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Vicia faba Peptide Network Supplementation Does Not Differ From Milk Protein in Modulating Changes in Muscle Size During Short-Term Immobilization and Subsequent Remobilization, but Increases Muscle Protein Synthesis Rates During Remobilization in Healthy Young Men☆

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A B S T R A C T

Background: Muscle mass and strength decrease during short periods of immobilization and slowly recover during remobilization. Recent artificial intelligence applications have identified peptides that appear to possess anabolic properties in *in vitro* assays and murine models.
Objectives: This study aimed to compare the impact of *Vicia faba* peptide network compared with milk protein supplementation on muscle mass and strength loss during limb immobilization and regain during remobilization.
Methods: Thirty young (24 ± 5 y) men were subjected to 7 d of one-legged knee immobilization followed by 14 d of ambulant recovery. Participants were randomly allocated to ingest either 10 g of the *Vicia faba* peptide network (NPN_1; $n = 15$) or an isonitrogenous control (milk protein concentrate; MPC; $n = 15$) twice daily throughout the study. Single-slice computed tomography scans were performed to assess quadriceps cross-sectional area (CSA). Deuterium oxide ingestion and muscle biopsy sampling were applied to measure myofibrillar protein synthesis rates.
Results: Leg immobilization decreased quadriceps CSA (primary outcome) from 81.9 ± 10.6 to 76.5 ± 9.2 cm^2 and from 74.8 ± 10.6 to 71.5 ± 9.8 cm^2 in the NPN_1 and MPC groups, respectively ($P < 0.001$). Remobilization partially recovered quadriceps CSA (77.3 ± 9.3 and 72.6 ± 10.0 cm^2, respectively; $P = 0.009$), with no differences between the groups ($P > 0.05$). During immobilization, myofibrillar protein synthesis rates (secondary outcome) were lower in the immobilized leg (1.07% ± 0.24% and 1.10% ± 0.24%/d, respectively) than in the non-immobilized leg (1.55% ± 0.27% and 1.52% ± 0.20%/d, respectively; $P < 0.001$), with no differences between the groups ($P > 0.05$). During remobilization, myofibrillar protein synthesis rates in the immobilized leg were greater with NPN_1 than those with MPC (1.53% ± 0.38% vs. 1.23% ± 0.36%/d, respectively; $P = 0.027$).
Conclusion: NPN_1 supplementation does not differ from milk protein in modulating the loss of muscle size during short-term immobilization and the regain during remobilization in young men. NPN_1 supplementation does not differ from milk protein supplementation in modulating the myofibrillar protein synthesis rates during immobilization but further increases myofibrillar protein synthesis rates during remobilization.

Keywords: immobilization, recovery, muscle protein synthesis, plant–derived protein supplementation, skeletal muscle

Introduction

Recovery from injury or illness often requires a period of inactivity. Periods of inactivity strongly reduce muscle mass and strength [1, 2]. Importantly, even short periods of immobilization (<7 d) induce a substantial decline in muscle mass and strength [3–6], which is accompanied by reduced insulin sensitivity and impacts the time course of recovery [7–10]. The loss of

Abbreviations: CSA, cross-sectional area; MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.

☆ This trial was registered at https://trialsearch.who.int/ (identifier: NL7645).

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muscle mass during disuse has been attributed to a decline in both basal [2, 11–13] and postprandial muscle protein synthesis rates [14], resulting in a negative net muscle protein balance.

So far, few nutritional strategies have been identified to preserve muscle mass during a period of immobilization. Amino acid supplementation and omega-3 fatty acid supplementation have been shown to attenuate muscle disuse atrophy during 7 and 14 d of limb immobilization [15, 16]. In contrast, protein, leucine, and creatine supplementation did not attenuate muscle disuse atrophy during 5–7 d of limb immobilization [4–6, 17]. Recently, a targeted and untargeted predictive machine learning approach has been applied to characterize phytopeptides with potentially bioactive properties, including anti-inflammation and protein synthesis activity [18]. In short, for peptides with protein synthesis activity, known protein–protein and peptide–peptide interactions were used to predict the likely binding to proteins of interest and predictive neural networks were used to predict antiinflammatory peptides. With this approach, a peptide network derived from *Vicia faba* (NPN_1) was identified and isolated for human consumption following enzymatic hydrolysis. Two potentially bioactive peptides within NPN_1 have been characterized *in vitro*, and it was observed that the specific peptides were capable of increasing protein synthesis while reducing the markers of protein degradation and reducing TNF-α secretion [18, 19]. Additionally, these peptides are resistant to simulated gastrointestinal digestion, can traverse a representative intestinal membrane, and exhibit good stability within human plasma, which creates a possibility for these peptides to reach skeletal muscles [18]. Follow-up work in mice has suggested that NPN_1 supplementation upregulated mTOR activity and attenuated soleus muscle atrophy in a disuse (hindlimb unloaded) model [19]. This implies that NPN_1 represents a plant–derived alternative protein source that may promote muscle health in vivo in humans. Consequently, we hypothesized that NPN_1 supplementation may attenuate the muscle size and strength loss by stimulating muscle protein synthesis rates during a short period of single-leg immobilization *in vivo* in humans.

Although a handful of studies have assessed the impact of a short period of disuse on muscle mass and strength, few studies have addressed the changes in muscle protein synthesis and muscle mass and strength regain during subsequent remobilization. Previous work from our group has showed that muscle mass and strength can return to baseline values following 6 wk of natural rehabilitation [20]. Shorter periods of ambulatory recovery [7–14 d] reported a partial recovery of muscle cross sectional area [4, 15, 21]. To date, the only nutritional supplement that was shown to accelerate recovery during active exercise rehabilitation is creatine [22]. Nutritional strategies failed to further augment muscle mass regain during ambulatory recovery [4, 15, 16, 21]. We hypothesized that NPN_1 supplementation during recovery from a period of immobilization may also support a more rapid regain of muscle size and strength by stimulating muscle protein synthesis rates in healthy adults. As most plant–derived proteins are generally considered to be less anabolic, we aimed to compare the impact of supplementing NPN_1 with a high-quality animal–derived protein reference (MPC) on the loss and regain of muscle size and strength during immobilization and subsequent remobilization.

To test our hypotheses, we recruited 30 healthy young volunteers to be subjected to 1 week of single-leg immobilization, followed by a 2-week recovery period. During this period, the participants were supplemented with the NPN_1 or an isonitrogenous control (milk protein) in a randomized, double-blind manner. Prior to and after immobilization and following 2 wk of recovery, the leg muscle size, leg muscle strength, and daily muscle protein synthesis rates were assessed.

Methods

Participants

Thirty healthy young men (24 ± 5 y) were included in the present study. The exclusion criteria were as follows: a (family) history of thrombosis; (family) history of factor V Leiden, or other known thrombophilia (such as protein C, protein S, and antithrombin deficiency); lower limb, back, or shoulder injuries that could interfere with the use of crutches; allergies to milk protein; participation in structured resistance exercise program; co-morbidities interacting with mobility and muscle metabolism of the lower limbs (e.g., arthritis, spasticity/rigidity, all neurological disorders, and paralysis); use of any medications known to (or that may) affect protein metabolism; diagnosis of diabetes or metabolic, cardiovascular, or intestinal disorders; a history of neuromuscular problems; use of anticoagulants; use of protein and/or fish-oil supplements; participation in a ^2H_2O study in the previous 6 months; and smoking. On the screening visit, all participants were fully informed about the nature and risks of the experimental procedures before providing informed consent. This study was approved by the local Medical Ethical Committee of Maastricht University Medical Centre+, and conforms to the principles outlined in the latest version of the Declaration of Helsinki for use of human subjects and tissue. This trial was registered at https://trialsearch.who.int/ under the identifier NL7645. The study was independently monitored by the Clinical Trial Center Maastricht.

