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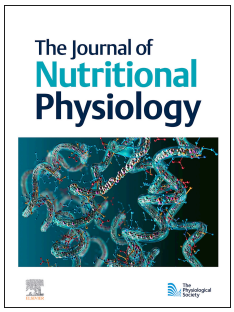

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Comparable amino acid & intramuscular signalling responses following consumption of microflora compared to whey protein post-resistance exercise in young adults

Research Article

Jedd Pratt[1], Jordan Acheson[1], Anna Lazaratou[1], Eve K. Greenhalgh[1], Oliver C. Witard[2], Craig Sale[1], Sarkis J. Hannaian[3,4], Ari Gritsas[3], Tyler A. Churchward-Venne[3,4,5], Mark Hearris[1], Nathan Hodson[1,6], Paul T. Morgan[1] *

[1] Department of Sport and Exercise Sciences, Manchester Metropolitan University Institute of Sport, 99 Oxford Road, Manchester, M1 7EL, United Kingdom.
[2] Centre for Human and Applied Physiological Sciences, Faculty of Life Sciences and Medicine, King's College London, London, United Kingdom.
[3] Research Institute of the McGill University Health Centre, Montreal, QC, Canada.
[4] Department of Kinesiology and Physical Education, McGill University, Montreal, QC, Canada.
[5] Division of Geriatric Medicine, McGill University, Montreal, QC, Canada.
[6] Faculty of Kinesiology & Physical Education, University of Toronto, ON, Canada

Address for Correspondence:
Dr Paul Morgan, PhD.
Department of Sport and Exercise Sciences, Institute of Sport,
99 Oxford Road, Manchester Metropolitan University, Manchester, M1 7EL, UK.
Tel: 0161 247 5100; **E-mail**: p.morgan@mmu.ac.uk
** Corresponding author*

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ABSTRACT

There is growing interest in alternative protein sources to reduce the intake of animal foods, although the quality of non-animal-based proteins has been questioned. In a double-blind, crossover, randomised and counter-balanced trial, we investigated the anabolic potential of a microflora (bacterial) protein designed to mimic 'high-quality' whey protein. Twelve, physically-active young adults (sex: M=6, F=6; age: 21.1 ± 3.0 years; body fat: 21.1 ± 7.3%; fasting glucose: 4.3 ± 0.4 mmol·L^{-1}) consumed 0.3 g·kg^{-1} (of body mass) of either whey protein (WHEY; dose: 27.7 ± 7.5 g) or microflora protein (MIC; dose: 29.8 ± 7.3 g) concentrate in an overnight postabsorptive state immediately following single-leg resistance exercise (~80% of 1 repetition maximum). The amino acid (AA) composition of protein supplements was independently analysed by mass spectrometry. Postprandial plasma AA concentrations (0-4 h, by liquid chromatography mass spectrometry) and intramuscular signalling responses (1 h postprandial, by Western Blot) to exercise were determined. Diet and physical activity were monitored prior to each experimental visit and replicated in a cross-over fashion. A comparable pattern of AA composition was observed between sources. Postprandial plasma AA, glucose and insulin concentrations and intramuscular signalling responses (i.e., p-mTOR, p-4E-BP1, p-Akt, p-RPS6) were largely not different between WHEY and MIC ($p>0.05$) with the exception of leucine whereby significantly higher plasma concentrations were observed with MIC across the postprandial period ($p=0.024$). Therefore, MIC represents a viable alternative protein source of comparable quality to traditional whey protein that may support skeletal muscle remodelling in young healthy adults.

INTRODUCTION

There is rapidly growing interest in alternative protein sources to reduce the intake of animal foods, owing to the purported increased environmental cost associated with animal foods and an overall increased global demand for food [1,2]. However, on a per gram (or iso-nitrogenous) basis plant-based proteins are *typically* less potent in stimulating muscle protein synthesis (MPS) compared with animal proteins [3–6]. This observation has been attributed to the typical lower essential amino acid (EAA) content and/or lower protein digestibility (referred to a 'lower quality' proteins) compared with 'higher quality' animal proteins [3–5], highlighting the need to identify higher-quality protein sources at a lower environmental cost.

Microflora refers to a group of micro-organisms including bacteria and fungi. Supplemental microflora protein products have recently become commercially available and have been marketed as environmentally friendly animal-free proteins that are identical in amino acid (AA) composition as dairy proteins. Hence, microflora protein is suggested to represent a promising innovative source of dietary protein to closely mimic the AA composition of high-quality proteins, whilst potentially mitigating some environmental (e.g., carbon-emissions) and ethical (e.g., animal welfare) consequences associated with large-scale intensive animal agriculture. This protein source is manufactured by providing microflora with sugars containing an exact copy of the genome corresponding to whey protein (considered one of the highest quality dietary proteins), as published by the Bovine Genome

Sequencing and Analysis Consortium [7,8]. The microflora cells incorporate the foreign DNA to repair breaks in its own DNA through homologous recombination [9]. Following fermentation and several purification and filtering steps, an 'animal-free whey protein' is produced and is claimed to be identical to whey protein derived from cows' milk. However, the ability for microflora to replicate the AA composition of whey, as well as the efficacy of these products in supporting skeletal muscle health remains untested.

Skeletal muscle mass is regulated by variations in rates of muscle protein synthesis (MPS) and muscle protein breakdown [10,11]. The MPS response and subsequent muscle remodelling is driven by exercise (predominantly resistance exercise) and in part, by the availability of AA into circulation following consumption of protein-rich foods which likely explains some of the superiority of animal proteins for MPS and skeletal muscle remodelling [3,10–12]. In addition, intracellular signalling pathways, such as the mammalian target of rapamycin complex 1 (mTORC1) cascade, are responsible for upregulating the MPS response to dietary protein ingestion and acute resistance exercise [13,14]. Therefore, the aims of the study were to: (i) characterise the composition of AA of an 'animal-free whey (microflora) protein' (MIC) in comparison to conventional whey protein (WHEY); and (ii) assess the efficacy of a matched protein dose of MIC on postprandial plasma AA concentrations and intracellular anabolic signalling pathways compared with WHEY in healthy adults following a single bout of resistance exercise.

Given that whey protein is considered one of the highest quality dietary protein sources due it's AA composition, branched-chain amino acid (BCAA) content, and superior digestibility, we hypothesised that: (i) WHEY would display a superior profile of AA composition compared with MIC; and (ii) postprandial plasma AA concentrations and intramuscular anabolic signalling pathways will be significantly elevated following the consumption of a matched protein dose of WHEY compared with MIC. Further, given that acute postprandial increases in glucose and insulin have important implications for skeletal muscle remodelling [15] and for metabolic health [16], we also assessed postprandial systemic concentrations of glucose and insulin.

