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Whole-food protein sources differentially modulate postprandial plasma dileucine concentration and muscle protein synthesis in humans

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ABBREVIATIONS

BCCA, branched-chain amino acids

CHO, carbohydrate control drink

COMP, complementary pairing of rice and black beans

C*max*, peak plasma amino acid concentration

DILEU, dileucine

EAA, plasma essential amino acids

FSR, fractional synthesis rate

GSLTPAQ, Godin-Shephard Leisure-Time Physical Activity Questionnaire

HFP, high fat pork

iAUC, incremental area under the curve

IPAQ, International Physical Activity Questionnaires

ISO, isolated mixture of crystallized amino acids, maltodextrin, cellulose, pectin, and soy oil.

LFP, low fat pork

MPB, muscle protein breakdown

MPS, muscle protein synthesis

mTOR1, mechanistic target of rapamycin complex 1

PepT1, peptide transporter 1

Tmax, time of peak plasma amino acid concentration

ΔMPS, change in myofibrillar protein synthesis rates from baseline

I. INTRODUCTION

Dietary protein ingestion increases circulating amino acid availability and thereby supports the postprandial stimulation of skeletal muscle protein synthesis (MPS) (Boirie et al., 1996; Groen et al., 2015a; Hodson et al., 2019; Pennings et al., 2011). Considerable research has focused on amino acids, particularly leucine, as key regulators of this response (Churchward-Venne et al., 2012, 2014; Lee et al., 2022). More recently, dileucine has emerged as a potentially relevant leucine-containing peptide, although its physiological role remains uncertain (J. A. Aguilera et al., 2025; Paulussen et al., 2021). Because most work to date has examined isolated proteins or nutrients rather than whole foods, it remains unclear whether dileucine increases meaningfully in the circulation after whole-food protein ingestion and whether such a response may be relevant to postprandial anabolic regulation. This work addresses that gap by examining the postprandial appearance of dileucine after ingestion of different whole-food protein sources and by evaluating its association with the post-exercise anabolic response. The present chapter therefore introduces the physiological and methodological concepts required to contextualize this work within the broader field of human protein metabolism.

1. Skeletal muscle and muscle protein turnover

Skeletal muscle is essential for locomotion and is a major determinant of whole-body metabolic health (Kim & Kim, 2020). It is the most abundant tissue by mass (commonly ~40% of the body weight of a young man) and represents the body's largest reservoir of protein and amino acids (Merz & Thurmond, 2020). As such, skeletal muscle contributes to the maintenance of systemic amino acid availability and provides amino acid substrates to other tissues during periods of reduced dietary amino acid supply (e.g., fasting) and during catabolic stress associated with illness (Wolfe, 2006).

Skeletal muscle also plays a central role in glucose homeostasis and insulin action. Under insulin-stimulated conditions, skeletal muscle accounts for the majority of whole-body glucose disposal, and it is responsible for over ~80% of glucose uptake following an oral glucose load in the postprandial state (Honka et al., 2018; Merz & Thurmond, 2020). Consequently, disturbances in skeletal muscle metabolism,

particularly skeletal muscle insulin resistance, are strongly implicated in impaired glucose regulation and the pathophysiology of obesity-related metabolic disease (DeFronzo & Tripathy, 2009). Beyond glucose handling, muscle quantity and quality are clinically important across a wide range of conditions: low lean mass and/or sarcopenia are associated with adverse outcomes, including increased risk of disability and mortality, and low muscle mass has been linked to poorer prognosis in clinical populations such as cardiovascular disease and cancer (Cruz-Jentoft et al., 2019; Wolfe, 2006). Because fat-free mass is a primary determinant of resting energy expenditure, and skeletal muscle comprises a large component of fat-free mass, preserving muscle mass and function contributes not only to physical capability but also to energy metabolism and body-composition regulation across the lifespan ((Heymsfield et al., 2021; Zurlo et al., 1990).

A main determinant of skeletal muscle health is muscle protein turnover, the continuous remodeling of muscle proteins through the dynamic balance between MPS and muscle protein breakdown (MPB) (Biolo et al., 1995; Kumar et al., 2009; Phillips et al., 1997). Net muscle protein balance, defined as the difference between synthesis and breakdown, determines whether muscle proteins are maintained, accrued (hypertrophy), or lost (atrophy) (Biolo et al., 1995; Kumar et al., 2009; Phillips et al., 1997). Importantly, MPS represents only one component of muscle protein turnover and does not, in isolation, indicate net anabolism without concurrent consideration of MPB. While simultaneous assessment of MPS and MPB would provide net muscle protein balance, quantifying MPB remains methodologically challenging, particularly during non-steady-state conditions such as acute feeding and the post-exercise period (Gwin et al., 2020). Moreover, relying on MPS alone can be misleading in catabolic stress states (e.g., severe energy deficit). Although MPS may be elevated secondary to increased breakdown-derived intracellular amino acid availability. However, net balance can still remain negative if MPB rises to a greater extent and amino acids are redirected toward efflux and/or oxidation rather than efficient reutilization (Gwin et al., 2020).

