Mycoprotein ingestion stimulates protein synthesis rates to a greater extent than milk protein in rested and exercised skeletal muscle of healthy young men: a randomised controlled trial.

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Data described in the manuscript, code book, and analytic code will be made available upon request pending approval by the corresponding author.

Abstract

Background: Mycoprotein is a fungal-derived sustainable protein-rich food source, and its ingestion results in systemic amino acid and leucine concentrations similar to that following milk protein ingestion.

Objective: We assessed the mixed skeletal muscle protein synthetic response to the ingestion of a single bolus of mycoprotein compared with a leucine matched bolus of milk protein, in rested and exercised muscle of resistance-trained young men.

Design: Twenty resistance-trained healthy young males (age: 22±1 y, body mass: 82±2 kg, BMI: 25±1 $kg \cdot m^{-2}$) took part in a randomized, double-blind, parallel-group study. Participants received primed, continuous infusions of L-[ring-$^{2}H_5$]phenylalanine and ingested either 31 g (26.2 g protein: 2.5 g leucine) milk protein (MILK) or 70 g (31.5 g protein: 2.5 g leucine) mycoprotein (MYCO) following a bout of unilateral resistance-type exercise (contralateral leg acting as resting control). Blood and *m. vastus lateralis* muscle samples were collected before exercise and protein ingestion, and following a 4 h postprandial period to assess mixed muscle fractional protein synthetic rates (FSR) and myocellular signalling in response to the protein beverages in resting and exercised muscle.

Results: Mixed muscle FSR increased following MILK ingestion (from 0.036±0.008 to 0.052±0.006%·h^{-1} in rested, and 0.035±0.008 to 0.056±0.005%·h^{-1} in exercised muscle; $P<0.01$) but to a greater extent following MYCO ingestion (from 0.025±0.006 to 0.057±0.004%·h^{-1} in rested, and 0.024±0.007 to 0.072±0.005%·h^{-1} in exercised muscle; $P<0.0001$) (treatment × time interaction effect; $P<0.05$). Postprandial FSRs trended to be greater in MYCO compared with MILK (0.065±0.004 vs 0.054±0.004%·h^{-1}, respectively; $P=0.093$) and the postprandial rise in FSR was greater in MYCO compared with MILK (Δ 0.040±0.006 vs Δ 0.018±0.005%·h^{-1}, respectively; $P<0.01$).

Conclusions: The ingestion of a single bolus of mycoprotein stimulates resting and post-exercise muscle protein synthesis rates, and to a greater extent compared with a leucine matched bolus of milk protein, in resistance-trained young men.

Introduction

Adequate dietary protein intake is required to maintain skeletal muscle mass and to facilitate the remodelling and/or hypertrophy of muscle tissue in response to exercise training. Mechanistically, this is largely achieved by dietary protein ingestion transiently (2-5 h) stimulating muscle protein synthesis rates (1-3), primarily due to a postprandial elevation of plasma essential amino acid concentrations (4), particularly leucine (5, 6). A single bout of resistance exercise also stimulates muscle protein synthesis rates for up to (and at least) 48 h (7, 8), whilst sensitising muscle tissue to the anabolic effects of dietary protein for at least 24 h (7, 8). Consequently, research has sought to identify aspects of protein nutrition (e.g. amount and timing) that can be manipulated to optimally support post-exercise muscle protein synthesis rates (1, 3, 9-12). However, information relating to the anabolic properties of non-animal derived dietary protein sources is lacking, which is concerning given the increasing emphasis on dietary sustainability.

Animal-derived dietary protein sources, such as whey (1, 2, 11, 13, 14), casein (13, 15), milk (16, 17) beef (17-20) and egg (3, 21) have all been shown to stimulate post-exercise muscle protein synthesis rates. It is assumed that plant-based dietary protein sources are inferior in their capacity to stimulate muscle protein synthesis rates, due to their typically slower digestibility, lower bioavailability, and lower essential amino acid and leucine content (22). Indeed, whey protein stimulates muscle protein synthesis rates to a greater extent than soy in young men (1, 14) and, compared to wheat protein in older men (13). To date, however, these are the only non-animal derived protein sources to be studied with respect to their impact on muscle protein synthesis.

Mycoprotein is a sustainably produced food source rich in protein (~45% of total mass) and essential amino acids (~44% of total protein) derived from the cultivation of the fungus *Fusarium venenatum.* We have reported that total postprandial essential amino acid (and

leucine) concentrations following mycoprotein ingestion are comparable to that seen following milk protein ingestion (23), a finding atypical of animal versus non-animal dietary protein comparisons (13, 24). Further, essential amino acid concentrations following mycoprotein ingestion increased in a dose-response fashion up to 60-80 g mycoprotein consumption (27-36 g protein, 2.1-2.9 g leucine) (23), suggesting mycoprotein would be capable of robustly stimulating muscle protein synthesis rates.

In the present work, we tested the hypothesis that the ingestion of a 70 g bolus of mycoprotein (31.5 g protein, 2.5 g leucine) would stimulate mixed muscle protein synthesis rates over a four h postprandial period in both rested and exercised skeletal muscle of resistance-trained, healthy young men. We compared the muscle protein synthetic response of mycoprotein ingestion to a leucine-matched bolus of milk protein. Here, we hypothesised that, despite equivalent leucine contents, due to slower aminoacidemia (23), muscle protein synthesis rates would increase to a lesser extent following mycoprotein ingestion. We chose to match the two beverages on leucine content as various lines of enquiry suggest leucine content, rather than total protein, is the primary factor determining the postprandial muscle protein synthetic response when sufficient protein is consumed (6, 25, 26).

Methods

Participants

Twenty young, healthy (age: 22±1 y, body mass: 82±2 kg, BMI: 25±1 $kg \cdot m^{-2}$) men volunteered to take part in the present study (**Supplementary Figure 1**). Participants' characteristics are displayed in **Table 1**. Participants were recreationally active and experienced with resistance training (at least 3 times per week for at least 3 months prior to participation). Participants were deemed healthy based on their blood pressure ($\leq$140/90mmHg), BMI (18-30 $kg \cdot m^{-2}$) and responses to a routine medical screening questionnaire (absence of any diagnosed metabolic impairment, cardiovascular disease, or motor disorders), and were informed of the experimental procedures, potential risks, and the purpose of the study prior to providing full written consent. Participants were all 'tracer naïve' having not undergone any previous stable isotope amino acid infusion protocols. The study was approved by the Sport and Health Sciences ethics committee of the University of Exeter (REF NO. 161026/B/05) in accordance with standards for human research as outlined in the declaration of Helsinki. Recruitment and data collection were completed between January 2017 and August 2017 at The University of Exeter.

Pre-testing

Following screening and acceptance onto the study, all participants underwent a single pre-testing session, which took place at least 5 days prior to the experimental trial. Participants were familiarised with the exercise equipment and exercise protocol, and body fat and lean mass were determined by Air Displacement Plethysmography (BodPod, Life Measurement, Inc. Concord, CA, USA). Participants were familiarised with the unilateral resistance-type exercise that was employed in the experimental protocol. This consisted of 5 sets of 30 repetitions of maximal concentric isokinetic leg extension and leg flexion contractions on a

Biodex System 3 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY, USA) at a speed of 60° per second over a central 80° range of motion using their self-reported dominant leg. Verbal encouragement was provided throughout the familiarisation and experimental testing to engender maximal effort through every repetition. Work done (J) was recorded for each completed set, and fatigue was calculated as the percentage decrement in work done between the first and last set. Participants were instructed to report their habitual dietary intake by recording a weighted food diary for two weekdays, and one weekend day prior to partaking in the study (Table 1) (Nutritics LTD, Dublin, Ireland).

Experimental protocol

Participants were randomly assigned to two parallel-groups, A or B, by the lead investigator and completed a single trial in a double-blind fashion. An overview of the experimental protocol is shown in **Figure 1**. Participants were directed to abstain from vigorous physical activity and alcohol consumption in the 48 h preceding the trial. All participants were provided with and consumed a standardised meal ~10.5 h prior to the start of the experimental trial (744 kcal [3.1 MJ], 29% energy (%En) fat, 20%En protein, 51%En carbohydrate). On the day of the trial, participants arrived at the laboratory between 07:00 - 08:00 after a 10 h overnight fast. A Teflon^{TM} cannula was inserted into an antecubital vein of one arm in preparation for stable isotope infusion, a baseline venous blood sample was taken from this site to measure background isotope enrichments prior to infusion. Following baseline blood sampling (t = -210 min) the phenylalanine and tyrosine pools were primed with a single intravenous dose of L-[ring-$^{2}H_5$]phenylalanine (2.12 µmol/kg) and L-[3,3-$^{2}H_2$]tyrosine (0.75 µmol/kg). Thereafter, continuous tracer infusion was initiated and maintained at a rate of 0.035 µmol·kg^{-1}·min^{-1} for L-[ring-$^{2}H_5$]phenylalanine and 0.012 µmol·kg^{-1}·min^{-1} for L-[3,3-$^{2}H_2$]tyrosine for the duration of the protocol. Once the infusion

had begun, a second Teflon cannula was inserted into a dorsal hand vein of the contralateral arm and placed in a warmed air hand unit (55°C) for arterialized venous blood sampling (27). Arterialized venous blood samples were collected throughout the experimental protocol at the following time points: t = -180, -120, -60, 0 (drink consumption), 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 min. A baseline muscle sample was collected after 90 min of the infusion (t -120 min) from the non-dominant leg (designated as the 'resting leg'). Muscle biopsies were collected from the mid-region of the *m. vastus lateralis* (approx. 15 cm above the patella) with a modified Bergström suction needle under local anaesthesia (2% lidocaine). All biopsy samples were immediately freed from any visible blood, adipose and connective tissue, frozen in liquid nitrogen (within 30 s), and stored at –80°C until subsequent analysis. Eighty-five min after the initial biopsy (t = -35 min), participants undertook the unilateral resistance-type exercise protocol, as previously described, which took 30 min. Immediately following exercise bilateral muscle biopsies were collected (i.e. from both the rested and exercised leg). Immediately post-biopsy (t = 0 min) participants consumed either a milk protein (MILK) or mycoprotein (MYCO) beverage, within an allotted 5 min period, with the experimental drinks administered in a double-blind manner. Thereafter, participants rested in a semi-supine position for 4 h, after which further bilateral biopsies were collected 1–2 cm proximal to the previous incisions (t = 240 min).