Experimental design

A schematic overview of the experimental design is depicted in Figure 1. Participants were randomly allocated to either the *Vicia faba*–derived peptide network (NPN_1; $n = 15$) or milk protein concentrate (MPC; $n = 15$) supplemented groups. All participants were subjected to 7 d of knee immobilization on a randomized leg, followed by 14 d of free-living (habitual) recovery. A series of measurements were performed 1 d prior to casting, directly after cast removal, and 14 d after cast removal. Single-slice computed tomography (CT) scans were performed at the mid-thigh of both legs, whole-body DXA scans were taken, muscle biopsies from both the immobilized and non-immobilized legs were taken, venous blood samples were collected, and 1-legged knee extension and leg press strength (1RM) was assessed for both legs separately. Participants underwent a deuterated water (^2H_2O)–dosing protocol, which started 2 d prior to casting and continued until the end of the experimental trial.

Leg immobilization and habitual recovery

Each participant was fitted for a full leg plaster cast in a randomized and counterbalanced (for leg) fashion to induce knee immobilization. The cast extended from 10 cm above the ankle to 25 cm above the patella, and was set at a 30° flexion

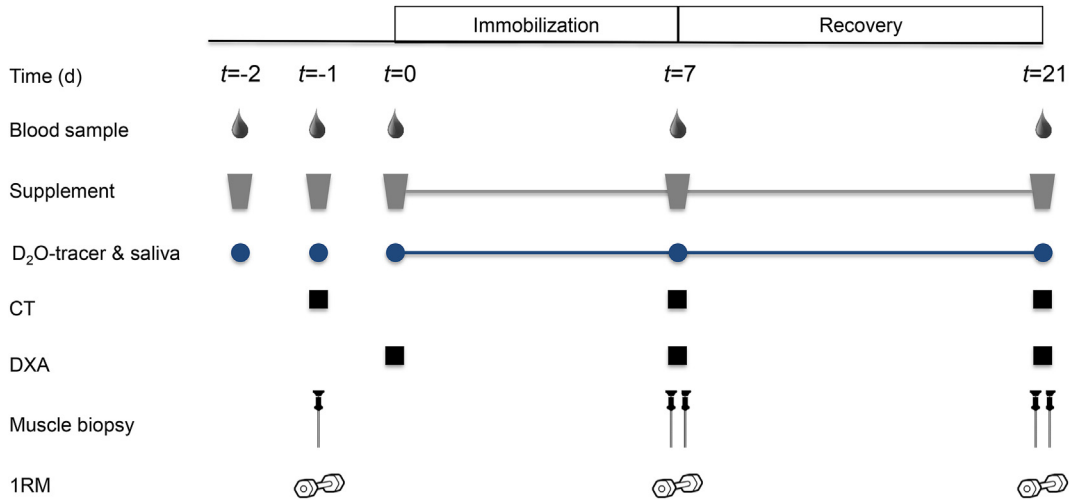

FIGURE 1. Schematic representation of the experimental protocol.

angle. Throughout the immobilization period, participants were provided with crutches to allow movement, but were instructed to not put any weight on the immobilized leg. They were instructed to perform a series of ankle exercises (i.e., plantar and dorsal flexion and circular foot movements) to avoid developing deep vein thrombosis. Following cast removal, participants were transported by wheelchair until the muscle biopsy sample was collected. After the post immobilization visit ($t = 7$ d), the 14-d recovery period started.

Protein supplementation

Supplementation began 2 d prior to casting. The NPN_1 group consumed a *Vicia faba*–derived peptide network (NPN_1, Nuritas). The MPC group received an isonitrogenous MPC; Friesland Campina). NPN_1 was prepared as described by Cal et al. with some modifications [19]. Briefly, the commercially available fava bean (*Vicia faba*) protein concentrate was resuspended in a food-grade buffer in a temperature-controlled bioreactor under constant agitation. Protein hydrolysis was initiated with the addition of a food-grade endoprotease. Hydrolysis progressed for a defined period, after which enzyme activity was inactivated by heating to 80°C for 10 min. The resulting hydrolysate suspension was then spray-dried to a fine, free-flowing powder. The amino acid composition for both supplements is displayed in Table 1. Supplements were consumed twice daily (after breakfast and before sleep) for the entirety of the experimental trial. Each serving of total 10 g contained ~6 g of protein resuspended in 200 mL of water.

Amino acid composition

Quantification of amino acids in the different powders was performed using ultraperformance liquid chromatograph mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters). At least 5 mg of the freeze-dried tissue was hydrolyzed in 3 mL of 6 M HCl for 12 h at 120°C and dried under a continuous N_2-stream. Five milliliters of 0.1 M HCl was used to reconstitute the hydrolysates after which 50 µL of each protein hydrolysate was deproteinized using 100 µL of 10% SSA with 50 µM of MSK-A2 internal standard (Cambridge Isotope Laboratories). Subsequently, 50 µL of ultrapure demineralized water was added and samples were centrifuged (15 min at 21000 rcf).

TABLE 1
Protein content and amino acid composition

	MPC	NPN_1
Protein content (%)	59	55
EAA (g/dose)		
Histidine	0.14	0.12
Isoleucine	0.28	0.21
Leucine	0.55	0.40
Lysine	0.46	0.32
Methionine	0.15	0.03
Phenylalanine	0.28	0.23
Threonine	0.24	0.18
Valine	0.35	0.23
∑EAA	2.46	1.71
NEAA (g/dose)		
Alanine	0.18	0.22
Arginine	0.19	0.57
Aspartic acid[1]	0.35	0.47
Cysteine	0.01	0.02
Glutamic acid[2]	1.26	0.89
Glycine	0.10	0.20
Proline	0.59	0.23
Serine	0.32	0.26
Tyrosine	0.31	0.19
∑NEAA	3.32	3.86

EAA, essential amino acids; MPC, milk protein concentrate; NEAA, non-essential amino acids; NPN_1, *Vicia faba* peptide network.
[1] Aspartic acid includes asparagine.
[2] Glutamic acid includes glutamine.

After centrifugation, 10 µL of the supernatant was added to 70 µL of Borate reaction buffer (Waters). In addition, 20 µL of AccQ-Tag derivatizing reagent solution (Waters) was added after which the solution was heated to 55°C for 10 minutes. Of this, the 100-µL derivative 1 µL was injected and measured using UPLC-MS.

Dietary intake and physical activity

Participants were instructed to refrain from strenuous physical activity, avoid alcohol intake, and keep their diet as constant as possible for 2 d prior to the first experimental test day until the final test visit ($t = 21$ d). All participants received a standardized meal prior to test days on $t = -1$, 7, and 21 d (2.9 MJ, 53 En%

carbohydrate, 31 En% fat, 16 En% protein). Dietary intake and physical activity records were completed by the participants for 3 d prior to the immobilization period and during the final 3 d of the immobilization and recovery periods. Dietary intake records were analyzed using the Dutch Food Consumption Database 2019 (NEVO; RIVM) [23]. Daily steps were recorded over the same 3-d periods using a triaxial accelerometer (Actigraph GT3X; Actigraph LLC) worn on the waist. Data were included in the analysis if participants wore the Actigraph for a minimum of 2 d and at least for 10 h/d.