METHODS

Participants

Twelve healthy young active (sex: M=6, F=6; age: 21.1 ± 3.0 years; body mass index: 24.6 ± 4.4 $kg \cdot m^{-2}$; body fat: 21.1 ± 7.3%; fasting glucose: 4.3 ± 0.4 $mmol \cdot L^{-1}$) adults participated in the present study. Participants were excluded if they had a history of lactose intolerance or allergies to milk protein, a daily protein intake of <0.7 g per kg body mass or > 2.2 g per kg body mass or were a regular smoker. Participants with type 2 diabetes mellitus, cardiovascular disease/complications, pulmonary disease, seizures, use or abuse of psychoactive medications or any medication or condition known to influence protein digestion and absorption were also excluded. Finally, participants with current antibiotic, anabolic steroid or corticosteroid use were also excluded. We did not assess

the menstrual status of our female participants as menstrual cycle phase does not seem to influence MPS or whole-body myofibrillar proteolysis in response to resistance exercise [17].

Justification of sample size

Based on previous research using an identical cross-over design, our sample size was calculated with differences in postprandial plasma EAA incremental area under the curve (iAUC) between protein sources as the primary outcome measure. A sample size of 12 participants was calculated with a power of 80% and a significance level of 0.05 to detect a physiologically relevant difference of ~15% in EAA iAUC between protein sources [18].

Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Science and Engineering Ethics Committee of Manchester Metropolitan University (Ref No. EthOS 58411). Written informed consent was obtained from all subjects. Recruitment and data collection were carried out in the Institute of Sport (Manchester Metropolitan University, Manchester, UK) between February 2024 and June 2024.

Pretesting

Prior to inclusion in the study, participants completed a screening session which consisted of assessments of body mass (to calculate body mass index and protein dosage consumed during the experimental trials), height, body composition (Bodystat 1500 Impedance Monitor Body Composition Analyser, Bodystat, Isle of Man, UK), the completion of a routine medical screening questionnaire and assessment of single-leg knee extension 1 repetition maximum (1RM). 1RM was assessed using standard National Strength and Conditioning Association guidelines, as previously described [19].

Study design

Following screening and consent, and in a randomised, double-blind, counterbalanced, cross-over design, participants completed two experimental test days involving an identical bout of resistance exercise, with WHEY or MIC consumption post-exercise. Randomisation was performed by an independent person using a computerised randomiser. Prior to each visit, participants were asked to complete a 3-d diet diary, with the final 24 h replicated for the subsequent visit. Dietary intake was assessed by nutrition software (Nutritics©, Dublin, Ireland) and can be viewed in **Supplementary Table 1**. Participants were advised to avoid vigorous activity in the 24 h prior to trials and to replicate physical activity patterns before their subsequent visit. The consumption of alcohol 24 h prior to each visit was prohibited and participants were instructed to arrive at the laboratory in an overnight fasted state, as verified on arrival. Test days were separated by ~7d to allow participants to re-establish habitual dietary patterns and recover from the resistance exercise.

Experimental procedures

Participants reported to the laboratory on test days at ~08.00 h after an >10 h overnight fast and were asked to rest in bed in a semi-supine position. The experimental protocol during each test day is shown in **Figure. 1**. Initially, a Teflon cannula was inserted into the antecubital vein, and the arm was placed in a heated blanket to facilitate arterialised blood sampling. Each blood sample was taken after the application of ~15 mins heating to the arm. A total number of 3 muscle biopsies were taken. A baseline vastus lateralis biopsy was obtained, either on the first or second experimental visit (randomised and counter-balanced), using a modified Bergström needle, as described in our previous work (*e.g.,* [20]). The procedure was conducted under 1% lidocaine and completed by a qualified practitioner (P.T.M., N.H). Participants then completed the single-leg knee extension exercise task (Kineo Intelligent Load System, Florida, USA). The first set of resistance exercise involved a warm-up of 12 repetitions at 40% 1RM. The next 3 sets consisted of 8-12 repetitions (or to failure) at ~80% 1RM (1RM: 30 ± 11 kg; 80% 1RM: 24 ± 9 kg). Resting periods of 2 min were implemented between all sets. The exercise load, and number of repetitions and sets were replicated for the subsequent visit. Immediately after exercise task completion, participants were given 2 minutes to consume a protein dose of 0.3 g·kg^{-1} of body mass serving of either WHEY or MIC (WHEY: 27.7 ± 7.5 g; MIC: 29.8 ± 7.3 g), which upon completion, indicated the start of the postprandial period (t = 0 min). The 0.3 g·kg^{-1} dose was selected in accordance with typical habitual dietary patterns for this population [21]. Consumption of the beverage was followed by a 4 h postprandial period in which a further 12 arterialised blood samples were collected at t = 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, and 240 min, while participants remained in a semi-supine position throughout. The 4 h postprandial period was selected to capture the initial increase in postprandial plasma AA concentrations, before their return to baseline levels. During both experimental visits, a further vastus lateralis biopsy was taken from the exercised leg 1 h post protein consumption for assessment of intracellular anabolic signalling.

********** Insert Figure 1 here **********

Experimental supplement preparations

Whey protein concentrate (Impact Whey®, MyProtein™, THG plc, Manchester, UK), and microflora protein concentrate (Perfect Day® Future Whey®, MyProtein™, THG plc, Manchester, UK) were provided by a commercial supplier. Protein sources were independently analysed (Premier Analytical Services, Buckinghamshire, UK) for energy, macronutrient, and AA composition in accordance with standard food industry accredited methods, with details presented in **Table 1**. Protein content was calculated as nitrogen (N) × 6·25 (N determined via the Kjeldahl method). While we concede the presence of non-protein nitrogen-containing factors potentially introduces error, we consider this to be minimal and consistent with what is typically accepted within the food industry. Protein beverages were prepared the morning of the test days by adding the amount of powder required to provide a total of 0.3 g·kg^{-1} of body mass of protein to 300 mL water. Beverages were mixed for ~2 min. For blinding purposes, protein drinks were flavour matched. Following drink consumption, an additional 100 mL of water was added to 'rinse' the bottle and ensure that all protein had been consumed,

making a total fluid volume of 400 mL consumed by participants on each occasion. Double blinding of drinks was achieved by having a different researcher to the one coordinating the trial prepare the drinks in a metal, non-transparent bottle ready for consumption.