Beyond regulating muscle mass, protein turnover is essential for maintaining proteostasis, ensuring the removal and replacement of damaged or dysfunctional proteins (Trommelen & Loon, 2021). Muscle proteins are continually exposed to structural and metabolic stressors, including oxidative and nitrosative modifications

(Jang et al., 2020). Damaged or misfolded proteins are normally cleared and replaced through proteostatic systems such as the ubiquitin-proteasome pathway and autophagy (Korovila et al., 2017). Age- and disease-associated impairments in these quality-control processes can contribute to accumulation of dysfunctional proteins and deterioration of muscle function.

Therefore, the regulation of muscle protein turnover is central to maintaining muscle mass and proteostasis, ultimately preserving functional capacity, and can be acutely modulated by feeding-related changes in circulating amino acid availability (Gwin et al., 2020; Korovila et al., 2017).

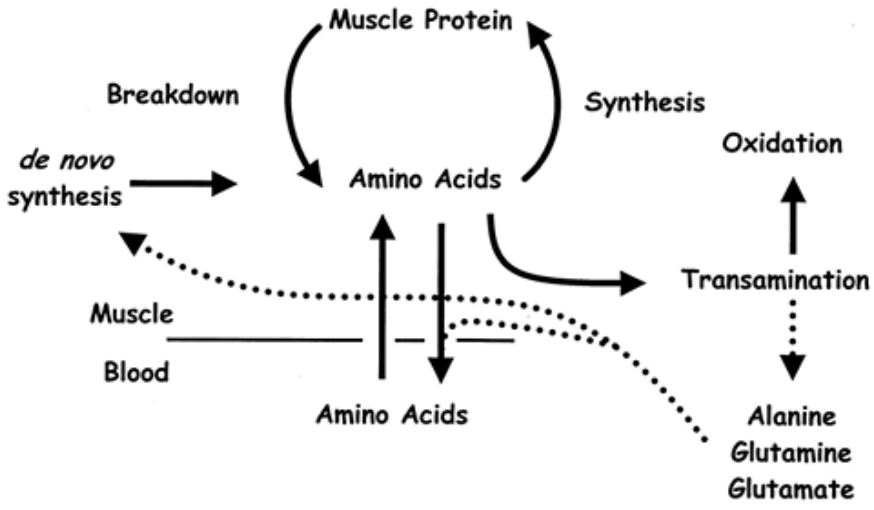

Figure 1. Schematic of protein turnover in skeletal muscle. From Philips, 2004.

2. From ingestion to systemic availability

Dietary protein elevates plasma amino acid concentration for several hours, and the rate of protein digestion and absorption has wide metabolic effects (Loveday, 2023). Following ingestion, protein digestion begins in the mouth where food is disrupted by chewing and mixed with saliva. In the stomach, acid and pepsin, together with peristaltic mixing, further break down the food matrix and initiate proteolysis (Loveday, 2023). In this framework, proteolysis in the stomach is described as limited and functioning primarily to release peptides (and some amino acids) that can contribute to gut sensing of meal composition. The ingested protein is then delivered to the duodenum via gastric emptying. As chyme empties into the

duodenum, bile and pancreatic secretions are delivered to the intestinal lumen, including bicarbonate that helps shift luminal pH toward neutral. Further secretions (including bicarbonate, water, and mucus) occur along the small intestine, and luminal proteases cleave dietary protein into short peptides and amino acids. In addition to luminal digestion, the villi at the apical surface of enterocytes ("brush border") contain anchored proteases and can contribute to continued peptide hydrolysis (Loveday, 2023; Trommelen et al., 2021).