Body composition

Body weight was measured with a digital balance with an accuracy of 0.1 kg (SECA GmbH). A single-slice CT scan (Siemens Definition Flash; Siemens) was performed to assess the upper leg muscle cross-sectional area (CSA) as described previously [24]. Briefly, a 2-mm-thick axial image was taken 15 cm proximal to the top of the patella with participants lying supine with their legs extended and feet secured. Image analysis was performed using ImageJ software (1.53k) and muscle CSA was determined for the whole thigh and the quadriceps. Body composition (fat, fat-free mass, and bone mineral content) was determined by a DXA scan (Hologic Discovery A). The system's software package APEX version 4.0.2 was used to determine whole-body and regional (e.g., legs) lean mass, fat mass, and bone mineral content.

Leg strength

At the end of the test day, the single-leg 1RM of participants was assessed. During screening, all participants were instructed and familiarized with the safe lifting technique for the leg extension and leg press exercise. On the test day, after warming up, the load was set at 90% of the estimated 1RM from the screening visit and increased after each successful lift until failure. Three-minute rest periods were allowed between the lifts. A repetition was considered valid when the participant was able to complete the entire lift in a controlled manner without assistance.

Deuterated water–dosing protocol

The deuterated water–dosing protocol consisted of 1 dosing day and 22 maintenance days. The dosing protocol was modified from previously published studies that have administered deuterated water in human participants [25–27]. Prior to dosing, the baseline blood and saliva samples were collected. Participants then ingested 2 doses of 100 mL of 70% deuterium oxide (Cambridge Isotopes Laboratories) with an interval of 30–60 min between the doses. Participants ingested 20 mL of 70% deuterium oxide every morning for the remainder of the trial. To assess body water enrichment (^2H_2O), participants collected saliva samples using a dental swab (Celluron) every evening for the entire experimental protocol. The participants were instructed to not eat or drink anything 30 min prior to saliva collection.

Blood samples were collected at $t = -2$, -1, 0, 7, and 21 d in EDTA-containing tubes and centrifuged at 1000 *g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C. Prior to the immobilization period, a muscle biopsy was collected from the *vastus lateralis* muscle of the leg identified as the leg that would not be immobilized (non-immobilized leg). On the visits following immobilization and recovery, muscle biopsies from both the immobilized leg and the non-immobilized leg were collected. Muscle biopsy samples were obtained from the middle region of the *vastus lateralis*, ~1–3 cm below the level where the CT scan was performed, by using the percutaneous needle biopsy technique [28]. Muscle samples were dissected carefully, freed from any visible non-muscle material, frozen in liquid nitrogen, and stored at −80°C until further analyses. A separate piece of muscle (~20 mg) was embedded in Tissue-Tek and immediately frozen in liquid nitrogen-cooled isopentane for later immunohistochemical analyses.

Serum, plasma, and saliva analyses

Creatinine, gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), triglycerides (TG), high-sensitivity C-reactive protein (hs-CRP), FFA, insulin, and glucose concentrations were assessed in serum samples collected in a rested state on $t = -2$, 0, 7, and 21 d. Serum creatinine, GGT, AST, ALT, LDH, CK, TG, and glucose concentrations were measured by using spectrophotometry (cobas 8000 instrument; Roche Diagnostics). hs-CRP was measured by using particle-enhanced immunonephelometry (BN ProSpec; Siemens Healthineers). FFA concentrations were measured by using spectrophotometry (Alinity ci; Abbott), and insulin concentrations were determined by using the chemiluminescent immunometric assay (XPi instrument; Siemens Medical Solutions Diagnostics). Plasma-free [^{2}H]alanine enrichments were determined by gas chromatography–mass spectrometry analysis (GC–MS; Agilent 5975C MSD, 7890A GC) on $t = -2$, -1, 0, 7, and 21 d as described previously [25]. Body water enrichments were analyzed using the saliva samples collected throughout the experimental protocol. The samples were diluted 35-fold with ddH_2O, and catalytic rods (Thermo Fisher Scientific) were placed inside 12-mL glass vials (Labco Exetainer). Then, the dilution samples were prepared and analyzed as described by Holwerda et al. [25].

Muscle analysis

Myofibrillar protein–enriched fractions were extracted from ~70 mg wet muscle tissue by hand-homogenizing on ice using a pestle in a standard extraction buffer (10 μL/mg). The samples were centrifuged at 700 *g* and 4°C for 15 min. The pellet was washed with 400 μL of extraction buffer and centrifuged at 700 *g* and 4°C for 10 min. The supernatant was removed and the pellet was washed with 500 μL of milliQ water before vortexing and centrifugation at 700 *g* and 4°C for 10 min. Then, the supernatant was removed and 1 mL of homogenization buffer was added, and the material was suspended by vortexing before transferring into microtubes containing 1.4 mm ceramic beads and Lysing Matrix D (MP Biomedicals). The samples were shaken 4 times for 45 s at 5.5 m/s (FastPrep-24 5G, MP Biomedicals) to mechanically lyze the protein network. Samples were left to rest at 4°C for 3 h before centrifuging at 800 *g* and 4°C for 20 min. The supernatant was discarded and 1 mL of the homogenization buffer was added. The microtubes were shaken once for 45 s and 5.5 m/s and left to rest at 4°C for 30 min before centrifuging at 800 *g* and 4°C for 20 min. Subsequently, the supernatant was discarded and 1 mL of KCl buffer was added to the pellet, and samples were left to rest overnight at 4°C. The next morning, the samples were vortexed, transferred to new microtubes, and centrifuged at

1600 *g* and 4°C for 20 min. The supernatant containing the myofibrillar proteins was collected. The myofibrillar protein was washed once with 100% ethanol, once with 70% ethanol, and hydrolyzed overnight in 2 mL of 6 M HCl at 110°C. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under a continuous nitrogen stream while heating them at 120°C. The free amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG 50W-X8 resin columns (mesh size: 100–200, ionic form: hydrogen; Bio-Rad Laboratories), and eluted with 2 M NH_4OH. Thereafter, the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters. The derivatized samples were measured using a gas chromatography–isotope ratio mass spectrometer (GC-IRMS; MAT 253; Thermo Fisher Scientific) equipped with a pyrolysis oven and a 60-m DB-17MS column (no. 122-4762; Agilent) and 5-m precolumn. Ion masses 2 and 3 were monitored to determine the $^2H/^1H$ ratios of muscle protein–bound alanine. A series of known standards was applied to assess the linearity of the mass spectrometer and to control for the loss of tracer.

Immunohistochemistry

From all biopsies, 7-μm-thick cryosections were cut at −20°C using a cryostat (CM 3050, Leica Biosystems). Samples were thaw-mounted onto uncoated precleaned glass slides, and samples of each individual subject were mounted on the same slide. Care was taken to properly align the samples for the cross-sectional orientation of the muscle fibers. Samples were stained for muscle fiber typing, CSA, and myonuclei, as described previously [29]. In short, samples were air-dried for 30 min after taking them out of the freezer. After 5 min fixation in acetone, the cryosections were incubated for 30 min with anti-myosin heavy chain type 1 (A4.840, 1:25, DSHB) and anti-Laminin (L9393, 1:50, Sigma-Aldrich) in a 0.05% Tween PBS. Slides were then washed 3 times in the Tween/PBS solution. Appropriate secondary antibodies were then applied – GAMIgM Alexa 488 (A21426, 1:500, Invitrogen) and GARIgG Alexa 647 (A21238, 1:400, Invitrogen) in combination with 4′,6-diamidino-2-phenylindole (DAPI; D1306, 1:100, Invitrogen) for 30 minutes. After a final triple washing with PBS, slides were mounted with Mowiol (Calbiochem).