********** Insert Table 1 here **********

Blood, serum, and plasma sample analysis

Eight mL of arterialised venous blood was collected at each sampling point. A 20 µL plastic capillary was filled from this and immediately analysed for blood glucose concentrations (Biosen C-Line GPþ, EKF Diagnostics, Cardiff, Wales, UK). Blood samples were collected in EDTA and SST II tubes (BD vacutainer; BD Diagnostics) and centrifuged at 11,000 g at 4°C for 10 min to separate plasma and serum samples, respectively. Prior to centrifugation, SST II tubes were left to clot at room temperature for at least 30 min. The plasma and serum supernatants were then removed, aliquoted and frozen at −80°C for subsequent analysis. Serum insulin concentrations were analysed using a commercially available enzyme-linked immunosorbent assay according to the manufacturer's instructions (#DINS00, R&D Systems Inc., Minneapolis, MN, USA). Insulin and glucose concentrations were assessed at all time points. Plasma concentrations of AAs were determined by ultra-performance liquid chromatography mass spectrometry (UPLC-MS) at timepoints 0, 15, 30, 60, 90, 120, 180, and 240 min only.

Plasma amino acid concentrations

Plasma AA concentrations were measured in collaboration with the Proteomics and Molecular Analysis platform at the Research Institute of the McGill University Health Centre (Montreal, Quebec, Canada). Plasma concentrations of EAA: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, and non-essential amino acids (NEAA): alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, proline, serine, and tyrosine were determined by UPLC-MS. AA were extracted from plasma using protein precipitation and derivatised with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC; Cayman Chemical, Ann Arbor, Michigan, USA) for analysis using reversed phase UPLC-MS. Plasma samples were extracted alongside a calibration curve of AA in 0.1N HCl with norvaline as an internal standard (all AA and norvaline purchased from Sigma-Aldrich; St. Louis, MO, USA). A calibration curve of 5 to 1000 µM was used for all AA except cystine (2.5 to 500 µM). An internal standard working solution (ISWS) containing 50 µM norvaline in 5% 5-sulfosalicylic acid was used to extract plasma and calibration samples. ISWS aliquots (25 µL) were added to sample aliquots (25 µL) in microcentrifuge tubes, vortexed and centrifuged at 15,000 x g at 10°C for 10 min. Supernatant aliquots (10 µL) were transferred into glass tubes containing 70 µL buffer solution (0.2M sodium borate pH 8.8) along with 20 µL derivatisation solution (10 mM AQC in acetonitrile), mixed and incubated for 10 min at 55°C. After cooling to room temperature, aliquots (10 µL) were transferred to autosampler vials containing 1000 µL Type-I water for UPLC-MS analysis. Extracts were analysed by UPLC-MS using an Agilent 6460 triple quadrupole mass spectrometer coupled with an Agilent 1290 UPLC system (Agilent; Santa

Clara, CA, USA). Extracts (5 µL) were injected onto an Agilent Eclipse Plus C18 100 x 2.1 mm (1.8 µm) column and chromatographed with a reverse phase gradient at 0.250 mL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The derivatised AA were detected using electrospray positive mode ionisation followed by MS/MS fragmentation. Data acquisition was performed using Agilent MassHunter Data Acquisition (version B.04.01) software. Peak area measurements from selected product ions, calibration curve regression analysis and resulting sample quantification were performed using Agilent MassHunter Quantitative Analysis (version B.05.00) software.

Intramuscular signalling

On extraction of all muscle tissue, biopsy samples were quickly rinsed in saline and blotted to remove any visible blood, fat, and connective tissue before being immediately snap-frozen in liquid nitrogen and stored at -80ºC until analysis. Anabolic (*i.e.,* mTOR-mediated and extracellular signal-regulated kinase [ERK] signalling) intramuscular signalling markers were determined for total (4E-BP1, Akt, eEF2, ERK1/2, mTOR, rpS6) and phosphorylation (4E-BP1$^{Thr37/46}$, Akt^{Ser473}, eEF2^{Thr56}, ERK1/2$^{Thr202/Tyr204}$, mTOR^{Ser2448}, rpS6$^{Ser235/236}$) protein content by Western Blot analysis on mixed muscle, as previously described (*e.g.,* [22–24]). Specifically, we assessed upstream (i.e., Akt), central (i.e., mTOR) and downstream (i.e., eEF2, rpS6, 4-EBP1) mTORC-1 proteins, as well as mTORC-1 independent responses (i.e., ERK1/2), which associate with the muscle anabolic response. First, snap-frozen muscle tissue was powdered and homogenised in ice-cold RIPA buffer (Thermo Fisher Scientific, Massachusetts, USA), supplemented with a complete protease and phosphatase inhibitor cocktail solution (Roche, West Sussex, UK) at 10 µL/µg per tissue using a FastPrep 24 Homogeniser (MP Biomedicals, Cambridge, UK). Mixed muscle homogenates were then spun at 8,000 g for 10 min at 4°C, and the supernatant was collected and frozen at −80°C for subsequent Western Blot analysis. Protein content was determined by BCA Protein Assay (Thermo Fisher Scientific, Massachusetts, USA). Western Blot aliquots were subsequently prepared at 3 µg/µL in 4 × Laemmli sample buffer and ddH_2O. Samples were boiled at 95° for 5 min and equal amounts of protein (18-30 µg) were loaded into 4–20% Mini-PROTEAN® TGX™ precast polyacrylamide gels (#4561096, Bio-Rad Laboratories, Inc., California, USA) and separated by SDS-PAGE for ~50 mins at 200 V in tris-glycine running buffer (Bio-Rad Laboratories, Inc., California, USA). Proteins were transferred to either a polyvinylidene difluoride or nitrocellulose membrane at 100 V for 1 h in tris-Methanol transfer buffer (Bio-Rad Laboratories, Inc., California, USA). Membranes were blocked in 5% low-fat milk (diluted in Tris-buffered saline and 0.1% Tween-20 [TBS-T]) for 1 h at room temperature and then incubated in a rocking device overnight at 4°C in the presence of the following primary antibodies, prepared in Tris Buffered Saline with TBS-T (1:1000): total 4E-BP1 in 5% BSA (CST 9452), total Akt (CST 4691), total eEF2 (CST 2332), total ERK1/2 (CST 4695), total mTOR (CST 2983), total rpS6 (CST 2217), p-4E-BP1$^{Thr37/46}$ (CST 9459), p-Akt^{Ser473} (CST 4060), p-eEF2^{Thr56} (CST 2331), p-ERK1/2$^{Thr202/Tyr204}$ (CST 4377), p-mTOR^{Ser2448} (CST 2971), p-rpS6$^{Ser235/236}$ (CST 4858). For each, membranes were washed 3 × 5 min in TBS-T, incubated in HRP-linked anti-rabbit (CST 7074, 1:10,000 in TBS-T) at room temperature for 1 h, before 3 × 5 min washes in TBS-T. Finally, membranes were exposed to

chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA) for 2-3 min and visualised using a ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Inc.). Bands were quantified using ImageJ software (National Institute of Health, Bethesda, MD). Relative arbitrary units were normalised to the total amount of protein loaded as visualised via Ponceau S staining. The phosphorylation of proteins as a proxy of their activation is expressed relative to the total content of protein.