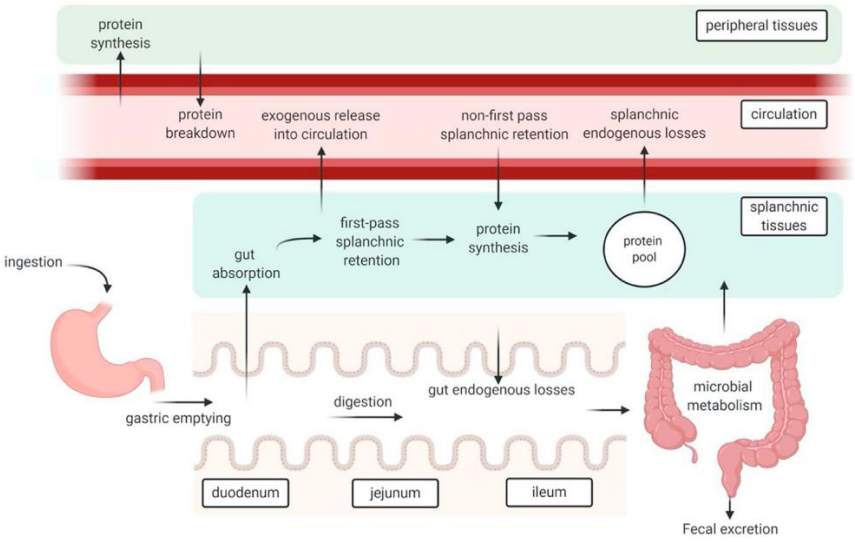

Figure 2. Schematic of protein digestion and absorption processes. From Simon M. Loveday, 2022.

The rate at which amino acids appear in the circulation after a protein meal is strongly influenced by digestion and absorption kinetics, which are modulated by protein source and food structure/processing (Loveday, 2023). In vivo evidence indicates that food-processing treatments (e.g., heating, gelling/aggregation, enzymatic hydrolysis) can substantially alter the kinetics of amino acid release and absorption; hydrolysis typically accelerates digestion/absorption, whereas aggregation/gelation effects can either slow or accelerate proteolysis depending on the resulting microstructure. A clear human example of kinetics shaping postprandial amino acid availability is the "fast versus slow" protein concept. Using intrinsically ^{13}C-labelled milk proteins, whey protein ingestion produced a dramatic but short rise in plasma amino acids, whereas casein ingestion produced a prolonged plateau of moderate hyperaminoacidaemia, interpreted by the authors

as probably reflecting slower gastric emptying (Boirie et al., 1997). Because changes in absorption rate influence the maximum concentration (C*max*), duration, and area under the curve (AUC) of postprandial plasma amino acid time courses, processing-driven differences in digestion/absorption kinetics can have downstream metabolic relevance.

After luminal digestion, amino acids and small peptides are taken up from the intestinal lumen into enterocytes through apical transport systems and subsequently released into portal venous blood via basolateral transport mechanisms (**Figure 3**) (Loveday, 2023). Free amino acids are absorbed through several apical amino acid transport systems, while di- and tripeptides are efficiently absorbed via the peptide transporter 1 (PepT1), encoded by the SLC15A gene (Loveday, 2023; Miner-Williams et al., 2014). PepT1 has broad substrate scope for di- and tripeptides and functions as a high-capacity transport of small peptide digestion products into enterocytes. Once inside enterocytes, di- and tripeptides are generally hydrolyzed rapidly by cytosolic peptidases, and the resulting amino acids are exported across the basolateral membrane to portal blood through amino acid transporters (Miner-Williams et al., 2014). Consistent with this processing logic, a critical evaluation of peptide bioavailability concludes that there is little unequivocal evidence that dietary bioactive peptides, other than di- and tripeptides, cross the healthy gut intact and enter the hepatic portal system in physiologically relevant concentrations (Miner-Williams et al., 2014).