Slides were viewed and automatically captured using a 10× objective on a modified Olympus BX51 fluorescence microscope with a customized disk-spinning unit (Olympus), computer-controlled excitation and emission filter wheels (Olympus), 3-axis high-accuracy computer-controlled stepping motor specimen stage (Grid Encoded Stage, Ludl Electronic Products), ultra-high sensitivity monochrome electron multiplier CCD camera (C9100-02, Hamamatsu Photonics), and controlling software (StereoInvestigator; MBF BioScience). Before analyses, slides were blinded for both intervention and time point. All areas selected for analysis were free of the "freeze fracture" artifact, and care was taken such that longitudinal fibers were not used in the analysis. Quantitative analyses were performed using ImageJ software package [version 1.52p, National Institutes of Health [30]]. On average, 213 ± 133 muscle fibers were analyzed per muscle biopsy sample collected to determine muscle fiber type distribution, CSA, myonuclear content, and domain size.

rtPCR

Total RNA was isolated and quantified from 10 to 20 mg of frozen muscle tissue using a TRIzol Reagent (Life Technologies, Invitrogen) according to the manufacturer's protocol, as described previously [3]. Taqman primer/probe sets were obtained from Applied Biosystems: MAFBx (Hs01041408_m1), MuRF1 (Hs00261590_m1), FOXO1 (Hs01054576_m1), and 18S (Hs03003631_g1). Relative quantification of the genes was performed using the ΔΔCT method ($2^{-\Delta\Delta Ct}$). Ct values of the target genes were normalized to Ct values of the internal control, and results were calculated as relative expression against the standard curve. The Ct values of all genes of interest were always within the lower and upper boundaries of the standard curve.

Calculations

The myofibrillar protein FSR was determined using the incorporation of [^{2}H]alanine into muscle proteins and the mean precursor [^{2}H]alanine enrichment. The precursor [^{2}H]alanine enrichment was estimated by correcting body water deuterium enrichments by a factor of 3.7 based on the deuterium labeling during *de novo* alanine synthesis [25]. As we assessed FSR for >14 d, the nonlinear equation was used to calculate FSR as described earlier [31, 32]:

$$FSR\ (\%/d) = \frac{-\ln(1-f)}{t} \times 100$$

where *f* is calculated as the change in muscle protein–bound [^{2}H] alanine enrichment divided by the mean precursor [^{2}H]alanine enrichment and *t* represents the time between biopsies on days −1 and 7 or between days 7 and 21.

Statistics

Data are expressed as mean ± SD, unless stated otherwise. Normality of the data was verified using visual inspection of QQ plots and Shapiro–Wilk tests. No major violations for specific 2-way ANOVA assumptions were observed; in case of non-sphericity, the Greenhouse–Geisser correction was used. An independent t test was used to assess the differences in baseline characteristics between participants in the MPC and NPN_1 groups. The primary outcome variable was *quadriceps* CSA. A two-way repeated measures ANOVA with time (2 levels, pre immobilization vs. post immobilization) as the within-subjects factor and treatment (MPC vs. NPN_1) as the between-subjects factor was applied to compare changes in *quadriceps* and whole thigh muscle CSA and muscle strength during immobilization. A two-way repeated measures ANOVA with time (3 levels, pre immobilization, post immobilization and post recovery) as the within-subjects factor and treatment (MPC vs. NPN_1) as between-subjects factor was applied to compare changes between *quadriceps* and whole thigh muscle CSA and strength during recovery. To compare changes in muscle protein synthesis rates during immobilization, a two-way repeated measures ANOVA with leg (immobilized vs. non-immobilized) as within-subjects factor and treatment (MPC vs. NPN_1) as between-subjects factor was applied. To compare changes in muscle protein synthesis rates over time, a two-way repeated measures ANOVA with time (immobilization vs. recovery) as

within-subjects factor and treatment (MPC vs. NPN_1) as between-subjects factor was applied for both legs (immobilized and non-immobilized). In case of significant interactions, separate analyses were performed in the NPN_1 and MPC groups, and in case of significant main time effects, a Bonferroni post hoc test was applied to locate differences. A power calculation was performed with the change in quadriceps CSA determined by CT scan as our primary endpoint. The sample size (N) was calculated with a power of 80% (1-β = 0.8) and a significance level of 5% (α = 0.05). Based on previous studies, we expected the standard deviation of the measurement of quadriceps CSA to be approximately 1.8% in both groups and we also expected that the muscle loss from baseline in the control group will be approximately 5% [3–5]. We consider a reduction in muscle loss by 40% (i.e., a total muscle CSA loss of 3% instead of 5%) as clinically relevant. The latter will likely lead to significant retention of muscle function and strength. Taking into consideration a drop-out rate of 10% during the experimental trial, the final number of subjects that should be recruited after screening is 15 per group. Since we included 2 groups, the study required recruitment of 30 subjects. Data were analyzed using SPSS version 27 (SPSS, IBM Corp). Statistical significance was set at $P < 0.05$.

Results

Participants

The baseline characteristics of participants did not differ between the MPC and NPN_1 groups (Table 2). Of the 30 participants, 29 completed the study [$n = 14$ (MPC), $n = 15$ (NPN_1)]. Data of one participant were excluded because of drop out during the recovery period in turn caused by the COVID-19 lockdown. A flowchart of participants is displayed in Supplemental Figure 1.

Muscle CSA

At baseline, *quadriceps* CSA did not differ between the groups ($P = 0.116$). Leg immobilization significantly decreased *quadriceps* CSA (Figure 2, $P < 0.001$), with no differences observed between the MPC (−4.2% ± 1.7%) and NPN_1 (−5.4% ± 2.4%; *P*-treatment = 0.136, *P*-time × treatment = 0.141) groups. Following recovery, quadriceps CSA was greater compared with post immobilization ($P = 0.009$) but remained lower compared with *quadriceps* CSA prior to immobilization ($P < 0.001$). The recovery in *quadriceps* CSA did not differ between the MPC (1.9% ± 2.4%) and NPN_1 groups (1.2% ± 2.3%; $P = 0.151$, *P*-time × treatment = 0.064).

At baseline, the whole thigh CSA did not differ between the groups (Table 2, $P = 179$). Leg immobilization significantly decreased whole thigh CSA ($P < 0.001$), with no observed differences between the MPC (−2.8% ± 1.8%) and NPN_1 (−4.4% ± 2.6%) groups ($P = 0.227$, *P*-time × treatment = 0.104). Following recovery, the whole thigh CSA was greater compared with that post immobilization ($P = 0.001$), but smaller than that prior to immobilization ($P = 0.010$). The recovery in whole thigh CSA did not differ between groups ($P = 0.293$).