Experimental beverage preparations

Freeze-dried isolated milk protein concentrate was obtained from a commercial supplier (Bulk Powders, Colchester, UK) and freeze-dried mycoprotein was produced by and obtained from Marlow Foods Ltd, Quorn Foods, Stokesley, UK. Both protein sources were independently analysed by a third party company for energy, macronutrient content and amino acid composition (Premier Analytical Services, High Wycombe, UK). The powdered

protein sources were prepared the evening before the experimental trial. The protein sources were assimilated with 400 mL water and 10 g of artificial energy-free flavouring (Myprotein, Manchester, UK), blended for approximately 2 min, topped up with water to make a total final beverage volume of 600 mL and refrigerated overnight. Drinks were enriched (2.5%) with L-[ring-$^{2}H_{5}$]phenylalanine to account for postprandial tracer dilution by non-labelled phenylalanine and to maintain a systemic isotopic steady-state following protein ingestion (1, 3). Following drink consumption by the participant, an additional 50 mL of water was then added to 'wash' the bottle and ensure that all protein had been consumed, making a total volume of 650 mL consumed by participants. All drinks were well tolerated, consumed within the allotted time (i.e. 5 min) and resulted in no adverse effects during or after the test day. Double blinding of the drinks was achieved by having a different researcher from the individual running the infusion trial prepare the drinks in an opaque bottle ready for consumption. Despite careful blinding, we cannot discount the possibility that participants allocated to MYCO may have perceived the unusual texture of mycoprotein. The milk protein beverage contained 31 g of milk protein powder which contained 26.2 g total protein (providing 2.5 g of leucine). The mycoprotein beverage contained 70 g of mycoprotein which contained 31.5 g total protein (providing 2.5 g of leucine). The detailed nutritional content and amino acid composition of the drinks are displayed in **Table 2.**

Blood sample collection and analyses

Ten mL of arterialised venous blood was collected into a syringe at each sampling point. For each blood sample, six mL was aliquoted into liquid heparin containing tubes (BD vacutainer LH; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged immediately (3000 *g*, 4°C, 10 min). Blood plasma was aliquoted and frozen at –80°C for subsequent analysis. The remaining 4 mL of blood was aliquoted into additional vacutainers

(BD vacutainers SST II, Becton, Dickinson and Company) which were left to clot at room temperature for at least 30 min and then centrifuged (3000 *g*, 4°C, 10 min) to obtain blood serum. Serum was aliquoted before freezing at –80°C for subsequent analyses. Serum insulin concentrations were analysed using a commercially available kit (DRG Insulin ELISA, EIA-2935, DRG International Inc, Springfield, IL, USA). Plasma branched chain amino acid (BCAA) (leucine, isoleucine and valine), phenylalanine and tyrosine concentrations, and L-[ring-$^{2}H_{5}$]-phenylalanine, L-[ring-3,5-$^{2}H_{2}$]-tyrosine, and L-[ring-$^{2}H_{4}$]-tyrosine enrichments were determined by gas chromatography-mass spectrometry (GC-MS) as described previously (28). Briefly, 10 µL internal standards of leucine, valine, phenylalanine and tyrosine were added to the samples. The plasma was deproteinised on ice with 500 µL of 15% 5-sulfosalicylic acid. Free amino acids were purified using acid-washed cation exchange columns (AG 50W-X8 resin; Bio-Rad Laboratories, Inc., CA, USA), with the amino acids being eluted from the column with 8 mL of 2N ammonium hydroxide. The eluate was then dried under vacuum with a Speed-Vac rotary dryer (Savant Instruments, Farmingdale, NY, USA). In order to derivatize the plasma sample, 40 µl MTBSTFA + 1% tert-butyl-dimethylchlorosilane and 40 µl acetonitrile were added to the dry samples, vortexed and heated at 95 °C for 40 min (29).The samples were analyzed by GC-MS (7890 GC coupled with a 5975 inert MSD; Agilent Technologies, Santa Clara, CA, USA) in duplicates using electron impact ionization and selected ion monitoring for measurement of isotope ratios (30). One microliter of the sample was injected in splitless mode (injector temp. 280°C). Peaks were resolved using an HP5-MS 30m × 0.25mm ID × 0.25µm capillary column (Agilent). Helium was used as carrier gas at 1.2ml/min constant flow rate. The temperature ramp was set from 80 – 245 °C at 11°C/min, then to 280 °C at 40 °C/min (30). Selected ion recording conditions were used to monitor fragments m/z 336, 341 and 346 for

phenylalanine, m/z 288 and 296 for valine, m/z 274 and 280 for leucine and isoleucine, and m/z 466 and 475 for tyrosine.

Skeletal muscle tissue analyses

Muscle biopsy tissue samples were analysed for protein-bound and free intracellular L-[ring-$^2H_5$]phenylalanine, as previously described (28). Briefly, 20-30 mg of frozen muscle tissue was weighed and precipitated in 600 µL 10% perchloric acid. The tissue was homogenized by a mechanical tissue grinder. The supernatant, for determination of intracellular L-[ring-$^2H_5$]phenylalanine enrichment, was subsequently transferred following centrifugation (4000 rpm, 20 min, 4 °C) and stored at −80 °C. This procedure was repeated with an additional 800 µL wash with 10% perchloric acid. The remaining pellet of muscle tissue was washed three times in 2% perchloric acid, twice in ethanol, and then once in ethyl ether, before being oven-dried overnight at 50 °C. The following day, the dried muscle pellet was hydrolysed in 6N hydrochloric acid at 110 °C for 24 h. The hydrolysate, representing the bound protein pool of amino acids, was subsequently used to determine the enrichment of bound L-[ring-$^2H_5$]phenylalanine. The protein hydrolysate was deionized using ion-exchange columns as described for blood analyses. The supernatant, for determination of intracellular labelled phenylalanine enrichment, was prepared in the same manner as the protein-bound acid hydrolysates. In order to derivatize the muscle sample, 50 µl MTBSTFA + 1% tert-butyl-dimethylchlorosilane and 50 µl acetonitrile were added to the dry samples, vortex mixed and heated at 95 °C for 45 min (29). The samples were analyzed by GC-MS (7890 GC coupled with a 5975 inert MSD; Agilent Technologies) in duplicates using electron impact ionization and selected ion monitoring for measurement of isotope ratios (30). One microliter of the sample was injected in splitless mode (injector temp. 280 °C). Peaks were resolved using an HP5-MS 30m × 0.25mm ID × 0.25µm capillary column (Agilent). Helium was used as

carrier gas at 1.2ml/min constant flow rate. The temperature ramp was set from 80 – 245 °C at 11 °C/min, then to 280 °C at 40 °C/min (30). Selected ion recording conditions were used to monitor fragments m/z 237 and 239 for the m+3 and m+5 fragments of phenylalanine bound protein and m/z 336 and 341 for phenylalanine free fraction.

Muscle biopsy tissue samples were analysed for total and phosphorylated forms of mechanistic target of rapamycin (mTORSer^{2448} and pmTOR Ser^{2448}). Briefly, ~10 mg of whole frozen muscle was mechanically homogenised using steel beads (Qiagen, Hilden, Germany) in 20 volumes of buffer (Tris-HCl 50 mM, Triton X-100 1%, EDTA 1 mM, EGTA 1 mM, NaF 50 mM, β-glycerophosphate 10 mM, sodium pyrophosphate 5 mM, 2-mercaptoethanol 0.1%, sodium orthovanadate 0.5 mM, okadaic acid 100 nM and complete Mini protease inhibitor cocktail (Roche Holding AG, Basel, Switzerland). Following centrifugation (10000 *g*, 4°C, 10 min) the supernatant protein content was determined by colorimetric assay (DC protein assay, Bio-Rad Laboratories, Inc.). Proteins were unfolded by incubating for 5 minutes at 95 °C in XT sample buffer (Bio-Rad Laboratories, Inc.). Twenty µg protein per lane were loaded onto 3-8% tris acetate polyacrylamide gels, and separated by electrophoresis in XT tricine running buffer for 65 min at 150 V. Proteins were transferred to 0.2 µM nitrocellulose membranes using a Trans-blot turbo transfer system (Bio-Rad Laboratories, Inc.), at 2.5 A and 25 V for 10 min. Membranes were blocked in 5% BSA in TBST (pH 7.6) for 1 h, before overnight incubation at 4 °C with rabbit anti-phospho-mTOR Ser^{2448} monoclonal antibody (5536, Cell Signaling Technology, Inc., Danvers, Mass, USA; 1:1000 in TBST) and rabbit anti-α-tubulin (11H10, Cell Signaling Technology, Inc.; 1:20000 in TBST) loading control. Following 3 × 10 min washes in TBST, membranes were incubated for 1 h at room temperature in secondary HRP conjugated anti-rabbit IgG antibody (ab6721, Abcam PLC, Cambridge, UK; 1:3000 in TBST). Following 3 × 10 min washes in TBST, membranes were then exposed for 5 min in Clarity Western chemiluminescent

detector solution (Bio-Rad Laboratories, Inc.), visualised using a Chemidoc scanner (Bio-Rad Laboratories, Inc.), and band density quantified using Image Lab software (Bio-Rad Laboratories, Inc.). The expected migration of phospho-mTOR (~289 kDa) and α-tubulin (~52 kDa) was confirmed using a kaleidoscope protein ladder (Bio-Rad Laboratories, Inc.). For total mTOR, membranes were incubated for 15 min in Restore stripping buffer (Thermo Fisher Scientific, Waltham, MA, USA), blocked for 1 h in 5 % BSA in TBST and re-probed overnight with an anti-mTOR monoclonal primary antibody (2972, Cell Signaling Technology, Inc.; 1:1000 in TBST) plus anti-α-tubulin, and the above steps were repeated to obtain corresponding bands for total mTOR. The band density for phospho-mTOR was calculated as a ratio against the band density for α-tubulin, within each lane. This was divided by the ratio of mTOR against α-tubulin to give an overall ratio for 'mTOR phosphorylation status', which was finally expressed as a fold change from the rested, fasted, baseline.