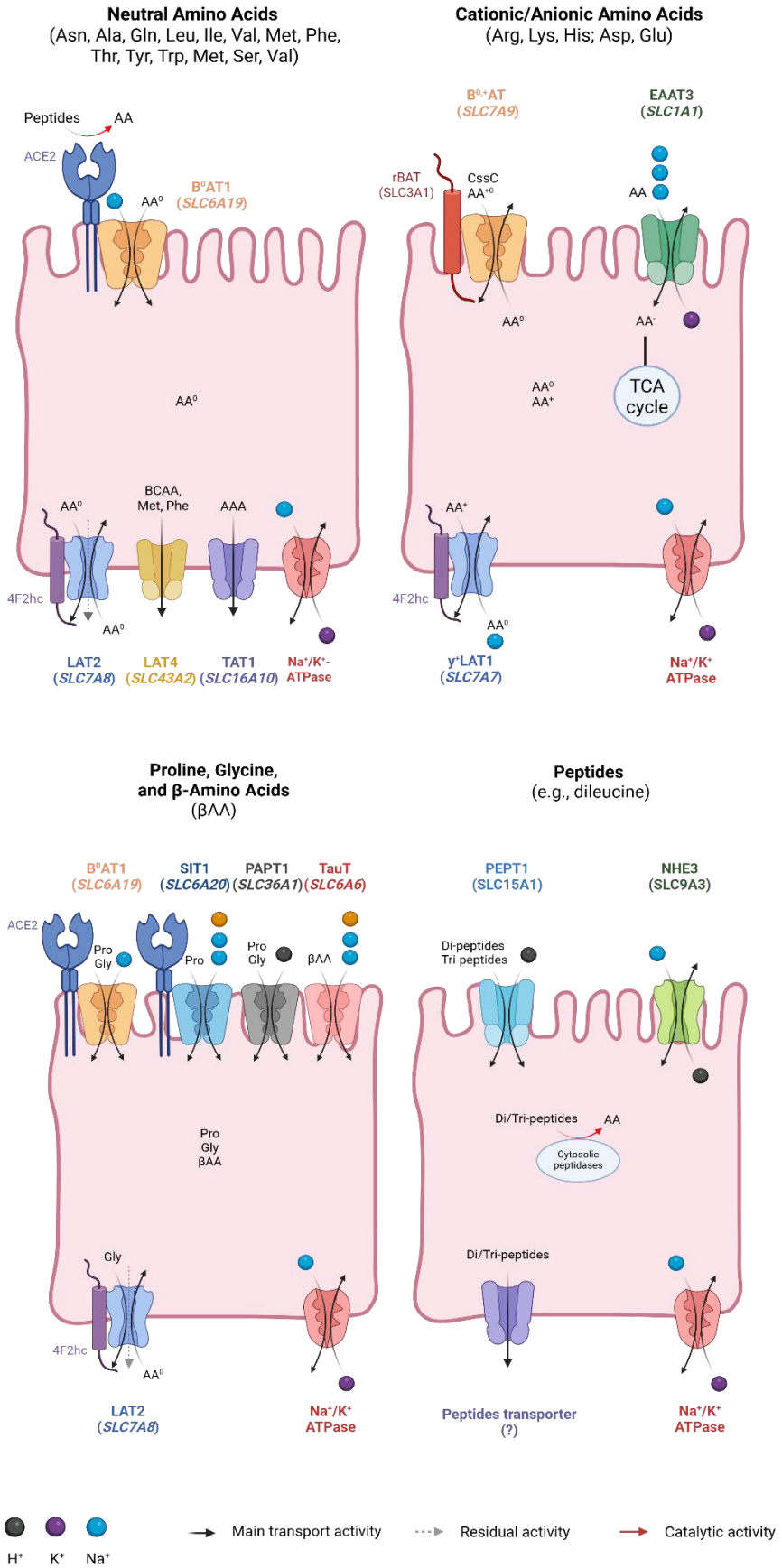

Figure 3. Schematic overview of intestinal amino acid and peptide transport systems. Adapted from Bröer, 2023, and Miner-Williams et al., 2014. Created in BioRender.

Importantly, not all absorbed dietary amino acids become systemically available due to first-pass splanchnic extraction (**Figure 2**) (Loveday, 2023). Using intrinsically labelled casein to quantify dietary amino acid appearance in vivo, ingestion of 20 g casein resulted in only ~50% of protein-derived phenylalanine being released into the circulation over a 5-h postprandial period. The remaining fraction was interpreted as being retained within the splanchnic region (Gorissen et al., 2020). This illustrates that systemic amino acid availability reflects not only the ingested dose and digestion/absorption kinetics, but also the extent of first-pass utilization by splanchnic tissues before amino acids reach peripheral circulation.

3. Protein source, food matrix, and postprandial anabolic responses

Postprandial aminoacidaemia and the anabolic response are determined not only by the quantity of protein ingested, but also by the protein source, its indispensable amino acid composition, digestibility, and the food matrix in which it is consumed. In general, animal-derived proteins produce a greater and/or more rapid rise in circulating essential amino acids, particularly leucine, than many plant-derived proteins, which has often been associated with a more robust stimulation of muscle protein synthesis (MPS) (P. J. M. Pinckaers et al., 2021). This difference is commonly attributed to the lower digestibility of many plant proteins and their lower content of essential amino acids, including leucine, lysine, and methionine (P. J. M. Pinckaers et al., 2021). However, the anabolic inferiority of plant proteins should not be considered absolute. Recent human studies show that some plant-derived proteins or plant-protein blends can stimulate postprandial MPS to a similar extent as milk protein when provided in sufficient amounts, even when the aminoacidaemic response remains lower (Pinckaers et al., 2023). Likewise, leucine fortification of plant protein can increase MPS to levels comparable to whey protein, indicating that amino acid composition, particularly leucine availability, could be a major determinant of the anabolic response (Lim et al., 2024).