TABLE 2
Participants' characteristics

	MPC (n = 14)	NPN_1 (n = 15)
Age (y)	26 ± 5	23 ± 4
Body weight (kg)	75.9 ± 14.9	72.0 ± 7.9
BMI (kg/m^2)	24.4 ± 3.1	22.6 ± 1.3
Total lean mass (kg)	53.6 ± 8.7	54.3 ± 6.7
Leg (non-immobilized) lean mass (kg)	9.1 ± 1.6	9.3 ± 1.3
Leg (immobilized) lean mass (kg)	9.2 ± 1.4	9.1 ± 1.1
Whole thigh (non-immobilized) CSA (cm^2)	140 ± 22	150 ± 16
Whole thigh (immobilized) CSA (cm^2)	139 ± 22	150 ± 17
Fat (%)	26.0 ± 6.4	21.6 ± 3.7
Fasted glucose (mmol/L)	4.9 ± 0.4	4.8 ± 0.3
Systolic blood pressure (mmHg)	122 ± 13	124 ± 9
Diastolic blood pressure (mmHg)	70 ± 10	67 ± 9
Resting heart rate (beats/min)	64 ± 7	65 ± 9

CSA, cross-sectional area; MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.

Muscle strength

Baseline, single-legged 1RM leg extension strength did not differ between groups ($P = 0.646$). Leg immobilization significantly decreased the 1RM leg extension strength (Figure 3, $P < 0.001$), with no differences between the MPC (−13% ± 8%) and NPN_1 (−13% ± 7%) groups ($P = 0.611$, *P*-time × treatment = 0.864). Following recovery, single-leg 1RM leg extension was greater compared with post immobilization ($P = 0.008$) but remained lower compared with prior to immobilization ($P = 0.002$). No differences were detected on the increase in single-leg 1RM leg extension between the MPC (2.2% ± 6.7%) and NPN_1 groups (1.5% ± 7.3%, $P = 0.738$, *P*-time × treatment = 0.985) during recovery. Similar findings were observed for the 1RM leg press strength, with no differences in the decline following immobilization or in the increase following recovery.

Muscle fractional synthesis rates

During immobilization, myofibrillar protein synthesis rates were lower in the immobilized leg than in the non-immobilized leg in the MPC and NPN_1 groups, respectively ($P < 0.001$), with no differences between the groups ($P = 0.955$, *P*-time × treatment = 0.415; Figure 4). During remobilization, myofibrillar protein synthesis rates were lower in the non-immobilized leg (1.31% ± 0.34% and 1.42% ± 0.28%/d in the MPC and NPN_1 groups, respectively) when compared with the immobilization period ($P = 0.030$), with no differences between groups ($P = 0.371$, *P*-time × treatment = 0.565). During remobilization, myofibrillar protein synthesis rates in the previously immobilized leg were higher when compared with the period during immobilization ($P = 0.001$). The increase in myofibrillar protein synthesis rates following remobilization was greater following NPN_1 (50% ± 46%; from 1.07% ± 0.24% to 1.53% ± 0.38%/d) when compared with MPC supplementation (13% ± 38%; from 1.12% ± 0.24% to 1.23% ± 0.36%/d, *P*-time × treatment = 0.027; Figure 5).

Muscle fiber characteristics

Muscle fiber characteristics are displayed in Table 3. Data of 8 participants were excluded or missing because of low fiber count or missing samples. Prior to immobilization, no differences were observed in type I, type II, and mixed muscle fiber CSA between groups (all *P* values > 0.05). Leg immobilization tended to reduce the mixed muscle fiber CSA ($P = 0.091$). Following

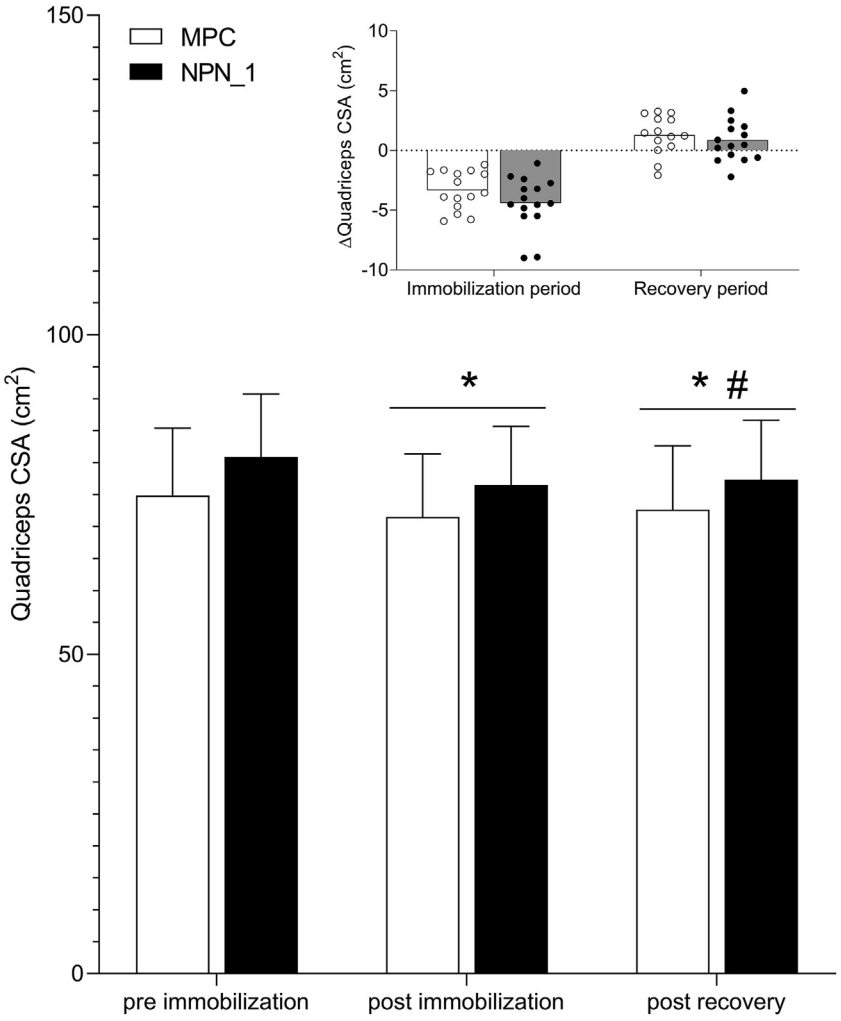

FIGURE 2. CSA of *M. quadriceps* in the MPC and NPN_1 group before and after 7 d of single-legged immobilization and after 14 d of free-living recovery. Data are expressed as mean ± SD, $n = 29$. *Significantly different from pre immobilization. #Significantly different from post immobilization. CSA, cross-sectional area; MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.

recovery, the mixed muscle fiber CSA was lower than in baseline ($P = 0.010$) but not different when compared with the post immobilization timepoint ($P = 0.999$). No differences in mixed muscle fiber CSA were detected between groups ($P = 0.365$, P-time × treatment = 0.404).

mRNA expression

Following immobilization, FOXO (Figure 6A), MuRF1 (Figure 6B), and MAFBx (Figure 6C) mRNA expressions were increased compared with those in baseline (all $P < 0.001$), with no differences between groups (all $P > 0.05$). Following recovery, FOXO, MuRF1, and MAFBx mRNA expressions were lower compared with those post immobilization ($P < 0.001$), with no differences detected between the groups ($P > 0.05$). FOXO, MuRF1, and MAFBx mRNA expressions returned to baseline levels following recovery ($P > 0.05$).