Skeletal muscle mRNA expression of 48 genes was analysed as previously described (31). In brief, total RNA was extracted from ~20 mg frozen muscle tissue using TRIzol® Reagent (Thermo Fisher Scientific) (32), according to the manufacturer's protocol. Total RNA quantification was carried out spectrophotometrically at 260 nm (NanoDrop ND-2000 Spectrophotometer; Thermo Fisher Scientific) and RNA purity was determined as the ratio of readings at 260/280 nm. Reverse transcription of RNA was carried out using a commercially available kit (SuperScript™ III First-Strand Synthesis SuperMix, Thermo Fischer Scientific) (33). Taqman low-density custom-designed array cards (Thermo Fisher Scientific) were used for the relative quantification of the expression of genes involved in the regulation of cellular amino acid transport, protein synthesis and protein breakdown. Each card allowed for eight samples to be run in parallel against 48 Taqman gene expression assay targets that had been preloaded into each well on the card (**Table 4**). In short, 50 μL Taqman Universal Master Mix II (Thermo Fisher Scientific) was added to 150 ng of RNA equivalent cDNA into an

RNAse-free Eppendorf tube, and RNAse-free water was added to make the total reaction volume up to 100 μl. The reaction mixture was vortexed, centrifuged and loaded into one of the fill reservoirs of the Micro Fluidic card, after which the cards were centrifuged (Hereaus 3 S-RMicrofuge, Thermo Fisher Scientific) and run on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). Relative quantification of the genes of interest was performed using the delta-delta Ct method, with the fold change in mRNA amplification expressed relative to the rested fasted leg and the geometric mean of the two housekeeping genes, for which purpose GAPDH and B2M were selected.

Calculations

The fractional synthetic rates (FSR) of mixed muscle proteins were calculated using the standard precursor-product equation (28):

$$FSR\ (\% \cdot h^{-1}) = \left[\frac{\Delta E\mathrm{p}}{E_{\mathrm{precursor}} \times t}\right] \times 100$$

Where ΔEp is the increment in L-[ring-$^{2}H_5$]phenylalanine enrichment in mixed muscle protein between two biopsies, $E_{\mathrm{precursor}}$ is the average L-[ring-$^{2}H_5$]phenylalanine enrichment in the plasma or intracellular precursor pool over time, and t indicates the tracer incorporation time (h) between two muscle biopsies.

Intravenous infusion of L-[ring-$^{2}H_5$]-phenylalanine and L-[ring-3,5-$^{2}H_2$]-tyrosine and arterialized venous blood sampling were used to assess whole-body amino acid kinetics under non-steady state conditions (34). Whole-body total phenylalanine rates of appearance (R_a), rates of disappearance (R_d), and hydroxylation rates (the initial step in phenylalanine oxidation) were calculated using modified Steele's equations (34), as follows:

$$Total\ R_a = \frac{F_{iv} - \left[pV \times C(t) \times \frac{dE_{iv}}{dt}\right]}{E_{iv}(t)}$$

$$Total\ R_d = R_a - pV \times \frac{dc}{dt}$$

$$\text{Phe Hydroxylation} = \text{Tyr } R_a \times \frac{E_{tyr}(t)}{E_{phe}(t)} \times \frac{\text{Phe } R_d}{F_{phe} + \text{Phe } R_d}$$

Where F_{iv} is the intravenous tracer infusion rate (μmol·kg^{-1}·min^{-1}), pV (0.125 L·kg^{-1}) is the distribution volume for phenylalanine (34), C(t) is the mean plasma phenylalanine concentration between two consecutive time points, dE_{iv}/dt represents the time-dependent variations of plasma phenylalanine enrichments derived from the intravenous tracer, and $E_{iv}(t)$ is the mean plasma phenylalanine enrichment from the intravenous tracer between two consecutive time points. Tyr R_a is the total rate of appearance based on the L-[ring-3,5-$^2H_2$]-tyrosine infusion and plasma enrichment of tyrosine. $E_{tyr}(t)$ and $E_{phe}(t)$ are the mean plasma L-[ring-$^2H_4$]-tyrosine and L-[ring-$^2H_5$]-phenylalanine enrichments between 2 consecutive time points, respectively, and F_{phe} is the intravenous infusion rate of L-[ring-$^2H_5$]-phenylalanine (μmol·kg^{-1}·min^{-1}).

The absence of an additional (unique) tracer within the protein drinks precluded us being able to differentiate between endogenous and exogenous R_a. Furthermore, the necessity to enrich the experimental drinks with the same tracer as was intravenously infused, to minimize disturbance in precursor isotopic steady states, precluded the calculation of whole body protein synthesis and breakdown, and provides a degree of error which we presume to be equivalent across the two groups.

Statistical analyses

A two-sided power analysis based on previous research (1) showed that n= 9 per condition was sufficient to detect expected differences in postprandial muscle protein synthesis rates between protein conditions (MILK vs MYCO) when using a repeated measures ANOVA ($P < 0.05$, 90% power, f = 0.67; G*power version 3.1.9.2). Our primary measure was postprandial muscle protein synthesis rates. The delta change in muscle protein synthesis rates from fasted to fed, plasma amino acid concentrations (including C_{max} and t_{max}), serum insulin concentrations, whole body amino acid kinetics, intracellular and mixed muscle protein-bound L-[ring-$^2H_5$]phenylalanine represent secondary measures, and skeletal muscle cell signalling responses represent exploratory variables. Differences in participant characteristics and background mixed muscle protein-bound L-[ring-$^2H_5$]phenylalanine were analysed using independent t-tests. Plasma amino acid and serum insulin concentrations, plasma L-[ring-$^2H_5$]-phenylalanine, L-[ring-3,5-$^2H_2$]-tyrosine, and L-[ring-$^2H_4$]-tyrosine enrichments, and muscle mTOR phosphorylation were tested by two-factor (treatment [milk protein vs mycoprotein] × time) repeated-measures analysis of variance (ANOVA). Mixed muscle FSRs, mixed muscle protein-bound L-[ring-$^2H_5$]phenylalanine enrichments, and muscle gene expression were analysed using a three-factor (treatment × time × exercise/rest) ANOVA. Data were tested for sphericity, and where violations occurred the Greenhouse-Geisser correction was automatically applied. Violations of normality were tested for using the Shapiro-Wilk test, and no considerable violations were found. When significant interaction effects were observed Sidak post hoc tests were performed to correct for multiple comparisons and locate individual differences. Total postprandial amino acid concentrations were calculated as incremental area under curve (iAUC), with baseline set as t = 0, and analysed using independent t-tests. Individual C_{max} (peak concentration) and t_{max} (time to peak concentration) were analysed using independent t-tests. Where plasma time point data were absent, missing data analyses was performed using regression imputation. Statistical significance was set at $P<0.05$. Calculations

were performed using GraphPad Prism version 7.1 (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± SEM.

342 **Results**

343

344 *Participant characteristics*

345 No differences in age, weight, height, BMI, body composition, or habitual nutritional intake
346 were detected between groups (all $P>0.05$; **Table 1**). No differences in total work performed
347 during the experimental resistance exercise bout (29540±1782 J in MILK vs 30722±2059 J in
348 MYCO; $P>0.05$) or in fatigue (19±3% in MILK vs 26±4% in MYCO; $P>0.05$) were detected
349 between groups.

350

351 *Plasma amino acid and serum insulin concentrations*

352 Plasma total and individual BCAA concentrations during the experimental period are shown
353 in **Figure 2**. Plasma total BCAA concentrations and each of the individual BCAAs all
354 showed similar kinetic responses. Specifically, from similar fasting values across conditions,
355 all parameters increased with protein ingestion (time effect; $P<0.0001$) but to differing
356 degrees between conditions (treatment × time interaction effect; $P<0.0001$). Plasma BCAA
357 concentrations (**A**) peaked more rapidly and to a greater peak magnitude following the
358 ingestion of MILK compared with MYCO. Plasma BCAA C_{max} was significantly greater in
359 MILK compared with MYCO (791±51 vs 646±25.9 μmol·L^{-1}; $P<0.05$), mean t_{max} was
360 82.5±15 min in MILK and 103±10 min in MYCO ($P>0.05$), and modal t_{max} was 90 min in
361 MILK and 120 min in MYCO. Plasma leucine concentrations (**C** and **D**) were different
362 between conditions (treatment effect; $P<0.05$), and were greater at 15, 30, and 45 min in
363 MILK compared with MYCO ($P<0.01$). Plasma leucine concentrations peaked at 30 min in
364 MILK at 299±36 μmol·L^{-1} and at 75 min in MYCO at 243±11 μmol·L^{-1}. Plasma leucine C_{max}
365 was significantly greater in MILK compared with MYCO (340±27 vs 258±9 μmol·L^{-}
366 ^{1}$P<0.01$), mean t_{max} was 80±16 min in MILK and 89±10 min in MYCO ($P>0.05$), and modal

t_{max} was 30 min in MILK and 75 min in MYCO. Total postprandial plasma leucine concentrations were 19±8% greater in MILK compared with MYCO (24420±2333 in MILK vs 20831±1279 μmol·L^{-1} × 4h in MYCO; *P*>0.05) (**D**), whereas isoleucine and valine postprandial concentrations did not differ between conditions (*P*>0.05) (**F** & **H**). Plasma phenylalanine and tyrosine concentrations increased with protein ingestion (time effect; *P*<0.0001) but also to differing degrees between conditions (treatment × time interaction effect; *P*<0.0001). Plasma phenylalanine concentrations (**K** and **L**) were greater in MILK compared with MYCO at 15 and 30 min (*P*<0.01). Plasma tyrosine concentrations (**I** and **J**) were different between conditions (treatment effect; *P*<0.001), and were greater in MILK compared with MYCO from 15-120 min (*P*<0.05).