Beyond protein source itself, the food matrix can further influence the postprandial response to feeding (Elliot et al., 2006; Pinckaers et al., 2021). The United States Department of Agriculture (USDA) defines the food matrix as the nutrient and non-nutrient components of food and their molecular relationships to one another. In this context of protein metabolism, this concept is important because whole foods

cannot be reduced to protein content alone. Structural properties of the matrix, processing characteristics, and interactions with co-ingested nutrients can all influence digestion, absorption, and the temporal pattern of postprandial amino acid availability (J. M. Aguilera, 2025; Fouillet et al., 2009; Meade et al., 2005). Therefore, food-matrix effects may alter postprandial aminoacidaemia. However, they may also influence the anabolic response more directly, such that differences in MPS are not always fully explained by differences in circulating amino acid availability alone (van Vliet et al., 2017).

This has been illustrated in several human studies. Following resistance exercise, whole milk promoted greater net uptake of some amino acids across the leg than fat-free milk, despite the latter providing more protein (Elliot et al., 2006). This result suggest that the matrix of whole milk may have enhanced the anabolic response. Likewise, ingestion of whole eggs stimulated post-exercise MPS more than an isonitrogenous amount of egg whites. This was observed despite broadly similar postprandial amino acid availability, indicating that non-protein components of the whole-food matrix can influence the anabolic response beyond amino acid availability alone (van Vliet et al., 2017).

This concept is also relevant to meat-based whole foods. High-fat pork blunted post-exercise myofibrillar protein synthesis compared with low-fat pork, despite matched protein and essential amino acid intake. In parallel, postprandial plasma essential amino acid and leucine concentrations were generally higher after low-fat pork than after high-fat pork (Zupančič et al., 2025).

Thus, the postprandial response to dietary protein (postprandial amino acids availability and MPS) reflects more than protein dose alone. It depends on the interaction between protein quality, digestion and absorption kinetics, and food matrix characteristics. This point is particularly important when interpreting anabolic responses to whole-food protein sources.

4. Postprandial amino acid availability stimulates MPS

Skeletal muscle is highly responsive to postprandial amino acid availability (Church et al., 2020). Following protein ingestion, dietary amino acids are released during digestion, absorbed into the circulation, and subsequently taken up by skeletal muscle, where they serve both as substrates for de novo protein synthesis and as signaling molecules regulating anabolic pathways (Groen et al., 2015b). Stable isotope tracer studies in humans have demonstrated that dietary protein-derived amino acids are directly incorporated into newly synthesized myofibrillar proteins during the postprandial period, confirming that ingested protein contributes directly to muscle remodeling and accretion (Holwerda et al., 2019).

The postprandial stimulation of MPS is therefore closely linked to the magnitude and duration of aminoacidaemia, particularly the availability of essential amino acids (Church et al., 2020; Trommelen et al., 2023). Among these, leucine appears to play a central signaling role through activation of anabolic pathways such as the mechanistic target of rapamycin complex 1 (mTORC1), although the anabolic response to feeding cannot be attributed to leucine alone, as sustained protein synthesis ultimately requires the coordinated availability of all indispensable amino acids (Ely et al., 2023). In this context, postprandial aminoacidaemia should be viewed not simply as a reflection of protein intake, but as a key determinant of the extent to which feeding can stimulate MPS.

Earlier dose-response studies suggested that ingestion of approximately 20-25 g of high-quality protein is sufficient to maximize post-exercise MPS in healthy young adults over the relatively short postprandial periods typically assessed, whereas larger doses are associated with greater amino acid oxidation and ureagenesis (Moore et al., 2009; Witard et al., 2014). However, more recent evidence indicates that this concept may be overly simplistic (**Figure 4**). When larger protein boluses are ingested (e.g. 100g of proteins) and the postprandial period is assessed over a longer duration, both the magnitude and duration of amino acid availability increase, resulting in greater whole-body net protein balance and continued incorporation of dietary amino acids into muscle protein (Trommelen et al., 2023). Furthermore, protein ingestion appears to exert only a negligible effect on whole-body protein breakdown rates (Trommelen et al., 2023).

This earlier concept that larger protein boluses do not further increase muscle protein synthesis has been based on metabolic tracer studies in vivo in humans confined to relatively short postprandial assessment periods (≤6 h) (Trommelen et al., 2023). Such a short time frame is likely insufficient to capture the full anabolic response to larger protein doses, as complete digestion and amino acid absorption require a more prolonged duration (Trommelen et al., 2023). Furthermore, intrinsically labeled proteins were not employed in those earlier studies, preventing direct tracking of dietary amino acid incorporation into muscle protein and potentially leading to an underestimation of the true anabolic potential of larger protein boluses. Indeed, by combining both a prolonged assessment period and the use of intrinsically labeled proteins, it was demonstrated that the ingestion of a large protein bolus requires an extended postprandial period to allow complete protein digestion, amino acid absorption, exogenous-protein-derived amino acid release into the circulation, and subsequent incorporation into tissue protein pools, with the duration of the postprandial state potentially exceeding 12 h (Trommelen et al., 2023).