Blood parameters

Blood parameters were assessed at baseline prior to and following immobilization and after remobilization. Data were analyzed for 20 participants [$n = 9$ (MPC), $n = 11$ (NPN_1)]. Data for 10 participants were excluded because of missing samples or participants not being fasted. Creatinine, GGT, AST, ALT, LDH, and CK concentrations are displayed in Supplemental Figure 2. TG, hs-CRP, FFA, insulin, and glucose concentrations are displayed in Supplemental Figure 3. There were no changes over time and between groups for GGT, AST, ALT, LDH, CK, TG, hs-CRP, FFA, and insulin (all P values > 0.05, all P-time × treatment >0.05). Creatinine concentrations were lower following immobilization when compared with baseline and prior to immobilization (both P values < 0.03), but not different when compared with post recovery. Creatinine was higher in the NPN_1 group when compared with the MPC group ($P < 0.001$). Glucose concentrations were lower following immobilization when compared with baseline ($P = 0.040$), with no differences between the groups ($P > 0.05$).

Dietary intake and physical activity

Dietary intake and step count data are displayed in Table 4. Data of 9 participants were excluded because of insufficient wear time. Prior to immobilization, no differences were observed in energy and protein intake between groups (both P values > 0.05). During immobilization, the total energy intake tended to be lower ($P = 0.089$) and the total protein intake was lower ($P =$

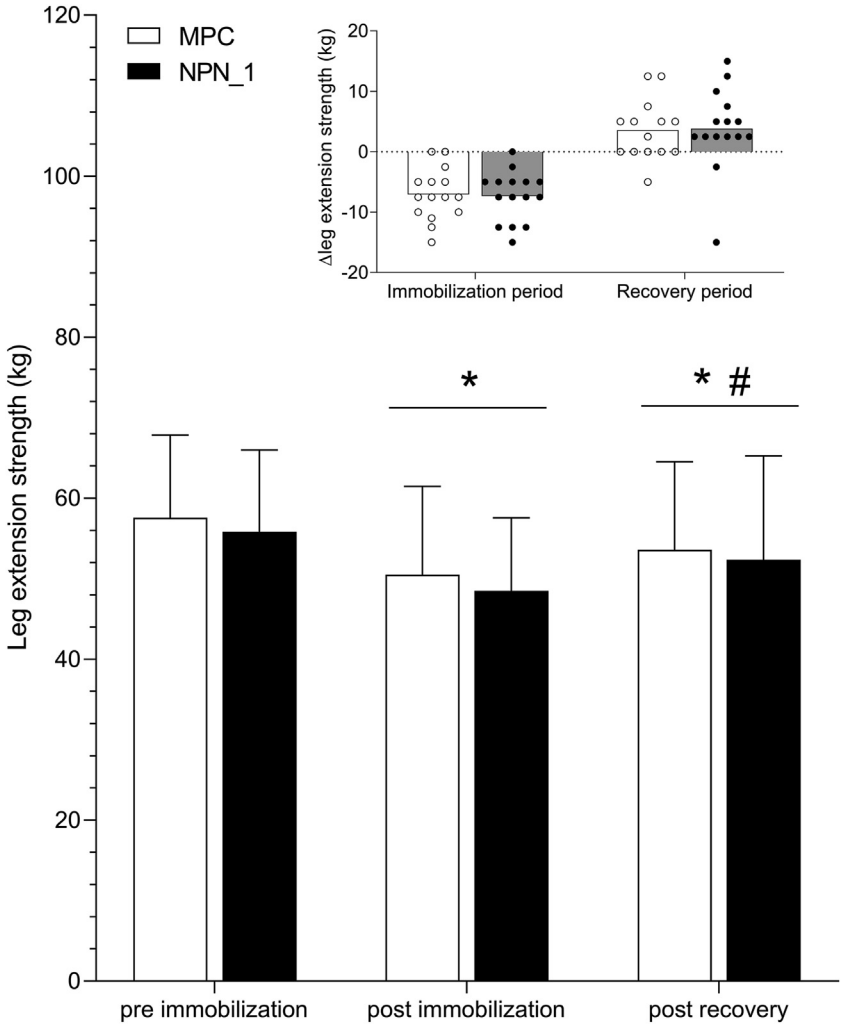

FIGURE 3. Leg extension strength (kg) in the MPC and NPN_1 groups before and after 7 d of single-legged immobilization and after 14 d of free-living recovery. Data are expressed as mean ± SD, $n = 29$. *Significantly different from pre immobilization; #Significantly different from post immobilization. MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.

0.022) when compared with that prior to immobilization. During recovery, the total energy ($P = 0.032$) and protein intake ($P = 0.008$) were lower when compared with pre immobilization, but not different from those during immobilization (both $P > 0.05$). There were no differences between the groups for both energy intake ($P = 0.162$, P-time × treatment = 0.402) and total protein intake ($P = 0.075$, P-time × treatment = 0.449) during the study period. Prior to immobilization, no differences were observed in the step count between groups ($P = 0.376$). During immobilization, step count reduced from 6060 ± 1920 to 1980 ± 1310 steps in the MPC group and from 6600 ± 2070 to 2400 ± 1340 steps in the NPN_1 group ($P < 0.001$). During recovery, the step count improved back to pre immobilization levels ($P = 0.706$). No differences in step count were detected between groups ($P = 0.292$, P-time × treatment = 0.830).

Discussion

The present study shows that 7 d of single-leg immobilization resulted in a substantial decline in daily muscle protein synthesis rates and a loss of muscle size and strength, which were only partially recovered following 14 d of remobilization. No differences were observed between the *Vicia faba* peptide network (NPN_1) or milk protein (MPC) supplementation for the loss of muscle size and strength during short-term immobilization or the regain of muscle size and strength during subsequent remobilization. However, NPN_1 supplementation resulted in significantly higher muscle protein synthesis rates during the remobilization period when compared with milk protein supplementation.

In the present study, 7 d of single-leg immobilization resulted in a substantial loss of muscle size (−5% ± 2%) and strength (−13% ± 7%). These observed rates of muscle size loss (−0.7% ± 0.3% per day) and strength loss (−1.8% ± 1.0% per day) confirm previous observations over similar durations of limb immobilization in our laboratory as well as others [3, 4, 13, 15, 17, 24, 33]. Our data clearly illustrate the impact of short periods of muscle disuse on muscle size and strength. Recently, a predictive machine learning approach has been applied to identify plant–derived peptides from *Vicia faba* with anabolic and anti-catabolic properties observed in *in vitro* and rodent models [18]. As most plant–derived proteins are generally considered to have lesser anabolic properties, we aimed to compare the impact of supplementing NPN_1 with a high-quality

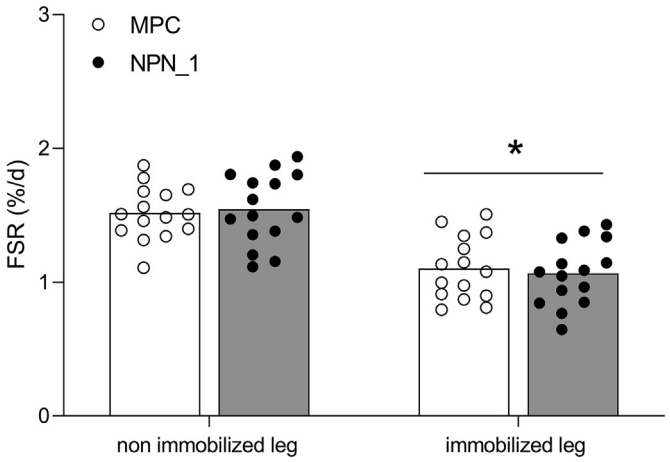

FIGURE 4. Myofibrillar protein FSRs (%/day) calculated from saliva precursor pools. Myofibrillar FSR assessed during 7 d of single-leg immobilization in the non-immobilized and immobilized legs in the MPC and NPN_1 groups. Bars represent means (MPC: $n = 15$; NPN_1: $n = 15$) and dots represent individual values. *Significantly different from non-immobilized leg. MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.