Statistical analyses

The differences in AA composition between WHEY and MIC were assessed descriptively. Two-way [time x condition (WHEY *vs* MIC)] repeated measures ANOVAs were used to determine differences in postprandial AA concentrations and markers of intracellular anabolic signalling. Where the result of the ANOVA revealed an interaction effect, Sidak-corrected t-tests conducted to establish at what timepoint differences were detected whilst correcting for multiple comparisons. Postprandial AA, blood glucose and serum insulin were calculated and represented as iAUC using the trapezoid rule. Differences in iAUCs as well as maximum concentrations (Cmax) and time to reach maximum concentrations (Tmax) were analysed with paired t-tests. Data were tested for normal distribution using Shapiro-Wilk tests. Where sphericity violations were incurred, the Greenhouse-Geisser correction was applied. Statistical significance was set at $P<0.05$. All calculations were performed using SPSS Statistics version 28 (IBM Corp., Armonk, N.Y., USA), and all graphs were created using Graph Prism version 9.5.0. Data are expressed as means ± SD, unless otherwise stated.

RESULTS

Protein supplement analysis

The macronutrient and composition of AAs for each supplement is displayed in **Table 1**. The protein supplement analysis revealed comparable pattern of AA composition between sources, which was largely compatible with the AA content of human skeletal muscle protein (see Gorissen et al [25]). However, there were some notable differences between sources (**Table 1**). The sum of total AAs (TAA), EAAs, and BCAAs were lower by 8.9% (WHEY: 76.8g/100g vs. MIC: 69.9g/100g), 13.0% (WHEY: 34.78g/100g vs. MIC: 30.46g/100g) and 12.4% (WHEY: 16.48g/100g vs. MIC: 14.34g/100g), respectively, in MIC compared with WHEY. By contrast, leucine content was 17.1% higher in MIC compared with WHEY. When following a plant-based diet, particular attention to lysine is required as many plant proteins lack this EAA [26]. However, lysine content was similar between sources (WHEY: 7.66g/100g vs. MIC: 7.78g/100g). The remaining EAAs (Histidine, Methionine, Phenylalanine, Threonine) were generally lower in MIC except for methionine which was 26.1% higher in MIC compared with WHEY (WHEY: 1.61g/100g vs. MIC: 2.03g/100g). Readers are directed to Gorissen et al. [25]. for reference to the AA composition of commonly consumed plant- and animal-based protein sources, as well as human skeletal muscle protein.

Systemic insulin and glucose concentrations

Time course and iAUC are shown in **Figure 2** for serum insulin (panels A and B) and blood glucose concentrations (panels C and D) over the 4 h postprandial period. Serum insulin concentrations increased following ingestion of WHEY or MIC (time effect; $P<0.001$, $\eta_p^2=0.71$), with no differences between conditions (treatment effect; $P=0.690$). Serum insulin iAUC did not differ between groups ($P=0.211$). No time, group, or time × group effects in glucose concentrations were observed (all $P>0.05$), although blood glucose iAUC was ~58% higher following MIC ingestion compared with WHEY ($P=0.036$, $\eta_p^2=0.34$).

********** Insert Figure 2 here **********

Blood amino acid concentrations

Time course and iAUC are shown in **Figure 3** for plasma TAA (panels A and B), EAA (panels C and D), BCAA (panels E and F), and leucine (panels G and H) concentrations over the 4 h postprandial period. TAA, EAA, BCAA (leucine, isoleucine, valine) and leucine concentrations all increased following ingestion of WHEY or MIC (time effect; all $P<0.001$, η_p^2 ranged from 0.60-0.70). Consistent with the higher leucine content in MIC, the postprandial increase in plasma leucine was higher following MIC ingestion compared with WHEY (time ×group effect; $P=0.024$, $\eta_p^2=0.10$). Peak leucine concentrations (*i.e.,* Cmax) were ~30% higher following MIC ingestion compared with WHEY ($P=0.003$, $\eta_p^2=0.56$), and occurred (*i.e.,* Tmax) at 42 ± 5 min and 56 ± 9 min, respectively ($P=0.168$). Plasma leucine iAUC was ~39% higher following MIC vs. WHEY ($P=0.002$, $\eta_p^2=0.60$). No group, or time × group effects were observed for TAA, EAA or BCAA concentrations (all $P>0.05$).

********** Insert Figure 3 here **********

Intramuscular signalling responses

Intramuscular signalling responses following resistance exercise and protein ingestion are shown in **Figure 4.** p-mTOR^{Ser2448}, p-4E-BP1$^{Thr37/46}$, p-Akt^{Ser473} and p-rpS6$^{Ser235/236}$ content all increased at 1 h post-exercise/post protein consumption (time effect; all $p<0.05$, η_p^2 ranged from 0.38-0.58). However, no difference in intramuscular signalling responses were observed between WHEY and MIC following resistance exercise. No changes in content of p-eEF2^{Thr56} and p-ERK1/2$^{Thr202/Tyr204}$ were observed following resistance exercise in either condition (both $p>0.05$).

********** Insert Figure 4 here **********

DISCUSSION

The present study demonstrates, for the first time, that the AA composition of microflora protein manufactured based on the genome of whey protein presents a similar pattern to that of a high-quality conventional animal-based whey protein. In addition, the consumption of microflora protein after resistance exercise resulted in a similar increase in postprandial plasma TAA, BCAA, EAA concentrations, and stimulation of anabolic intramuscular signalling pathways compared with whey

protein in healthy, young adults. However, and in contrast to our hypothesis, a favourable increase in postprandial leucine (a particularly anabolic AA important for stimulation of MPS and as a substrate for skeletal muscle remodelling [27–30]) concentrations was observed with microflora protein, in accordance with the higher leucine content of the microflora protein supplement. Collectively, these data support the potential of microflora protein as an alternative animal-free protein source for supporting skeletal muscle remodelling in healthy and active young adult populations.

Amino acid composition

There is rapidly growing interest in alternative protein sources to reduce the intake of animal foods [1,2]. However, plant-based proteins are *typically*, albeit not always [5,31], less potent in stimulating MPS compared with animal proteins [3–6], which is assumed to be attributable to their 'lower quality' [3–5] and highlights the need to identify higher-quality alternative protein sources at a lower environmental cost. Herein, and in contrast with our primary hypothesis, we found comparable pattern of AA composition between microflora and conventional whey protein (**Table 1**), which was largely compatible with human skeletal muscle protein AA content (with potentially important implications for EAA requirements for human skeletal muscle, see Gorissen et al [25]). Indeed, the AA composition of the microflora protein represents a typical profile of a high-quality protein [25]. Importantly, leucine content was ~17% higher in the microflora protein vs. whey condition (9.13g vs. 7.80g per 100g) which may be considered important given the anabolic potency of leucine [27–30]. In addition, lysine content was similar between sources (7.66g vs. 7.78g per 100g), which is notable given that many non-animal proteins lack this EAA [26]. Taken together, these data indicate microflora protein is an alternative animal-free protein source which closely mimics the AA composition of traditional high-quality dairy protein, and theoretically could mitigate some of the environmental (i.e., carbon-emissions) and ethical (i.e., animal welfare) consequences associated with large-scale intensive animal agriculture.