Consequently, the anabolic response to feeding may be less limited than previously thought, and further research is warranted to determine whether increasing protein intake beyond a certain point result in a greater proportion of amino acids being directed toward oxidation.

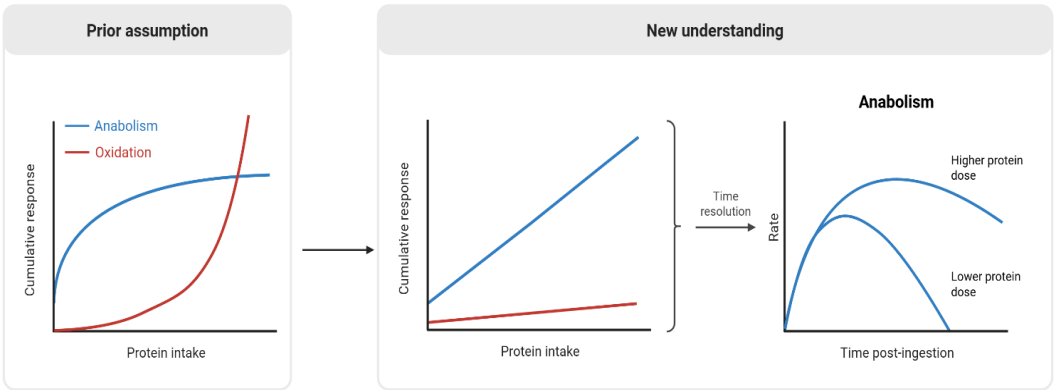

Figure 4. Conceptual model of the traditional and revised views of the anabolic response to protein ingestion. The traditional model suggests that anabolic responses plateau at moderate protein doses, with additional protein largely directed toward oxidation. The revised model proposes that higher protein doses may sustain anabolic processes over a longer postprandial period, thereby increasing the cumulative anabolic response. Adapted from Trommelen et al., 2023. Created in Biorender.

5. mTORC1 as a central integrator of nutrient and mechanical signals

At the molecular level, mTORC1 is a central regulator of translation that integrates nutrient, contractile signal, growth factor, and energy-related signals to control skeletal muscle protein synthesis (**Figure 5**) (Zheng et al., 2016). mTORC1 is composed of mTOR together with regulatory-associated protein of mTOR (Raptor), mLST8/GβL, PRAS40, and Deptor, and its best-characterized downstream targets in the control of translation are p70S6K/S6K1 and 4E-BP1. Phosphorylation of S6K1 regulates downstream effectors such as rpS6 and eEF2 kinase, whereas phosphorylation of 4E-BP1 releases eIF4E and promotes formation of the translation initiation complex (Drummond et al., 2009; Zheng et al., 2016). mTORC1 has also been implicated in ribosomal biogenesis (Lim et al., 2022). Amino acids provide a critical activation input to this pathway. Amino acid deprivation markedly suppresses phosphorylation of S6K and 4E-BP1, and this reduction cannot be fully compensated by growth factor or cellular energy signals alone, indicating that amino acids are indispensable for full mTORC1-dependent translational control (Drummond et al., 2009). Upstream of mTORC1, amino acid signaling is mediated in part through the Rag GTPases, which regulate lysosomal recruitment of mTORC1, and through Rheb, which contributes to its activation state (Zheng et al., 2016). Multiple amino acid sensing systems have been proposed to converge on this machinery, indicating that amino acid sensing is spatially distributed rather than mediated by a single sensor (Zheng et al., 2016).