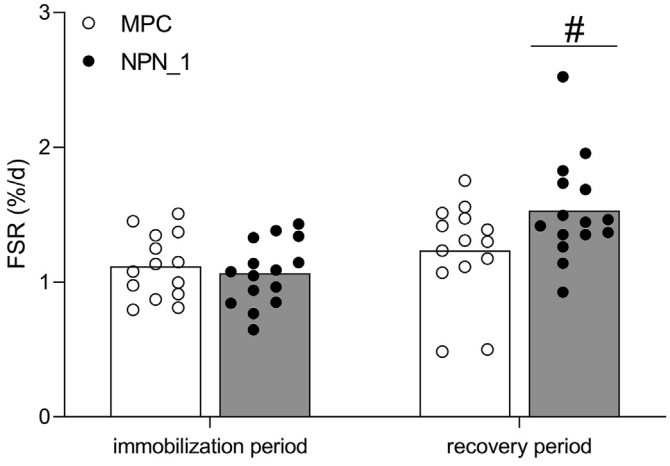

FIGURE 5. Myofibrillar protein FSRs (%/day) calculated from saliva precursor pools. Myofibrillar FSR assessed during 7 d of single-leg immobilization and 14 d of free-living recovery in the immobilized leg in the MPC and NPN_1 groups. Bars represent means (MPC: $n = 14$; NPN_1: $n = 15$) and dots represent individual values. #Significant time × treatment interaction. MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.

animal–derived protein reference (MPC) on the loss and regain of muscle size and strength during immobilization and subsequent remobilization.

In the current trial, participants were provided with either 6-g protein dose (gross product weight: 10 g) of NPN_1 or an isonitrogenous control consisting of milk protein concentrate, which was provided twice daily. The efficacy of NPN_1 at a low dose was determined based on the previous trial by Cal et al., which showed an effect with 650 mg/kg of NPN_1 [19]. This indicated the potential for a low human equivalent dose [34]. Furthermore, 2 potentially bioactive peptides within NPN_1 have been investigated in vitro, and it was observed that the specific peptides HLPSYSPSPQ and TIKIPAGT were capable of increasing protein synthesis and reducing TNF-α secretion, respectively [18]. In the current trial, daily NPN_1 consumption did not differ from MPC in the retention of muscle size or strength throughout the 7 d of disuse (Figure 2). These data are in line with earlier work demonstrating that supplementing high-quality protein sources alone do not preserve muscle size or strength throughout 5–7 d of limb immobilization in otherwise healthy adults [5, 6, 17]. Conversely, previous work reported that supplementation with selected essential and non-essential amino acids could mitigate muscle mass loss following 7 d of immobilization when compared with an energy-matched carbohydrate control [16]. The discrepancy between findings might be explained by the differences in the control treatment applied and/or the amount of amino acids provided in the studies, as we provided 12 g protein of the NPN_1, whereas Holloway et al. provided ~70 g of the amino acid supplement and preloaded for 7 d. However, the study by Kilroe et al. showed no differences in muscle loss when consuming either a high, low, or no protein diet during 3 d of single-leg immobilization [35]. Clearly, more work will be needed to establish the various factors that determine the functional food effect of amino acid and/or protein supplementation to attenuate muscle mass loss during muscle disuse.

Despite the clinical importance of recovery following such a short period of immobilization, few studies have assessed muscle size and strength regain during remobilization. Here, we observed only a partial regain in muscle size and leg extension strength following 14 d of ambulant recovery. This seems to be in line with previous work showing only partial recovery of muscle CSA following short periods [7–14 d] of ambulatory recovery [4, 15, 21]. When extrapolating the data on muscle size loss, muscle size would have returned to pre immobilization levels after ~7 wk of remobilization. This is in agreement with a previous work

TABLE 3
Muscle fiber characteristics

	Pre immobilization		Post immobilization		Post recovery	
	MPC ($n = 10$)	NPN_1 ($n = 12$)	MPC ($n = 10$)	NPN_1 ($n = 12$)	MPC ($n = 10$)	NPN_1 ($n = 12$)
Muscle fiber (n)						
Fiber type mixed	211 ± 78	186 ± 83	279 ± 100	247 ± 115	307 ± 77	230 ± 188
Fiber type I	60 ± 24	75 ± 41	93 ± 37	95 ± 61	106 ± 44	81 ± 68
Fiber type II	151 ± 65[1]	111 ± 52[1]	186 ± 80[1]	152 ± 59[1]	201 ± 67[1]	149 ± 127[1]
Muscle fiber CSA (μm^2)						
Fiber type mixed	5480 ± 704	5830 ± 1110	5110 ± 916	5090 ± 1000	4620 ± 854[2]	5230 ± 1260[2]
Fiber type I	5060 ± 1100	5690 ± 1250	5080 ± 1380	4940 ± 1140	4390 ± 1070[2]	5040 ± 1460[2]
Fiber type II	5640 ± 696	5920 ± 1130	5120 ± 837	5230 ± 1030	4610 ± 943[2]	5400 ± 1370[2]

MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.
[1] Significantly different from the fiber type-I value ($P < 0.05$).
[2] Significantly different from the preimmobilization value ($P < 0.05$).

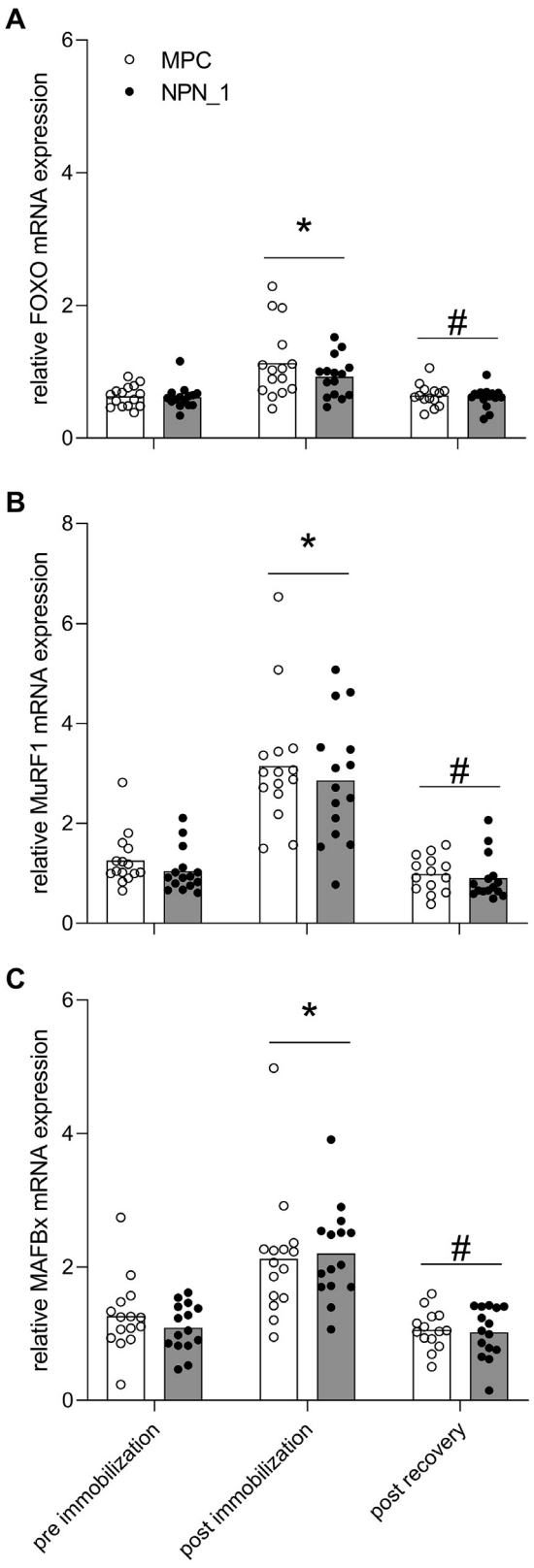

FIGURE 6. Skeletal muscle mRNA expression of selected genes of interest in the MPC and NPN_1 groups before and after 7 d of single-legged immobilization, and after 14 d of free-living recovery. Data are expressed as mean ± SD, $n = 29$. *Significantly different from pre immobilization. #Significantly different from post immobilization. MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.