Serum insulin concentrations during the experimental period are displayed in **Figure 3**. From similar fasting concentrations (15±3 and 16±2 mU·L^{-1} in MILK and MYCO, respectively) serum insulin concentrations increased with protein ingestion (time effect; P<0.0001) and to differing degrees between conditions (treatment × time interaction effect; *P*<0.0001). Milk protein ingestion resulted in a more rapid and transient increase in serum insulin concentrations that peaked at 15 min (42±7 mU·L^{-1}) and returned to fasting levels by 45 min (*P*<0.01). Mycoprotein ingestion induced a less rapid but more sustained increase in serum insulin concentrations that peaked at 30 min post-ingestion (36±4 mU·L^{-1}) and returned to baseline more slowly (60 min; *P*<0.0001). Serum insulin concentrations in MILK were only greater than MYCO at 15 min post-ingestion (*P*<0.05) and postprandial serum insulin AUC was not different between conditions (*P*>0.05). Serum insulin C_{max} was not different between conditions (45±6 vs 38±3 mU·L^{-1} in MILK and MYCO, respectively; *P*>0.05), mean t_{max} was 28±5 min in MILK and 33±4 min in MYCO (P>0.05), and modal t_{max} was 15 min in MILK and 30 min in MYCO.

Whole-body phenylalanine kinetics

The time-course of plasma L-[ring-$^2H_5$]phenylalanine, L-[3,3-$^2H_2$]tyrosine, and L-[ring-$^2H_4$]-tyrosine enrichments are illustrated in **Figure 4**. During the postabsorptive period, plasma L-[ring-$^2H_5$]phenylalanine remained in a steady state at ~4–5 MPE (mole percent excess) in both conditions. L-[ring-$^2H_5$] phenylalanine enrichments increased transiently after protein ingestion (time effect; $P<0.001$), with a greater increase in MYCO (treatment × time interaction effect, $P<0.01$). Specifically, plasma L-[ring-$^2H_5$]phenylalanine enrichments increased above postabsorptive levels for 30 min in MYCO only ($P<0.01$) before returning to baseline enrichments. This was presumably due to either the slower digestion of MYCO, or quicker entry of labelled L-[ring-2H5]phenylalanine into the circulation in the MYCO condition (Figure 4A). Two participants were excluded from the whole body kinetics analysis (final analysis therefore; MILK=9, MYCO=9) due to technical issues with the tyrosine tracer. L-[3,3-$^2H_2$]tyrosine decreased equivalently after the ingestion of protein (time effect; $P<0.0001$, treatment × time interaction effect; $P>0.05$) and remained below postabsorptive levels for 150 min following protein ingestion ($P<0.05$) (Figure 4B). Plasma L-[ring-$^2H_4$]-tyrosine increased following protein ingestion (time effect; $P<0.0001$), with a greater increase in MYCO (treatment × time interaction effect; $P<0.01$) which was elevated above postabsorptive enrichments for 30 min ($P<0.0001$) (Figure 4C). Phenylalanine hydroxylation increased following protein ingestion (time effect; $P<0.0001$), and remained elevated throughout the postprandial period, with no differences between conditions (treatment × time effects; $P>0.05$) (Figure 5A). Plasma phenylalanine total R_a and R_d changed divergently over time in MILK and MYCO (time and treatment × time effects; $P>0.001$). In MILK phenylalanine total R_a and R_d were elevated above postabsorptive values at 90 min ($P<0.05$), whereas in MYCO phenylalanine total R_a and R_d were suppressed below postabsorptive values at 30 min ($P<0.01$) (Figure 5B-C).

Skeletal muscle tracer analyses

One participants' samples were excluded from the MYCO condition due to insufficient tissue. Intracellular L-[ring-$^{2}H_5$]phenylalanine enrichments increased over time (time effect; $P<0.01$) with no differences between conditions or interaction effects (both $P>0.05$) (**Supplementary Figure 2**). Mixed muscle protein-bound L-[ring-$^{2}H_5$]phenylalanine enrichments did not differ between conditions at baseline (0.0030±0.0007 and 0.0034±0.0006 MPE in MYCO and MILK conditions, respectively; $P>0.05$). Mixed muscle protein L-[ring-$^{2}H_5$]phenylalanine enrichments increased during the fasting period in the rested leg (from 0.0030±0.0007 to 0.0065±0.0007 in MILK and 0.0034±0.0006 to 0.0058±0.0006 MPE in MYCO; time effect; $P<0.0001$) to the same extent in each condition (treatment and treatment × time effects; both $P>0.05$). Mixed muscle protein L-[ring-$^{2}H_5$]phenylalanine enrichments increased with protein ingestion (from 0.0065±0.0007 to 0.0161±0.001 MPE in rested, and 0.0061±0.0008 to 0.0164±0.0012 MPE in exercised muscle in MILK, and from 0.0058±0.0006 to 0.0167±0.0012 MPE in rested, and 0.0055±0.0006 to 0.0187±0.0011 MPE in exercised muscle in MYCO; $P<0.0001$) and to a greater extent in MYCO compared with MILK (treatment × time interaction effect; $P<0.05$) (**Supplementary Figure 3**).

Mixed muscle FSRs calculated using the average plasma L-[ring-$^{2}H_5$]phenylalanine plasma enrichment as the precursor pool are displayed in **Figure 6**. Exercise did not affect mixed muscle FSR (exercise effect; $P=0.0640$), nor did exercise interact with protein ingestion ($P=0.1251$) or condition ($P=0.2223$). Protein ingestion increased mixed muscle FSRs in rested and exercised muscle in both conditions (time effect; $P<0.0001$). Mixed muscle FSR increased from 0.036±0.008 to 0.052±0.006%·h^{-1} and 0.035±0.008 to 0.056±0.005%·h^{-1} in rested and exercised muscle, respectively, in MILK, and from 0.025±0.006 to 0.057±0.004%·h^{-1} and 0.024±0.007 to 0.072±0.005%·h^{-1} in rested and exercised muscle,

respectively, in MYCO. The increase in mixed muscle FSR was greater in the MYCO condition compared with MILK condition (treatment × time interaction effect; *P*=0.0199), with the divergence located as trends for a difference between conditions in the postabsorptive (*P*=0.0890) and postprandial FSRs (*P*=0.0930) (**A**). These trends resulted in the delta postabsorptive to postprandial rise in mixed muscle FSR being greater in MYCO compared with MILK (treatment effect; *P*=0.0084) **(B)**. The group differences became more pronounced when comparing muscle FSRs derived from the L-[ring-$^{2}H_{5}$]phenylalanine intracellular precursor pool. While main effects of exercise or any exercise interactions were still absent (all *P*>0.05), protein ingestion increased FSR (time effect; *P*=0.0003) and to a greater extent in MYCO compared with MILK (treatment × time interaction effect; *P*=0.0225). Specifically, MYCO ingestion stimulated mixed muscle FSR (from to 0.031±0.007 to 0.070±0.006%·h^{-1} in rested, and 0.028±0.008 to 0.082±0.008%·h^{-1} in exercised muscle; *P*<0.0001), whereas MILK ingestion only trended to stimulate FSR (from 0.040±0.010 to 0.060±0.007%·h^{-1} in rested, and 0.050±0.012 to 0.058±0.007%·h^{-1} in exercised muscle; *P*=0.060).

Skeletal muscle cell signalling responses

Skeletal muscle mTOR phosphorylation status was determined in n=15 due to restrictions on remaining muscle tissue (final analysis therefore; MILK=7, MYCO=8) (**Figure 7**). Fold change with protein ingestion in muscle mTOR phosphorylation status did not differ between conditions (treatment effect; *P*>0.05), was unaffected by exercise (*P*>0.05), and did not show an interaction effect (*P*>0.05).

Of the 46 genes analysed for their muscle mRNA expression (see **Table 4**), 19 genes showed no changes with exercise, protein ingestion, protein condition, or any interactions (all *P*>0.05; data not shown). Twenty seven genes responded to protein ingestion and/or exercise,

and the muscle mRNA expression of these genes are displayed in **Figure 8**. Specifically, protein ingestion either decreased (IRS1, TSC1, TSC2, CASTOR1, FOXO3, CAPN1, SRF, SLC7A8, SLC38A10, DDIT4, TRIM63 and SLC38A2) or increased (AKT1S1 and SLC38A9) muscle mRNA expression of some genes. Similarly, exercise decreased (SMAD2, PIK3R1, RPS6KB1, TFEB, MSTN, SLC38A2 and TRIM32) or increased (FBXO32, SLC7A1, and TRIM63) the muscle mRNA expression of some genes. Fifteen genes exhibited a time × exercise interaction ($P<0.05$) such that EIF4E and TGFb1 mRNA expression increased in exercised muscle only, and DDIT4L and MSTN mRNA expression increased in rested muscle only ($P<0.05$). FBXO32, MSTN, TRIM63 mRNA expression decreased in rested muscle only, and SLC38A10, DDIT4, SLC38A2, and TRIM32 mRNA expression decreased in exercised muscle only ($P<0.05$). Only a single gene, TRIM32, showed a differential response between nutritional conditions, with its muscle mRNA expression greater in MILK compared with MYCO (treatment effect; $P<0.05$), which was driven by a greater expression in MILK compared with MYCO in the postprandial state ($P<0.01$).

Discussion

We assessed *in vivo* protein synthetic responses to the ingestion of leucine matched boluses of milk protein and mycoprotein within resting and exercised skeletal muscle, in healthy and trained, young men. In support of our initial hypothesis, mycoprotein ingestion robustly stimulated protein synthesis rates in resting and exercised muscle. Mycoprotein ingestion resulted in slower and lower rises in plasma amino acid (and leucine) concentrations compared with the ingestion of milk protein. Despite this, and contrary to our secondary hypothesis, we report that mycoprotein ingestion stimulated muscle protein synthesis rates to a greater extent when compared with milk protein.