Resistance exercise provides an additional anabolic stimulus through mechanotransduction (**Figure 5**), whereby mechanical loading is translated into intracellular signals that ultimately regulate skeletal muscle mass (Lim et al., 2022). In this context, resistance exercise is considered the most potent nonpharmacological external stimulus for activating the internal biological processes that support skeletal muscle hypertrophy. Evidence from human studies further indicates that acute resistance exercise increases muscle protein synthesis during recovery and is associated with activation of mTORC1-related signaling, particularly increased phosphorylation of p70S6K/S6K1 (Lim et al., 2022; Song et al., 2017). Resistance exercise also enhances the anabolic sensitivity of skeletal muscle to amino acids. Prior exercise increases the utilization of dietary amino acids for de novo muscle protein synthesis, and this sensitizing effect can persist well

beyond the immediate recovery window (D'Hulst et al., 2022). More recent mechanistic work further suggests that leucine sensitivity may remain elevated for up to 48 h after exercise, together with increased leucine uptake, higher expression of amino acid transport- and sensing-related proteins such as LARS, and activation of ATF4-dependent pathways involved in amino acid handling (D'Hulst et al., 2022). Taken together, these findings support the view that mTORC1 should not be considered merely a nutrient-sensitive pathway, but rather a central integrative hub through which amino acid availability and mechanical loading jointly regulate skeletal muscle anabolism.