Skeletal muscle anabolism

Skeletal muscle hypertrophy is regulated by multiple mechanisms but is underpinned by a positive net protein balance due to an increased stimulation of MPS [32–35]. However, in the absence of a sufficient exogenous supply of all EAAs (i.e., failing to achieve a well-balanced EAA profile), the muscle remodelling process will be limited as all EAAs are required to synthesise skeletal muscle [36]. Although the AA composition of microflora protein was broadly comparable to that of whey protein, this may not translate to equal bioavailability and anabolic potency [37], and it was therefore important to also assess the anabolic effects of microflora protein *in vivo*. Perhaps the most notable differences in the AA composition were that leucine and methionine content, as key EAA supporting the postexercise MPS response [1], were ~17% and ~26% higher in microflora compared with whey protein. Therefore, microflora protein appears to potentially offer a favourable profile of AA composition for supporting skeletal muscle remodelling. This notion is supported by our postprandial AA data showing that microflora protein ingestion elicited a significant increase in TAA, EAA, and BCAA plasma concentrations which was not significantly different with that of whey protein. In

addition, a more favourable increase in plasma leucine concentrations was observed with microflora protein ingestion, likely a consequence of the higher leucine content in this supplement. The postprandial AA response to microflora protein ingestion following resistance exercise supports its use as an alternative, novel, animal-free protein source that may support skeletal muscle remodelling to a similar extent to high-quality conventional whey protein. To establish the anabolic potential of microflora protein, we also examined the phosphorylation status of several key intramuscular anabolic signalling markers implicated in the regulation of MPS (*i.e.,* mTORC1-mediated and ERK signalling pathways) [38,39]. In accordance with the comparable AA response between supplements, both microflora and whey protein supplementation upregulated intramuscular anabolic signalling (i.e., p-mTOR, p-4E-BP1, p-Akt, p-RPS6) to a similar degree (**Figure 4**), despite the higher content and plasma availability of leucine in MIC. Whilst we might expect a dietary protein source with higher leucine content to demonstrate anabolic superiority and enhanced mTORC1 signal activation following exercise [40,41], our findings are perhaps hardly surprising given that muscle loading, particularly in the form of resistance exercise, represents the most potent stimulator of MPS and skeletal muscle remodelling [33,34,42,43]. In addition, increased provision and blood concentrations of leucine do not always correspond to superior muscle anabolic responses, particularly when compared to sources of high-quality protein (e.g., [44]). However, it is also important to acknowledge the known independent effects of nutrition and exercise on mTORC1 signalling [45]. Therefore, the disparity in leucine without any apparent differences in signalling may also be due to the exercise and relatively high dose of protein administered (i.e., 0.3 $g \cdot kg^{-1}$), revealing likely maximal muscle anabolic responses. Nevertheless, based on data presented herein, there is no evidence to suggest an anabolic inferiority of MIC compared with gold-standard, high-quality conventional whey protein in the context of the study presented herein. Therefore, our findings indicate that ingestion of microflora protein following a bout of resistance exercise in young, healthy adults is accompanied by a pronounced aminoacidemia and activation of several anabolic signalling pathways, which may support a subsequent skeletal muscle remodelling response that is not significantly different to high-quality whey protein.

Experimental considerations and future directions

Our findings should be interpreted with several important considerations in mind. First, we focussed on healthy young adults and hence the generalisability of our findings to other populations such as older adults remain to be established. This consideration is particularly important in the context of populations associated with impaired nutrient digestion and absorption (e.g., ageing). In this regard, it is noteworthy that the fibre content of microflora is relatively high (7.0g vs. 4.5 g per 100 g protein) compared to conventional whey protein. Whilst postprandial AA availability did not seem to be affected by this in the present study of young healthy adults, a reduced digestibility and absorption of nutrients is possible in populations with compromised digestive health. Future studies are also warranted to explore the long-term health effects of microflora ingestion, which remains unknown. This is particularly relevant considering the higher carbohydrate content of microflora protein, which was associated with statistically significant elevations in postprandial glucose concentrations in the present

study, and has potentially important implications for metabolic health [16]. In addition, it is pertinent to note that we did not assess the effects of the protein supplements in fed-only compared with exercise and fed-states and were unable to differentiate between the independent effects of the exercise and protein sources. Although we assessed the content of several phosphorylated proteins involved in anabolic signalling pathways, whose quantitative association with MPS is equivocal [38,39], we did not assess muscle protein turnover directly and therefore cannot comment on the impact of microflora protein on MPS following resistance exercise. Given that cellular signalling pathways are dynamic, the use of stable isotopes to measure changes in MPS would have provided greater insight into the effects of microflora protein on muscle protein turnover. Nevertheless, acute molecular responses to exercise and dietary provision can be informative for understanding alterations to MPS and skeletal muscle adaptation [46]. The use of stable isotope tracers, and more specifically intrinsically labelled proteins, would also have allowed us to accurately determine true digestion and absorption of the dietary proteins studied herein. Indeed, assessment of plasma AA concentrations is not a direct measurement of AA flux (i.e., exogenous protein availability), but instead represents plasma AA concentrations are influenced by both endogenous and exogenous rates of AA appearance and rates of disappearance [47]. Nonetheless, given the similar profiles of the two supplements, we wouldn't expect to observe differences between protein sources in postprandial exogenous or endogenous AA appearance [47]. Therefore, it is likely that the WHEY and MIC were extremely close in their digestibility and, thus, bioavailability.

It is also noteworthy that, whilst the superior plasma leucine response with microflora protein was an interesting finding, the leucine content measured in whey (7.8g per 100g) was lower than expected (typically ~10g per 100g) [25]. The reason for this notably lower leucine (and methionine) content in whey is difficult to reconcile but may reflect typical variance observed with nutritional supplements due to different manufacturing processes and warrants further attention [48]. We also examined responses to the ingestion of an *isolated* microflora protein, and as such, the effects of the product when integrated as part of a whole-food diet remain to be elucidated. With a broader view, the scalability of microflora protein production, together with product cost, consumer acceptance and precise estimates of environmental impact compared with other emerging alternatives (e.g., insect, algae), will be key determining factors of its feasibility for widespread use amongst the general population. Indeed, microflora proteins are associated with their own GMO (i.e., genetically modified organism) considerations, which may influence dietary choices independent of findings on human health. While it should be acknowledged that complementary blending of plant protein sources, as well as alterations in food processing, have gained credence as a strategy to potentially overcome the barriers associated with the typically unfavourable profile of AA composition of plant proteins [4,49,50], our findings demonstrate how an alternative approach, based upon genome replication of high-quality proteins, may provide another strategy for overcoming the anabolic inferiority of lower-quality dietary proteins.