demonstrating full recovery of muscle mass and strength following 6 wks of natural rehabilitation [20]. This illustrates that there are opportunities to shorten recovery periods via exercise and/or nutritional interventions. To date, there are no nutritional strategies that have proven effective in accelerating muscle mass regain during ambulatory recovery. Here, we assessed the impact of supplementing a plant–derived peptide network that was shown preclinically to possess anabolic properties [19]. No differences were observed in muscle mass or strength regain when comparing NPN_1 supplementation with an isonitrogenous amount of milk protein. Whether or not the provided protein per se accelerated muscle size or strength regain cannot be determined within the applied study design. However, we consider this unlikely under conditions where ample amounts of protein are consumed throughout the day in otherwise healthy, active young adults not involved in any exercise rehabilitation program.

In addition to our assessments of skeletal muscle size and strength, we also provided participants with deuterium oxide to assess muscle protein synthesis rates during both immobilization and subsequent remobilization. We observed ~30% lower myofibrillar protein synthesis rates in the immobilized leg during short-term disuse when compared with the non-immobilized leg. These data are in line with the study of Kilroe et al. showing a similar reduction in myofibrillar protein synthesis rates during 7 d of disuse [13, 35]. Although muscle loss during disuse has been attributed to declines in both postabsorptive [2, 11, 36] and postprandial [12, 14, 36] protein synthesis rates, there are also indications of greater muscle protein breakdown during short periods of immobilization [21]. Similarly, we demonstrated a robust upregulation of protein breakdown markers in muscles (FOXO, MuRF1, and MAFBx; Figure 6). Again, no differences were observed in mRNA expression following the immobilization period between treatments. Of course, it should be noted that changes in mRNA expression are transient and merely represent snapshots in time, implying that changes in mRNA expression may have occurred during the early stages of immobilization. With protein intake levels having shown to not impact the decline in daily muscle protein synthesis rates during a short period of single-leg immobilization [35], it seems evident that other more intrinsic anabolic stimuli are required to preserve muscle size during a period of disuse [24, 37–39]. Local muscle contraction during immobilization, by active stimulation or the application of exercise mimetics, is likely an essential component in effective strategies to attenuate muscle size and strength loss during immobilization.

During subsequent 14 d of ambulant remobilization, daily myofibrillar protein synthesis rates increased when compared with the levels observed during immobilization. Despite the absence of differences in muscle size and strength regains between treatments, we observed significantly (50% vs. 13%) higher myofibrillar protein synthesis rates following NPN_1 supplementation when compared with MPC (Figure 5). We can only speculate on the relevance of these findings, but it is possible that the remobilization period was too short for us to allow for the detection of small, but clinically relevant, increases in muscle size and/or strength regain following NPN_1 supplementation. Higher myofibrillar protein synthesis rates during recovery from a period of immobilization are indicative of greater or more rapid reconditioning of muscles. However, whether these differences are specific for myofibrillar and/or other muscle protein fractions and

TABLE 4
Nutrition and physical activity

	Pre immobilization		Immobilization period		Recovery period	
	MPC	NPN_1	MPC	NPN_1	MPC	NPN_1
Nutrition[1]						
Energy intake (MJ/d)	9.7 ± 2.9	10.6 ± 2.7	8.1 ± 1.7	9.7 ± 2.2	8.6 ± 1.8[3]	8.9 ± 2.3[3]
Carbohydrate (En%)	45 ± 5	43 ± 10	48 ± 7	45 ± 5	46 ± 6	50 ± 8
Fat (En%)	36 ± 7	34 ± 5	34 ± 9	37 ± 5	36 ± 7	32 ± 7
Protein (En%)	16 ± 3	17 ± 4	16 ± 4	16 ± 4	16 ± 4	16 ± 3
Protein intake (g/d)	93 ± 32	106 ± 23	73 ± 20[3]	89 ± 33[3]	79 ± 22[3]	81 ± 21[3]
Protein intake (g/kg/d)	1.2 ± 0.4	1.5 ± 0.4	1.0 ± 0.3[3]	1.3 ± 0.5[3]	1.1 ± 0.3[3]	1.2 ± 0.2[3]
Physical activity[2]						
Step count	6060 ± 1920	6600 ± 2070	1980 ± 1310[3]	2400 ± 1340[3]	4950 ± 2960[4]	6080 ± 1910[4]

MPC, milk protein concentrate; NPN_1, *Vicia faba* peptide network.
[1] Nutrition data is displayed for $n = 14$ (MPC) and $n = 15$ (NPN_1); nutritional data excluding supplements.
[2] Physical activity data are displayed for $n = 8$ (MPC) and $n = 9$ (NPN_1).
[3] Significantly different from the pre immobilization value ($P < 0.05$).
[4] Significantly different from the post immobilization value ($P < 0.05$).

whether they can translate to clinical benefits remain to be addressed in further studies. Although most pharmacological and nutritional strategies to preserve muscle size and strength during a period of disuse and accelerate muscle size and strength regain during remobilization have been unsuccessful [4–6, 17, 21, 40], it is evident that such strategies may be more effective in more clinically compromised populations where habitual physical activity and daily food intake are less than optimal. In the present study, habitual physical activity and sufficient daily protein consumption were evident in our healthy, young adults (Table 4). This may compromise the ability to detect relevant improvements in the rate of muscle size and strength regain following nutritional and/or pharmacological interventions in a research model of limb immobilization.

In conclusion, NPN_1 supplementation does not differ from an isonitrogenous amount of milk protein to modulate muscle size or strength loss during short-term immobilization or to augment muscle size and strength regain during remobilization. NPN_1 supplementation does not differ from milk protein supplementation in modulating myofibrillar protein synthesis rates during immobilization but further increases myofibrillar protein synthesis rates during subsequent remobilization. The relevance of the latter findings will require further investigation.

Author disclosures

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Author contribution

The authors' responsibilities were as follows – MW, AH, HD, BK, NK, LvL: designed the research; AK: produced the supplements; MW, AH, GJ, LH: conducted the experimental trials; MW, AH: performed the sample analysis; MW, AH, LV: analyzed data; MW, AH, LV, LvL: interpreted results of experiments; MW, AH, LvL: had the primary responsibility for the final content and drafted the manuscript; and all authors: read and approved the final manuscript.

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This project was supported by Nuritas Ltd. The researchers are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The industrial partners have contributed to the project through regular discussions.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.01.014.

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