Previous work has suggested that the rate and/or magnitude of plasma essential aminoacidemia and leucinemia following dietary protein ingestion are the key determinants that modulate postprandial muscle protein synthesis rates (2, 6, 24, 35-37). As expected (23), we observed more rapid protein digestion and intestinal amino acid absorption rates following milk compared with mycoprotein ingestion, as evidenced by larger and more rapid postprandial aminoacidemia and leucinemia (Figures 2A-D). As a consequence, milk protein ingestion resulted in a quicker rise in circulating insulin concentrations (Figure 3A), also a postprandial systemic condition expected to facilitate muscle protein anabolism (38). In line with our hypothesis, we observed a robust stimulation of mixed muscle protein synthesis rates in response to both protein sources (Figure 6A). However, we report a greater stimulation of mixed muscle protein synthesis rates following mycoprotein compared with milk protein ingestion (Figure 6A). Moreover, when expressing these data as the change in muscle protein synthesis rates from postabsorptive to postprandial (Figure 6B), the response to mycoprotein ingestion was more than double that of milk protein ingestion. Given this response occurred despite ‘inferior’ postprandial plasma amino acid kinetics, it is of interest to consider *why* this was observed.

In studies where isolated protein sources are ingested (i.e. with little or no additional macronutrients), the rate and/or magnitude of leucinemia generally predicts subsequent rates of muscle protein synthesis (2, 24, 35, 36). However, in studies where protein was co-ingested with carbohydrate or fat, plasma leucine concentrations were less predictive of the subsequent muscle protein synthetic response. For example, carbohydrate co-ingestion can markedly attenuate the peak (and total) magnitude of postprandial leucinemia, but does not inhibit the muscle protein synthetic response compared with protein ingestion alone (39-42). This suggests, within the context of a mixed meal, plasma leucine kinetics alone do not dictate the postprandial muscle protein synthetic response. Further, co-ingestion of additional macronutrients *per se* do not confer additional stimulation of muscle protein synthesis rates (39-42), and therefore neither the additional macronutrients nor energy content of the mycoprotein can (solely) explain its *greater* anabolic response in the present study. In line with our findings, a recent study reported that protein consumed within a 'whole food' matrix (i.e. whole eggs) led to a delayed and lower peak magnitude of postprandial leucinemia, but *greater* rates of postprandial muscle protein synthesis compared with the protein ingested within the egg white only (21). It is therefore possible that the present data, and those from van Vliet and colleagues (21), are a result of a potentiating effect of consuming protein within a more complete food matrix/whole food meal. Whether this is an effect of the food matrix *per se* (42), a combined effect of the presence of additional macronutrients (or their specific subclasses (43-45)), fibre (mycoprotein is high in fibre composed of a 2:1 ratio of β-glucan and chitin), higher energy content, or an effect of other (micronutrient) factors (46-48) is unclear but clearly warrants future investigation.

To investigate how mycoprotein enabled such a potent muscle protein synthetic response, we used the remaining muscle tissue to probe various myocellular signalling responses (Figure 7 and 8). Aside from amino acids, it has been suggested that systemic rises in non-protein

nutrients (e.g. specific lipids (43, 45)), micronutrients (46)) may also exert influence over mTOR, the central molecular signalling pathway controlling muscle protein synthesis (49). Despite the divergent amino acid (and presumably other nutrients) profiles across the two nutritional conditions we observed no differences between groups in postprandial mTOR phosphorylation. We cannot discount the possibility that mTOR phosphorylation may have differed during the early postprandial period (50-52), that the translocation and subcellular location of mTOR may have differed across conditions (44), that investigating different phosphorylation sites may have yielded different results (53), that downstream targets of mTOR may have differed independently, or that our data are not appropriately powered to detect small, but physiologically relevant differences in phosphorylation status (particularly given we did not have a complete dataset due to limited tissue availability). Moreover, it is possible that the translocation and/or subcellular location of mTOR may have differed across conditions (44). Indeed, recent data suggest the subcellular locality of mTOR is important in regulating the (postprandial) muscle protein synthetic response (54, 55), and that non-protein dietary components may influence translocation and the regulation of post-exercise mRNA translation (44).

To gain insight into the potential adaptive response of the cell we examined the muscle postprandial and post-exercise transcriptional response. Exercise and protein ingestion resulted in a coordinated transcriptional response, demonstrated by the mRNA expression of 27 of the 46 genes of interest changing 4 h post protein ingestion with/without exercise. The upregulation in expression of genes involved in amino acid transport (e.g. CAT1, LAT2, SNAT2) and protein synthesis (e.g. TSC1, TSC2, DDIT4) underline the rapid transcriptional responses that occur with nutrition and exercise (10, 56-60) which, at least in part, restore cellular homeostasis and direct the adaptive response. Additionally, the early inhibition of myostatin expression with exercise supports the concept that low myostatin expression

facilitates an anabolic environment (61). Only a single gene, the E3 ubiquitin ligase TRIM32, differentially responded to the different protein sources; being expressed to a lesser degree following mycoprotein ingestion. TRIM32 preferentially ubiquitinates actin and desmin filaments, with reduced levels of TRIM32 reducing the loss of these proteins (62, 63). This may be indicative of a more potent ability of mycoprotein to suppress excessive post-exercise muscle protein breakdown, although given the lack of a coordinated response of other proteolyctic genes (e.g. MAFBx, MuRF1 etc.) this remains conjectural. Clearly, the ability of prolonged mycoprotein consumption to support resistance training-induced hypertrophy warrants future investigation. Worthy of note is one caveat of interpreting our cell signalling data in the present work is the increased risk of making a type 1 error due to due to multiple testing outcomes.

Two other factors that could have contributed to our findings are worthy of consideration. First, the mycoprotein drink contained ~20% more protein than the milk protein beverage (31.5 vs 26.2 g). In young men the muscle protein synthetic response to dietary protein ingestion plateaus at ~20 g protein (3, 11) (or 0.3 $g \cdot kg^{-1}$; (26)), which appears to be more due to leucine content, rather than total protein *per se* (64), at least when ample protein is provided (65). We therefore assume, since we fed in excess of 20 g / 0.3 $g \cdot kg^{-1}$ in both conditions, that ample protein was available to negate protein *amount* being a significant contributing factor. In agreement, systemic amino acid concentrations were *lower* following mycoprotein compared with milk protein ingestion, again indicating non-protein/amino acid factors are likely responsible for the greater muscle protein synthetic effect of mycoprotein. Second, whilst it is generally assumed that leucine (and the other BCAAs) is the primary nutritional anabolic trigger (6, 25, 64, 66), it is clear that other essential amino acids (50), such as arginine (67-69), may also play a role in initiating muscle protein synthesis rates. Therefore, it is also plausible that the amino acid profile of mycoprotein was simply more

581 anabolic than milk protein. This, however, would be contrary to the consensus of *in vivo*
582 human data (22), and necessitate that specific amino acids, with a greater preponderance in
583 the mycoprotein drink, possess anabolic signalling roles that are as yet undocumented in
584 humans.
585 To conclude, the bolus consumption of mycoprotein stimulated resting and post-exercise
586 muscle protein synthesis rates in young men, and to a greater extent than a leucine-matched
587 bolus of milk protein. These novel data show mycoprotein represents a viable, sustainably
588 produced non-animal derived alternative dietary protein source to support acute tissue
589 remodelling in response to exercise. Our work implies that mycoprotein could be
590 incorporated into the habitual diet of those undertaking prolonged resistance training to
591 facilitate muscle hypertrophic responses.
592

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606 primary responsibility for the final content. All authors have read and approved the final
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References

1. Tang JE, Moore DR, Kujbida GW, Tarnopolsky MA, Phillips SM. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. Journal of Applied Physiology 2009;107(3):987-92. doi: 10.1152/japplphysiol.00076.2009.
2. Pennings B, Boirie Y, Senden JM, Gijsen AP, Kuipers H, van Loon LJ. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. The American Journal of Clinical Nutrition 2011;93(5):997-1005. doi: 10.3945/ajcn.110.008102.
3. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. The American Journal of Clinical Nutrition 2008;89(1):161-8.
4. Tipton KD, Gurkin BE, Matin S, Wolfe RR. Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in healthy volunteers. The Journal of Nutritional Biochemistry 1999;10(2):89-95.
5. Wall BT, Hamer HM, de Lange A, Kiskini A, Groen BBL, Senden JMG, Gijsen AP, Verdijk LB, van Loon LJC. Leucine co-ingestion improves post-prandial muscle protein accretion in elderly men. Clinical Nutrition 2013;32(3):412-9. doi: 10.1016/j.clnu.2012.09.002.
6. Norton LE, Layman DK, Bunpo P, Anthony TG, Brana DV, Garlick PJ. The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats. The Journal of Nutrition 2009;139(6):1103-9. doi: 10.3945/jn.108.103853.

7. Phillips SM, Tipton KD, Aarsland A, Wolf SE, Wolfe RR. Mixed muscle protein synthesis and breakdown after resistance exercise in humans. American Journal of Physiology-Endocrinology and Metabolism 1997;273(1):E99-E107.
8. Biolo G, Maggi SP, Williams BD, Tipton KD, Wolfe RR. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. The American Journal of Physiology 1995;268(3 Pt 1):E514-20. doi: 10.1152/ajpendo.1995.268.3.E514.
9. Areta JL, Burke LM, Ross ML, Camera DM, West DW, Broad EM, Jeacocke NA, Moore DR, Stellingwerff T, Phillips SM, et al. Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. The Journal of Physiology 2013;591(9):2319-31. doi: 10.1113/jphysiol.2012.244897.
10. Wall BT, Burd NA, Franssen R, Gorissen SH, Snijders T, Senden JM, Gijsen AP, van Loon LJ. Presleep protein ingestion does not compromise the muscle protein synthetic response to protein ingested the following morning. American Journal of Physiology Endocrinology and Metabolism 2016;311(6):E964-e73. doi: 10.1152/ajpendo.00325.2016.
11. Witard OC, Jackman SR, Breen L, Smith K, Selby A, Tipton KD. Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. The American Journal of Clinical Nutrition 2013;99(1):86-95.
12. Rasmussen BB, Tipton KD, Miller SL, Wolf SE, Wolfe RR. An oral essential amino acid-carbohydrate supplement enhances muscle protein anabolism after resistance exercise. Journal of Applied Physiology 2000;88(2):386-92. doi: 10.1152/jappl.2000.88.2.386.