Conclusion

We show that the AA composition of microflora protein, manufactured based on the genome of whey protein, was comparable to that of a high-quality conventional animal-based whey protein. The consumption of microflora protein after resistance exercise resulted in a similar increase in postprandial AA concentrations in healthy young adults, including a favourable increase in leucine, and comparable stimulation of anabolic intramuscular signalling pathways compared with high-quality animal-based whey protein. Collectively, these data support the potential of microflora protein as an alternative animal-free protein source for supporting muscle anabolism in heathy young adults.

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[End of script]

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Glossary

1RM, 1 repetition maximum; AA, amino acid; BCAA, branched-chain amino acid; EAA, essential amino acid; ERK, extracellular signal-regulated kinase; iAUC, incremental area under the curve; ISWS, internal standard working solution; MIC, microflora protein; MPS, muscle protein synthesis; mTORC1, mammalian target of rapamycin complex 1; NEAA, non-essential amino acid; TAA, total amino acid; UPLC-MS, ultra-performance liquid chromatography mass spectrometry; WHEY, whey protein.

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Authorship

Conceptualization, P.T.M., and M.H.; **Data Curation**, P.T.M.; **Formal Analysis**, J.P., J.A., A.L., E.K.G., O.C.W., S.J.H., A.G., T.A.C.V., N.H., and P.T.M.; **Funding Acquisition**, P.T.M.; **Investigation**, J.P., J.A., A.L., E.K.G., N.H., and P.T.M.; **Methodology**, M.H., T.A.C.V., N.H., and P.T.M.; **Project administration**, P.T.M., and J.P.; **Resources,** n/a; **Software,** n/a; **Supervision**, P.T.M.; **Validation,** n/a; **Visualization**, P.T.M., and J.P.; **Writing – Original Draft**, P.T.M., J.A., and J.P.; **Writing – Review & Editing**, A.L., E.K.G., O.C.W., C.S., S.J.H., T.A.C.V., M.H., and N.H. All authors made sufficient contributions to either the conception and design of the study, or acquisition of data, or analysis and interpretation of data. All authors (Jedd Pratt, Jordan Acheson, Anna Lazaratou, Eve K. Greenhalgh, Oliver C. Witard, Craig Sale, Sarkis J. Hannaian, Ari Gritsas, Tyler A. Churchward-Venne, Mark Hearris, Nathan Hodson, Paul T. Morgan) contributed to drafting the article or revising it critically for important intellectual content. All authors contributed to the content of the manuscript. All authors

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481
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Table 1.

Table 1. Nutritional content of the protein test beverages.

	MP Impact® Whey Protein Concentrate (Chocolate)	MP Future Whey® Microflora Protein (Natural Chocolate)
Macronutrients (per 100 g)		
Protein (g) [N x 6.25]	70.3	71.0
Carbohydrates (g)	10.2	14.1
Of which sugars (g)	5.5	0.8
Fat (g)	3.8	3.9
Of which saturates	2.2	0.7
Fibre (g)	4.5	7.0
Energy (kcal)	365	356
Energy (kJ)	1,544	1,504
Typical amino acid content (per 100 g)		
Alanine (Total)	3.88	4.93
Arginine (Total)	1.95	1.52
Aspartic Acid (Asparagine, Total)	8.51	7.72
Cystine (Total)	1.64	1.88
Glutamic Acid (Glutamine, Total)	13.60	13.40
Glycine (Total)	1.41	1.36
Histidine (Total)	1.28	0.91
Isoleucine (BCAA, Total)	4.35	2.72
Leucine (BCAA, Total)	7.80	9.13
Lysine (Total)	7.66	7.78
Methionine (Total)	1.61	2.03
Phenylalanine (Total)	2.46	2.17
Proline (Total)	4.68	3.61
Serine (Total)	4.14	2.77
Threonine (Total)	5.29	3.23
Tryptophan (Total)	Not determined	Not determined
Tyrosine (Total)	2.15	2.18
Valine (BCAA, Total)	4.33	2.49
Sum Of Determined Amino Acids	76.80	69.90
Sum Of Determined BCAA	16.48	14.34
Sum Of Determined EAA	34.78	30.46

BCAA, branched-chain amino acids; EAA, essential amino acids; TAA, total amino acids; N, nitrogen. Macronutrient and typical amino acid content presented as grams of amino acid per 100 g.

