects on human blood biomarkers were identified in this study. Despite the lack of a ghrelinergic response, protein intake significantly increases in the UL-2-141 arm, suggesting that the bioactive component can modulate appetite, potentially *via* an indirect stimulation of the ghrelin receptor or a delayed agonism, which is undetected in our limited follow-up period. The lack of significance in energy intake is likely attributable to the small sample size. However, since healthy adults are often not in tune with their physiological hunger, they may not respond strongly to simple physiological modulators, and repeating the study in subjects with established anorexia may be prudent. As outlined above, several methodological considerations were highlighted as a result of this study, which should inform the protocol development of subsequent studies using dairy-derived hydrolysates for appetite stimulation.

Author contributions

Conceptualization: HS, JFC, BGT, AMR; data curation: ESS, DD; formal Analysis: ESS, DD; funding acquisition: HS, JFC, BGT, AMR; investigation: ESS, SJC, KH; methodology: ESS, SJC, KH, HS, JFC, BGT, DD, NN, AMR; project administration: ESS, SJC, AMR; resources: HS, JFC, BGT, DD, NN, AMR; software: DD; supervision: AMR; validation: ESS, HS, DD; visualization: ESS, DD; writing – original draft: ESS, AMR; writing – review & editing: ESS, NN, HS, DD, AMR.

Conflicts of interest

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Data availability

Raw data for this study are not available for sharing, as participants did not provide informed consent for sharing of individual data at the time of enrolment, and we do not have ethical approval for sharing of study data without explicit consent.

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