13. Gorissen SH, Horstman AM, Franssen R, Crombag JJ, Langer H, Bierau J, Respondek F, van Loon LJ. Ingestion of Wheat Protein Increases In Vivo Muscle Protein Synthesis Rates in Healthy Older Men in a Randomized Trial. The Journal of Nutrition 2016;146(9):1651-9. doi: 10.3945/jn.116.231340.

14. Yang Y, Churchward-Venne TA, Burd NA, Breen L, Tarnopolsky MA, Phillips SM. Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men. Nutrition & Metabolism 2012;9(1):57. doi: 10.1186/1743-7075-9-57.

15. Groen BB, Horstman AM, Hamer HM, de Haan M, van Kranenburg J, Bierau J, Poeze M, Wodzig WK, Rasmussen BB, van Loon LJ. Post-Prandial Protein Handling: You Are What You Just Ate. PloS one 2015;10(11):e0141582. doi: 10.1371/journal.pone.0141582.

16. Mitchell CJ, McGregor RA, D'Souza RF, Thorstensen EB, Markworth JF, Fanning AC, Poppitt SD, Cameron-Smith D. Consumption of Milk Protein or Whey Protein Results in a Similar Increase in Muscle Protein Synthesis in Middle Aged Men. Nutrients 2015;7(10):8685-99. doi: 10.3390/nu7105420.

17. Burd NA, Gorissen SH, van Vliet S, Snijders T, van Loon LJ. Differences in postprandial protein handling after beef compared with milk ingestion during postexercise recovery: a randomized controlled trial. The American Journal of Clinical Nutrition 2015;102(4):828-36. doi: 10.3945/ajcn.114.103184.

18. Symons TB, Sheffield-Moore M, Wolfe RR, Paddon-Jones D. A moderate serving of high-quality protein maximally stimulates skeletal muscle protein synthesis in young and elderly subjects. Journal of the American Dietetic Association 2009;109(9):1582-6. doi: 10.1016/j.jada.2009.06.369.

19. Robinson MJ, Burd NA, Breen L, Rerecich T, Yang Y, Hector AJ, Baker SK, Phillips SM. Dose-dependent responses of myofibrillar protein synthesis with beef ingestion are enhanced with resistance exercise in middle-aged men. Applied Physiology, Nutrition, and Metabolism 2013;38(2):120-5. doi: 10.1139/apnm-2012-0092.
20. Pennings B, Groen BB, van Dijk JW, de Lange A, Kiskini A, Kuklinski M, Senden JM, van Loon LJ. Minced beef is more rapidly digested and absorbed than beef steak, resulting in greater postprandial protein retention in older men. The American Journal of Clinical Nutrition 2013;98(1):121-8. doi: 10.3945/ajcn.112.051201.
21. van Vliet S, Shy EL, Abou Sawan S, Beals JW, West DW, Skinner SK, Ulanov AV, Li Z, Paluska SA, Parsons CM, et al. Consumption of whole eggs promotes greater stimulation of postexercise muscle protein synthesis than consumption of isonitrogenous amounts of egg whites in young men. The American Journal of Clinical Nutrition 2017;106(6):1401-12. doi: 10.3945/ajcn.117.159855.
22. van Vliet S, Burd NA, van Loon LJ. The Skeletal Muscle Anabolic Response to Plant- versus Animal-Based Protein Consumption. The Journal of Nutrition 2015;145(9):1981-91. doi: 10.3945/jn.114.204305.
23. Dunlop MV, Kilroe SP, Bowtell JL, Finnigan TJ, Salmon DL, Wall BT. Mycoprotein represents a bioavailable and insulinotropic non-animal-derived dietary protein source: a dose–response study. British Journal of Nutrition 2017;118(9):673-85.
24. Tang JE, Moore DR, Kujbida GW, Tarnopolsky MA, Phillips SM. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. Journal of Applied Physiology 2009;107(3):987-92. doi: 10.1152/japplphysiol.00076.2009.
25. Churchward-Venne TA, Breen L, Di Donato DM, Hector AJ, Mitchell CJ, Moore DR, Stellingwerff T, Breuille D, Offord EA, Baker SK, et al. Leucine supplementation of a

low-protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double-blind, randomized trial. The American Journal of Clinical Nutrition 2014;99(2):276-86. doi: 10.3945/ajcn.113.068775.

26. Stokes T, Hector AJ, Morton RW, McGlory C, Phillips SM. Recent Perspectives Regarding the Role of Dietary Protein for the Promotion of Muscle Hypertrophy with Resistance Exercise Training. Nutrients 2018;10(2). doi: 10.3390/nu10020180.

27. Abumrad NN, Rabin D, Diamond MP, Lacy WW. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. Metabolism: Clinical and Experimental 1981;30(9):936-40.

28. Wolfe RR, Chinkes DL. Isotope tracers in metabolic research: principles and practice of kinetic analysis: John Wiley & Sons, 2005.

29. Bornø A, Hulston CJ, van Hall G. Determination of human muscle protein fractional synthesis rate: an evaluation of different mass spectrometry techniques and considerations for tracer choice. Journal of Mass Spectrometry 2014;49(8):674-80. doi: 10.1002/jms.3387.

30. Zabielski P, Ford GC, Persson XM, Jaleel A, Dewey JD, Nair KS. Comparison of different mass spectrometry techniques in the measurement of L-[ring-13C6]phenylalanine incorporation into mixed muscle proteins. Journal of Mass Spectrometry 2013;48(2):269-75. doi: 10.1002/jms.3120.

31. Dirks ML, Stephens FB, Jackman SR, Galera Gordo J, Machin DJ, Pulsford RM, van Loon LJC, Wall BT. A single day of bed rest, irrespective of energy balance, does not affect skeletal muscle gene expression or insulin sensitivity. Experimental Physiology 2018;103(6):860-75. doi: 10.1113/ep086961.

32. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 1987;162(1):156-9. doi: 10.1006/abio.1987.9999.

33. Tsintzas K, Jewell K, Kamran M, Laithwaite D, Boonsong T, Littlewood J, Macdonald I, Bennett A. Differential regulation of metabolic genes in skeletal muscle during starvation and refeeding in humans. The Journal of Physiology 2006;575(Pt 1):291-303. doi: 10.1113/jphysiol.2006.109892.

34. Gorissen SHM, Burd NA, Kramer IF, van Kranenburg J, Gijsen AP, Rooyackers O, van Loon LJC. Co-ingesting milk fat with micellar casein does not affect postprandial protein handling in healthy older men. Clinical Nutrition 2017;36(2):429-37. doi: 10.1016/j.clnu.2015.12.011.

35. Koopman R, Crombach N, Gijsen AP, Walrand S, Fauquant J, Kies AK, Lemosquet S, Saris WH, Boirie Y, van Loon LJ. Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when compared with its intact protein. The American Journal of Clinical Nutrition 2009;90(1):106-15. doi: 10.3945/ajcn.2009.27474.

36. Burd NA, Yang Y, Moore DR, Tang JE, Tarnopolsky MA, Phillips SM. Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v. micellar casein at rest and after resistance exercise in elderly men. The British Journal of Nutrition 2012;108(6):958-62. doi: 10.1017/s0007114511006271.

37. Phillips SM. The impact of protein quality on the promotion of resistance exercise-induced changes in muscle mass. Nutrition & Metabolism 2016;13:64. doi: 10.1186/s12986-016-0124-8.

38. Abdulla H, Smith K, Atherton PJ, Idris I. Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis. Diabetologia 2016;59(1):44-55. doi: 10.1007/s00125-015-3751-0.

39. Gorissen SH, Burd NA, Hamer HM, Gijsen AP, Groen BB, van Loon LJ. Carbohydrate coingestion delays dietary protein digestion and absorption but does not modulate postprandial muscle protein accretion. The Journal of Clinical Endocrinology and Metabolism 2014;99(6):2250-8. doi: 10.1210/jc.2013-3970.

40. Hamer HM, Wall BT, Kiskini A, de Lange A, Groen BB, Bakker JA, Gijsen AP, Verdijk LB, van Loon LJ. Carbohydrate co-ingestion with protein does not further augment post-prandial muscle protein accretion in older men. Nutrition & Metabolism 2013;10(1):15. doi: 10.1186/1743-7075-10-15.

41. Koopman R, Beelen M, Stellingwerff T, Pennings B, Saris WH, Kies AK, Kuipers H, van Loon LJ. Coingestion of carbohydrate with protein does not further augment postexercise muscle protein synthesis. American Journal of Physiology Endocrinology and Metabolism 2007;293(3):E833-42. doi: 10.1152/ajpendo.00135.2007.

42. Churchward-Venne TA, Snijders T, Linkens AM, Hamer HM, van Kranenburg J, van Loon LJ. Ingestion of Casein in a Milk Matrix Modulates Dietary Protein Digestion and Absorption Kinetics but Does Not Modulate Postprandial Muscle Protein Synthesis in Older Men. Journal of Nutrition 2015;145(7):1438-45. doi: 10.3945/jn.115.213710.

43. Castellano BM, Thelen AM, Moldavski O, Feltes M, van der Welle RE, Mydock-McGrane L, Jiang X, van Eijkeren RJ, Davis OB, Louie SM, et al. Lysosomal cholesterol activates mTORC1 via an SLC38A9-Niemann-Pick C1 signaling complex. Science 2017;355(6331):1306-11. doi: 10.1126/science.aag1417.