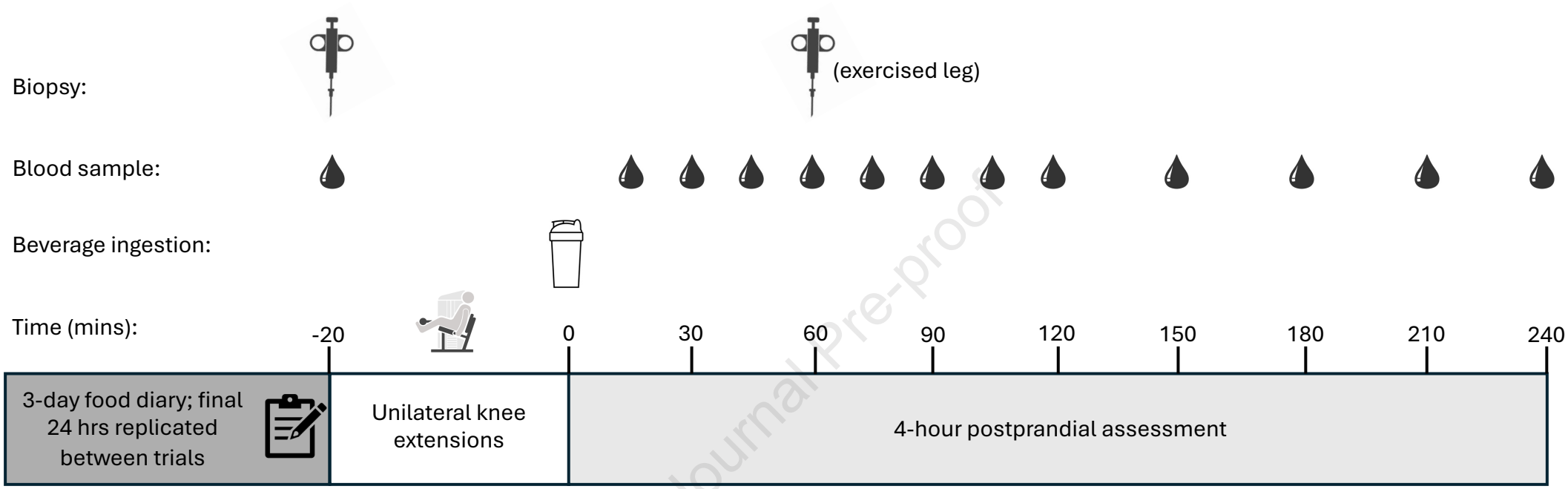
Figure legends

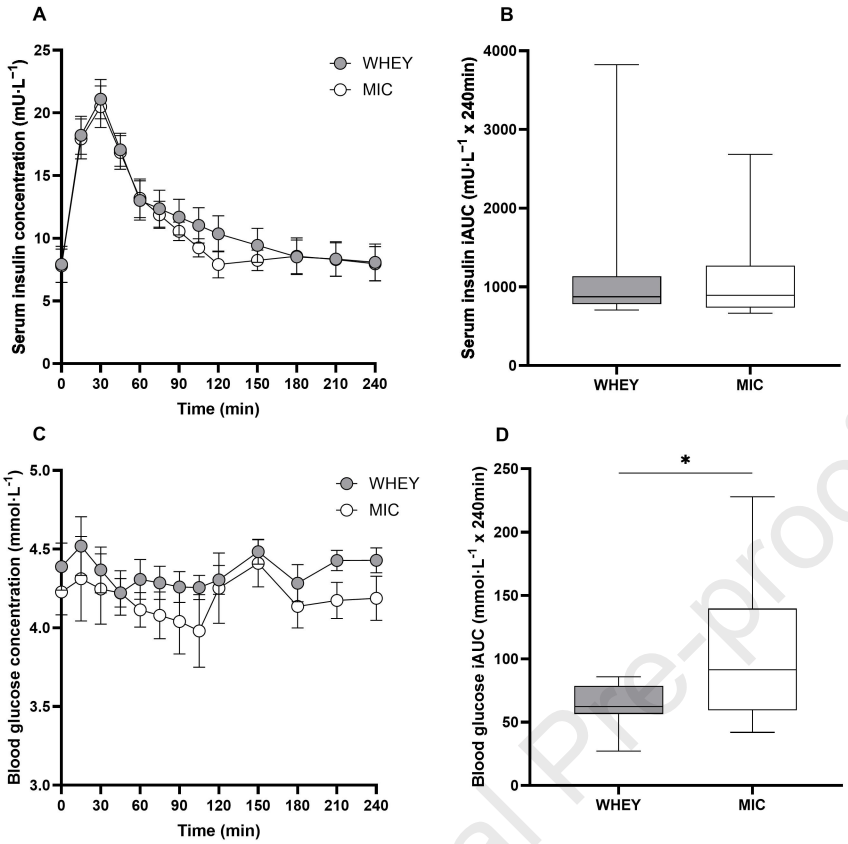
Figure 1. Protocol schematic for experimental test days.

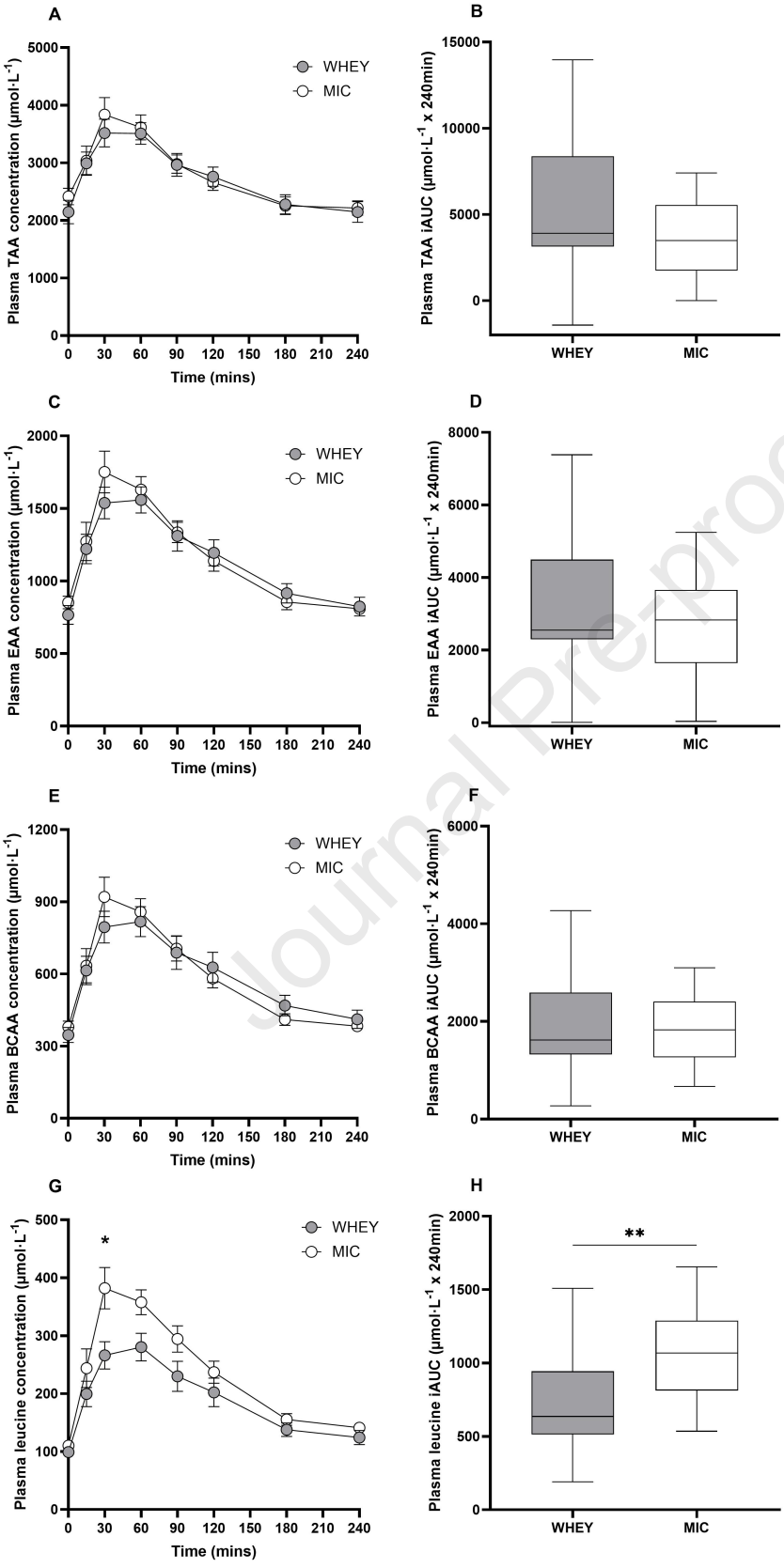
Figure 2. Time course and incremental area under the curve (AUC) for serum insulin (panels A and B) and blood glucose (panels C and D) across the 4 h postprandial period following the ingestion of 0.3 $g \cdot kg^{-1}$ of whey (WHEY: open circles) and microflora (MIC: grey filled circles) protein in healthy young (n=12) adults. Time course values are means ± SEMs, and box and whiskers denoted interquartile range and minimum/maximum values. Concentrations over time were analysed using a 2-way repeated-measures ANOVA [time × condition (WHEY *vs* MIC)] and Sidak post hoc tests. Significance was set at P<0.05. * denotes a difference between WHEY and MIC (P<0.05).

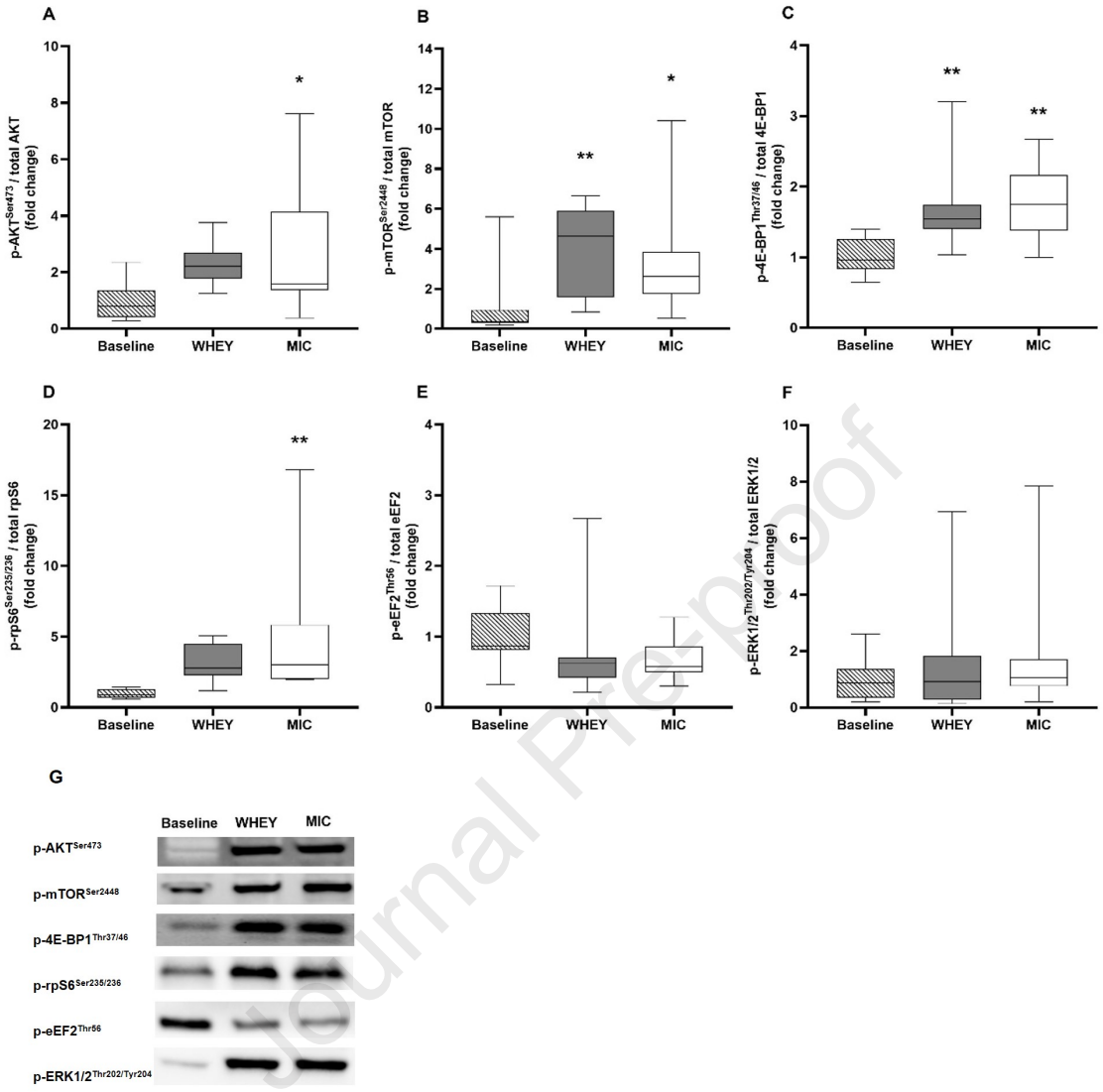
Figure 3. Time course and incremental area under the curve (AUC) for plasma TAA (panels A and B), EAA (panels C and D), BCAA (panels E and F) and leucine (panels G and H) across the 4 h postprandial period following the ingestion of 0.3 $g \cdot kg^{-1}$ of whey (WHEY: open circles) and microflora (MIC: grey filled circles) protein in healthy young (n=12) adults. Time course values are means ± SEMs and box and whiskers denoted median, interquartile range, and minimum/maximum values. Plasma concentrations over time were analysed with 2-way repeated-measures ANOVA [time × condition (WHEY *vs* MIC)] and Sidak post hoc tests. TAA, total amino acids; EAA, essential amino acids; BCAA, branched-chain amino acids. Significance was set at P<0.05. ** denotes an overall treatment difference between WHEY and MIC (P<0.01), * denotes an individual difference between WHEY and MIC at that specific time point (P<0.05).

Figure 4. Change in intramuscular signalling responses of p-Akt^{Ser473} (panel A), p-mTOR^{Ser2448} (panel B), p-4E-BP1$^{Thr37/46}$ (panel C), p-rpS6$^{Ser235/236}$ (panel D), p-eEF2^{Thr56} (panel E), and p-ERK1/2$^{Thr202/Tyr204}$ (panel F) following the completion of single-leg resistance exercise (~80% of 1 repetition maximum) and the consumption of (0.3 $g \cdot kg^{-1}$) whey (WHEY: clear plots) and microflora (MIC: grey filled plots) protein. Representative western blots are shown in panel G. Data are expressed as fold change from baseline levels (pattern filled plots) measured in the non-exercised control leg, which was normalised to a value of 1. Box and whiskers denote interquartile range and minimum/maximum values. Significance was set at P<0.05. ** and * denote differences from baseline (P<0.01 and P<0.05, respectively).









Declaration of Interests

The authors declare no competing interests. The microflora protein supplement was provided by Professor Leigh Breen via MyProtein (The Hut Group). However, MyProtein (The Hut Group) were not involved in any part of conceptualisation, investigation, data acquisition, analysis, interpretation, or dissemination. Funding was successfully awarded completely independent of any commercial partner. CS is the Editor-in-Chief of The Journal of Nutritional Physiology. We can confirm, however, that CS had no role in handling this manuscript on behalf of the journal and was neither involved nor consulted about any decisions to do with the outcomes of peer-review.