44. Abou Sawan S, van Vliet S, West DWD, Beals JW, Paluska SA, Burd NA, Moore DR. Whole egg, but not egg white, ingestion induces mTOR colocalization with the lysosome after resistance exercise. American Journal of Physiology-Cell physiology 2018;315(4):C537-c43. doi: 10.1152/ajpcell.00225.2018.

45. Yasuda M, Tanaka Y, Kume S, Morita Y, Chin-Kanasaki M, Araki H, Isshiki K, Araki S, Koya D, Haneda M, et al. Fatty acids are novel nutrient factors to regulate mTORC1 lysosomal localization and apoptosis in podocytes. Biochim Biophys Acta-Molecular Basis of Disease 2014;1842(7):1097-108. doi: 10.1016/j.bbadis.2014.04.001.

46. Salles J, Chanet A, Giraudet C, Patrac V, Pierre P, Jourdan M, Luiking YC, Verlaan S, Migne C, Boirie Y, et al. 1,25(OH)2-vitamin D3 enhances the stimulating effect of leucine and insulin on protein synthesis rate through Akt/PKB and mTOR mediated pathways in murine C2C12 skeletal myotubes. Molecular Nutrition and Food Research 2013;57(12):2137-46. doi: 10.1002/mnfr.201300074.

47. Zhao Z, Barcus M, Kim J, Lum KL, Mills C, Lei XG. High Dietary Selenium Intake Alters Lipid Metabolism and Protein Synthesis in Liver and Muscle of Pigs. The Journal of Nutrition 2016;146(9):1625-33. doi: 10.3945/jn.116.229955.

48. Marzani B, Balage M, Venien A, Astruc T, Papet I, Dardevet D, Mosoni L. Antioxidant supplementation restores defective leucine stimulation of protein synthesis in skeletal muscle from old rats. The Journal of Nutrition 2008;138(11):2205-11. doi: 10.3945/jn.108.094029.

49. Walker DK, Dickinson JM, Timmerman KL, Drummond MJ, Reidy PT, Fry CS, Gundermann DM, Rasmussen BB. Exercise, amino acids, and aging in the control of human muscle protein synthesis. Medicine and Science in Sports and Exercise 2011;43(12):2249-58. doi: 10.1249/MSS.0b013e318223b037.

50. Churchward-Venne TA, Burd NA, Mitchell CJ, West DW, Philp A, Marcotte GR, Baker SK, Baar K, Phillips SM. Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men. The Journal of Physiology 2012;590(11):2751-65. doi: 10.1113/jphysiol.2012.228833.

51. Drummond MJ, Dickinson JM, Fry CS, Walker DK, Gundermann DM, Reidy PT, Timmerman KL, Markofski MM, Paddon-Jones D, Rasmussen BB, et al. Bed rest impairs skeletal muscle amino acid transporter expression, mTORC1 signaling, and protein synthesis in response to essential amino acids in older adults. American Journal of Physiology Endocrinology and Metabolism 2012;302(9):E1113-22. doi: 10.1152/ajpendo.00603.2011.

52. West DW, Burd NA, Coffey VG, Baker SK, Burke LM, Hawley JA, Moore DR, Stellingwerff T, Phillips SM. Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise. The American Journal of Clinical Nutrition 2011;94(3):795-803. doi: 10.3945/ajcn.111.013722.

53. Figueiredo VC, Dungan CM, Peterson CA, McCarthy JJ. On the appropriateness of antibody selection to estimate mTORC1 activity. Acta Physiologica 2019:e13354. doi: 10.1111/apha.13354.

54. Hodson N, McGlory C, Oikawa SY, Jeromson S, Song Z, Ruegg MA, Hamilton DL, Phillips SM, Philp A. Differential localization and anabolic responsiveness of mTOR complexes in human skeletal muscle in response to feeding and exercise. American Journal of Physiology-Cell Physiology 2017;313(6):C604-c11. doi: 10.1152/ajpcell.00176.2017.

55. Abou Sawan S, van Vliet S, Parel JT, Beals JW, Mazzulla M, West DWD, Philp A, Li Z, Paluska SA, Burd NA, et al. Translocation and protein complex co-localization of mTOR is associated with postprandial myofibrillar protein synthesis at rest and after endurance exercise. Physiological Reports 2018;6(5). doi: 10.14814/phy2.13628.

56. Graber TG, Borack MS, Reidy PT, Volpi E, Rasmussen BB. Essential amino acid ingestion alters expression of genes associated with amino acid sensing, transport, and mTORC1 regulation in human skeletal muscle. Nutrition & Metabolism 2017;14:35. doi: 10.1186/s12986-017-0187-1.

57. Wall BT, Dirks ML, Snijders T, van Dijk JW, Fritsch M, Verdijk LB, van Loon LJ. Short-term muscle disuse lowers myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion. American Journal of Physiology Endocrinology and Metabolism 2016;310(2):E137-47. doi: 10.1152/ajpendo.00227.2015.

58. Drummond MJ, Fry CS, Glynn EL, Timmerman KL, Dickinson JM, Walker DK, Gundermann DM, Volpi E, Rasmussen BB. Skeletal muscle amino acid transporter expression is increased in young and older adults following resistance exercise. Journal of Applied Physiology 2011;111(1):135-42. doi: 10.1152/japplphysiol.01408.2010.

59. Reidy PT, Walker DK, Dickinson JM, Gundermann DM, Drummond MJ, Timmerman KL, Cope MB, Mukherjea R, Jennings K, Volpi E, et al. Soy-dairy protein blend and whey protein ingestion after resistance exercise increases amino acid transport and transporter expression in human skeletal muscle. Journal of Applied Physiology 2014;116(11):1353-64. doi: 10.1152/japplphysiol.01093.2013.

60. Mitchell CJ, Zeng N, D'Souza RF, Mitchell SM, Aasen K, Fanning AC, Poppitt SD, Cameron-Smith D. Minimal dose of milk protein concentrate to enhance the anabolic

signalling response to a single bout of resistance exercise; a randomised controlled trial. Journal of the International Society of Sports Nutrition 2017;14:17. doi: 10.1186/s12970-017-0175-x.

61. Rodriguez J, Vernus B, Chelh I, Cassar-Malek I, Gabillard JC, Hadj Sassi A, Seiliez I, Picard B, Bonnieu A. Myostatin and the skeletal muscle atrophy and hypertrophy signaling pathways. Cellular and Molecular Life Sciences : CMLS 2014;71(22):4361-71. doi: 10.1007/s00018-014-1689-x.

62. Cohen S, Zhai B, Gygi SP, Goldberg AL. Ubiquitylation by Trim32 causes coupled loss of desmin, Z-bands, and thin filaments in muscle atrophy. The Journal of Cell Biology 2012;198(4):575-89. doi: 10.1083/jcb.201110067.

63. Kudryashova E, Kudryashov D, Kramerova I, Spencer MJ. Trim32 is a ubiquitin ligase mutated in limb girdle muscular dystrophy type 2H that binds to skeletal muscle myosin and ubiquitinates actin. Journal of Molecular Biology 2005;354(2):413-24. doi: 10.1016/j.jmb.2005.09.068.

64. Devries MC, McGlory C, Bolster DR, Kamil A, Rahn M, Harkness L, Baker SK, Phillips SM. Leucine, Not Total Protein, Content of a Supplement Is the Primary Determinant of Muscle Protein Anabolic Responses in Healthy Older Women. The Journal of Nutrition 2018;148(7):1088-95. doi: 10.1093/jn/nxy091.

65. Fuchs CJ, Hermans WJH, Holwerda AM, Smeets JSJ, Senden JM, van Kranenburg J, Gijsen AP, Wodzig W, Schierbeek H, Verdijk LB, et al. Branched-chain amino acid and branched-chain ketoacid ingestion increases muscle protein synthesis rates in vivo in older adults: a double-blind, randomized trial. The American Journal of Clinical Nutrition 2019. doi: 10.1093/ajcn/nqz120.

66. Crozier SJ, Kimball SR, Emmert SW, Anthony JC, Jefferson LS. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. The Journal of Nutrition 2005;135(3):376-82. doi: 10.1093/jn/135.3.376.

67. Saxton RA, Chantranupong L, Knockenhauer KE, Schwartz TU, Sabatini DM. Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. Nature 2016;536(7615):229-33. doi: 10.1038/nature19079.

68. Chantranupong L, Scaria SM, Saxton RA, Gygi MP, Shen K, Wyant GA, Wang T, Harper JW, Gygi SP, Sabatini DM. The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway. Cell 2016;165(1):153-64. doi: 10.1016/j.cell.2016.02.035.

69. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell 2017;168(6):960-76. doi: 10.1016/j.cell.2017.02.004.

Tables

Table 1. Participant characteristics.

	MILK	**MYCO**
	(*n* = 10)	(*n* = 10)
Age (y)	22 ± 1	22 ± 1
Body mass (kg)	84 ± 3	81 ± 3
Height (cm)	178 ± 2	182 ± 3
BMI (kg·m^{-2})	26 ± 1	25 ± 1
Fat (% body mass)	12 ± 2	9 ± 2
Lean mass (kg)	73 ± 3	73 ± 2
Total work done (J)	29540 ± 1782	30722 ± 2059
Energy (MJ·day^{-1})	9.5 ± 0.8	11.6 ± 0.9
Protein (g·day^{-1})	150 ± 20	162 ± 17
Protein (g·kg^{-1}·day^{-1})	1.8 ± 0.2	2.1 ± 0.2

Values represent mean ± SEM. MILK, Milk protein ingestion condition; MYCO, mycoprotein ingestion condition, BMI, body mass index. Total work represents the amount of work done (in J) during the experimental exercise protocol. No statistically significant differences were observed between conditions ($P>0.05$).

Table 2. The nutritional content of the experimental drinks.

	MILK	MYCO
Macronutrients		
Protein (g)	26.2	31.5
Fat (g)	0.3	9
Carbohydrate (g)	1.7	7
Fibre (g)	<0.1	17.5
Energy (kcal)	108	238
Energy (kJ)	458	996
Amino acid content (g)		
Alanine	0.8	2.0
Arginine	0.9	2.2
Aspartic acid	1.9	3.3
Glutamic acid	5.8	3.9
Glycine	0.5	1.5
Histidine	0.8	0.8
Isoleucine	1.3	1.5
Leucine	2.5	2.5
Lysine	2.1	2.6
Phenylalanine	1.3	1.5
Proline	2.7	1.6
Serine	1.5	1.6
Threonine	1.1	1.7
Tryptophan	-	1.2
Tyrosine	1.3	1.2
Valine	1.7	1.9

Protein content (g) is calculated from the sum of amino acids measured after protein hydrolysis. The experimental drinks contained 31 g and 70 g of total product for MILK and MYCO, respectively.

Table 3. Muscle protein synthesis rates (FSR) before and after protein ingestion, and resistance exercise.

		Postabsorptive	**Postprandial**
MILK	Rested	0.036 ± 0.008	0.052 ± 0.006
(*n* = 10)	Exercised	0.035 ± 0.008	0.056 ± 0.005
MYCO	Rested	0.025 ± 0.006	0.057 ± 0.004
(*n* = 9)	Exercised	0.024 ± 0.007	0.072 ± 0.005

Values represent mean ± SEM. Mixed muscle protein fractional synthesis rates (FSRs; A) calculated from the plasma L-[ring-$^{2}H_{5}$]phenylalanine precursor pool in the postabsorptive (fasted) and postprandial (fed) state, in rested and exercised (single bout of unilateral concentric leg extensions) muscle in healthy young men. Postprandial state represents a 4 h period following the ingestion of 26.2g milk protein (MILK; *n*=10) or 31.5g mycoprotein (MYCO; *n*=9). Data were analysed with three-way ANOVA. Time effect, P<0.0001; treatment effect, P=0.99; exercise effect, P=0.06; treatment × time, P=0.02; treatment × exercise, P=0.22; exercise × time, P=0.13; treatment × time × exercise, P=0.42.

Table 4. Names and symbols of gene expression assay targets that were preloaded on to the microfluidic cards.

Gene name (human skeletal muscle)	Symbol
Insulin Receptor substrate 1	IRS1
AKT serine/threonine kinase 1	AKT1
AKT serine/threonine kinase 2	AKT2
phosphoinositide-3-kinase regulatory subunit 1	PIK3R1
mechanistic target of rapamycin	MTOR
ribosomal protein S6 kinase B1	RPS6KB1
eukaryotic translation initiation factor 4E binding protein 1	EIF4EBP1
eukaryotic translation initiation factor 4 gamma 1	EIF4G1
eukaryotic translation initiation factor 4E	EIF4E
sestrin 2	SESN2
GATS protein like 3	GATSL3
leucyl-tRNA synthetase	LARS
MAP kinase interacting serine/threonine kinase 2	MKNK2
transcription factor EB	TFEB
tuberous sclerosis 1	TSC1
tuberous sclerosis 2	TSC2
DNA damage inducible transcript 4	DDIT4
DNA damage inducible transcript 4 like	DDIT4L
regulatory associated protein of MTOR complex 1	RPTOR
NPR2-like, GATOR1 complex subunit	NPRL2
AKT1 substrate 1	AKT1S1
forkhead box O1	FOXO1
forkhead box O3	FOXO3
forkhead box O4	FOXO4
calpain 1	CAPN1
calpain 3	CAPN3
caspase 3	CASP3

nuclear factor kappa B subunit 1	NFKB1
F-box protein 32	FBXO32
tripartite motif containing 63	TRIM63/MuRF1
tripartite motif containing 32	TRIM32
activating transcription factor 4	ATF4
Myostatin	MSTN
growth differentiation factor 11	GDF11
SMAD family member 2	SMAD2
SMAD family member 3	SMAD3
transforming growth factor beta 1	TGFB1
inhibin beta A subunit	INHBA
solute carrier family 7 member 5	SLC7A5
solute carrier family 7 member 8	SLC7A8/LAT2
solute carrier family 36 member 1	SLC36A1
solute carrier family 7 member 1	SLC7A1
solute carrier family 38 member 2	SLC38A2
solute carrier family 38 member 9	SLC38A9/SNAT9
solute carrier family 38 member 10	SLC38A10
serum response factor	SRF
glyceraldehyde-3-phosphate dehydrogenase	GAPDH
beta-2-microglobulin	B2M

Figure Legends

Figure 1. Schematic representation of the experimental protocol.

Figure 2. The timecourse and incremental AUC (iAUC) of plasma total branched chain amino acid (A and B), leucine (C and D), isoleucine (E and F), valine (G and H), tyrosine (I and J) and phenylalanine (K and L) concentrations during a 3 h postabsorptive period (time-course graphs only) and a 4 h postprandial period in healthy young men. iAUC graphs represent total 4 h postprandial plasma concentrations above postabsorptive values. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; *n*=10) or 31.5g mycoprotein (MYCO; *n*=10), where a single bout of unilateral leg extension exercise was also performed. Time-course and iAUC data were analysed with a repeated measures two-way ANOVA and independent t-tests, respectively, with Sidak's post hoc tests applied to locate individual differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. * indicates individual differences between conditions at these time points, and a difference between conditions on the bar graphs. Treatment × time interaction effect; all $P < 0.0001$.

Figure 3. The timecourse and incremental AUC (iAUC) of serum insulin concentrations during a 3 h postabsorptive period (time-course graph only) and a 4 h postprandial period in healthy young men, with iAUCs representing total 4 h postprandial plasma concentrations above postabsorptive values. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; *n*=10) or 31.5g mycoprotein (MYCO; *n*=10), where a single bout of unilateral leg extension exercise was also performed. Data were analysed with a repeated measures two-way ANOVA and

independent t-tests, respectively, with Sidak's post hoc tests applied to locate individual differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. * indicates individual differences between conditions at these time points, and a difference between conditions on the bar graph. Treatment × time interaction effect; $P < 0.0001$.

Figure 4. L-[ring-$^{2}H_5$]phenylalanine (A), L-[3,3-$^{2}H_2$]tyrosine (B), and L-[ring-$^{2}H_4$]-tyrosine (C) enrichments during a stable isotope experimental test day in healthy young men. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; *n*=10) or 31.5g mycoprotein (MYCO; *n*=10), where a single bout of unilateral leg extension exercise was also performed. Data were analysed with a repeated measures two-way ANOVA, with Sidak's post hoc tests applied to locate individual differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. * indicates different from fasting (t = 0 min) for MYCO. Horizontal bar indicates a change from t=0 across conditions. Treatment × time interaction effect; A, P=0.0018; B, P=0.5357; C, P=0.0026.

Figure 5. Total phenylalanine rate of appearance (Ra; A), total phenylalanine rate of disappearance (Rd; B), and phenylalanine hydroxylation (C) during a 3 h postabsorptive period and a 4 h postprandial period in healthy young men. The vertical line on each graph indicates the transition from postabsorptive to postprandial conditions via the ingestion of 26.2g milk protein (MILK; *n*=9) or 31.5g mycoprotein (MYCO; *n*=9), where a single bout of unilateral leg extension exercise was also performed. Data were analysed with a repeated measures two-way ANOVA, with Sidak's post hoc tests applied to locate individual differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. Treatment × time interaction effect; A, P=0.2659; B, P=0.0003; C, P<0.0001.

Figure 6. Mixed muscle protein fractional synthesis rates (FSRs; A) calculated from the plasma L-[ring-$^{2}H_{5}$]phenylalanine precursor pool in the postabsorptive (fasted) and postprandial (fed) state, in rested and exercised (single bout of unilateral concentric leg extensions) muscle in healthy young men. Postprandial state represents a 4 h period following the ingestion of 26.2g milk protein (MILK; *n*=10) or 31.5g mycoprotein (MYCO; *n*=9). Data were analysed with three-way ANOVA, with Sidak post hoc tests applied to locate individual differences. The delta change in FSR in response to protein ingestion (B), representing the transition from postabsorptive to postprandial conditions in both groups is also presented. Data were analysed with two-way ANOVA, with Sidak's post hoc tests applied to locate individual differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. † indicates a main effect of protein ingestion. There was a trend for a difference in postprandial muscle protein synthesis rates between protein conditions (P=0.093). # represents a main effect of condition.

Figure 7. Skeletal muscle mechanistic target of rapamycin (mTOR) phosphorylation status, presented as a ratio of phosphorylated (p) to total protein, in the postabsorptive and postprandial state, in rested and exercised legs, after the ingestion of 26.2g milk protein (MILK; *n*=7) or 31.5g mycoprotein (MYCO; *n*=8), in young men. Values are means, with their standard errors represented by vertical bars. Data were analysed with two-way ANOVA. No significant effects were detected.

Figure 8. Skeletal muscle mRNA expression of genes involved in muscle protein synthesis, muscle protein breakdown, and amino acid transport in the postabsorptive and postprandial state, in rested and exercised legs, after the ingestion of 26.2g milk protein (MILK; *n*=10) or

31.5g mycoprotein (MYCO; *n*=10), in young men. Data were analysed using three-way ANOVA, with Sidak's post hoc tests used to detect differences ($P \leq 0.05$). Values are means, with their standard errors represented by vertical bars. There was a main effect of protein ingestion for IRS1, TSC1, TSC2, GATSL3, AKT1S1, CAPN1, FOXO3, SRF, LAT2, SNAT9, SLC38A10, DDIT4, TRIM63, and SLC38A2 ($P<0.05$). There was a time × exercise interaction effect for MSTN, DDIT4L, FBXO32, TRIM63, SLC38A10, DDIT4, SLC38A2, TRIM32, EIF4E, LARS, TGFB1, TFEB, PIK3R1, RPS6KB1, and SLC7A1 ($P<0.05$). SMAD2 showed a main effect of exercise ($P<0.05$). RPTOR and TSC1 showed a three way interaction (time × condition × exercise; $P<0.05$).