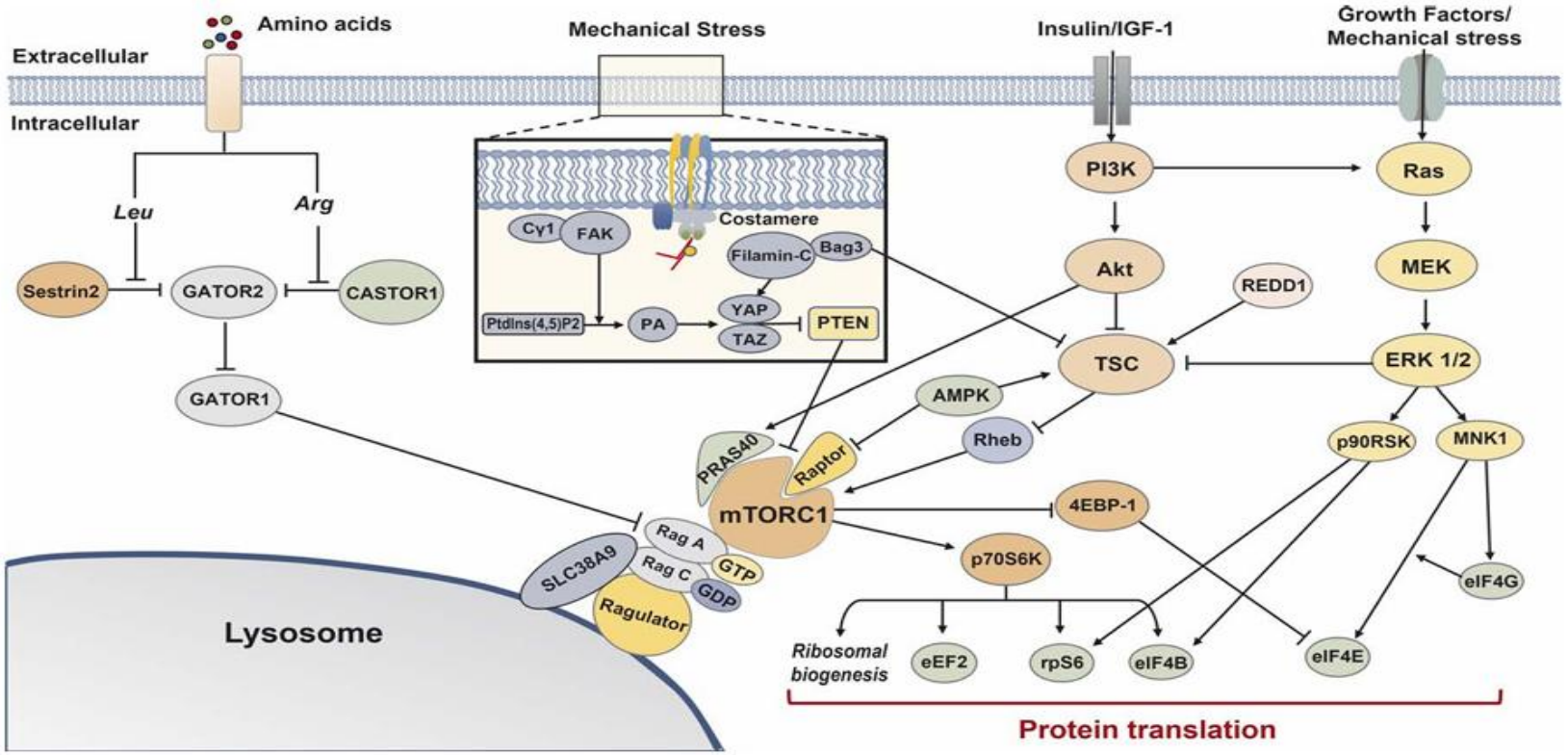

Figure 5. Schematic overview of the major anabolic signaling pathways controlling skeletal muscle protein synthesis. Multiple upstream signals converge on mTORC1 to regulate protein translation. Cytosolic leucine and arginine activate mTORC1 via the GATOR2-GATOR1-Rag signaling pathway. Mechanical stress and insulin/IGF-1 signal through distinct cascades involving the HIPPO pathway (YAP and TAZ) and PI3K-Akt-TSC, respectively. Growth factors further converge on mTORC1 via the Ras-MEK-ERK1/2 pathway. Once activated, mTORC1 promotes protein synthesis through downstream targets including p70S6K and 4EBP-1. Akt, protein kinase B; ERK ½, extracellular signal-related kinase ½; IGF-1, insulin-like growth factor 1; Leu, leucine; MEK, mitogen-activated protein kinase; mTORC1, mechanistic target of rapamycin complex 1; p70S6K, p70 S6 kinase 1; PI3K, phosphoinositide 3-kinase; TAZ, transcriptional coactivator with PDZ-binding motif; TSC, tuberous sclerosis complex; YAP, Yes-associated protein 1; 4EBP-1, eukaryotic translation initiation factor 4E-binding protein 1. From Lim et al., 2022.

6. Leucine as a key amino acid signal

Among the amino acids, leucine has received particular attention because of its prominent role in the nutritional regulation of skeletal muscle protein synthesis. Beyond serving as a substrate for protein accretion, leucine acts as a potent anabolic signal that contributes to the activation of mTORC1 and its downstream translational machinery (**Figure 5**). As a result, the leucine content of an ingested protein source is an important determinant of its anabolic potential after feeding (Ely et al., 2023; Wolfson et al., 2016).

One of the best-supported mechanisms by which leucine regulates MPS involves intracellular amino acid sensing upstream of mTORC1. In particular, leucine binding has been shown to disrupt the inhibitory interaction between Sestrin2 and GATOR2 (**Figure 5**), thereby promoting mTORC1 activation (Wolfson et al., 2016; Zheng et al., 2016). This framework supports the view that leucine availability is not simply permissive for protein synthesis but actively participates in the signaling events that regulate translation initiation. For intracellular sensing to operate, leucine must first be delivered into the relevant cellular compartment. Leucine transport is strongly influenced by amino acid transporters such as LAT1 (SLC7A5), which functions in complex with SLC3A2/4F2hc and contributes to the regulation of intracellular amino acid availability (Wolfson et al., 2016; Zheng et al., 2016).

In addition to intracellular sensing, emerging evidence supports the existence of extracellular or membrane-initiated amino acid sensing (Wauson et al., 2012). The G protein-coupled taste receptor T1R1/T1R3 has been identified as a sensor of amino acid availability, and reduced expression of this receptor impairs amino acid signaling to mTORC1, alters mTORC1 localization, blocks translation initiation, and promotes autophagy in the studied model systems (Wauson et al., 2012; Zheng et al., 2016). Accordingly, amino acid sensing appears to involve both intracellular sensors and plasma membrane-associated mechanisms, including the possibility that some transporters act as nutrient “transceptors” in a context-dependent manner (Zheng et al., 2016).

The physiological relevance of leucine is also supported by human feeding studies. Acute leucine supplementation can stimulate MPS, and leucine enrichment of lower-protein feeds has been shown to augment post-exercise MPS in both young

and older men (Atherton et al., 2017; Ely et al., 2023). However, leucine should not be viewed as acting independently of the other indispensable amino acids. Although leucine can trigger anabolic signaling, sustained protein synthesis still requires adequate availability of the full complement of essential amino acids, suggesting that leucine alone is unlikely to support prolonged muscle anabolism if other substrates become limiting (Ely et al., 2023). More generally, recent review conclude that leucine enrichment can improve the anabolic potential of suboptimal protein doses, supporting its potential value as a fortification strategy (Ely et al., 2023).

7. Dileucine

Beyond amino acids, dietary proteins also contain small peptides generated during digestion, including dipeptides and tripeptides, which may enter the circulation intact. Specifically, di- and tripeptides are transported across the intestinal epithelium via Pept1 (Miner-Williams et al., 2014). Importantly, the uptake capacity of Pept1 is high - the rate of absorption through PepT1 is estimated to be 70–80% faster than for free amino acids through classical AA transporters. Once inside the enterocyte, most di- and tripeptides are hydrolyzed by cytosolic peptidases, but some evidence suggests that a fraction might escape hydrolysis and cross the basolateral membrane intact, potentially reaching the circulation (Miner-Williams et al., 2014). Given the established anabolic potency of leucine, the leucine-containing dipeptide dileucine (Leu–Leu) has received significant interest, particularly for its potential to bypass amino acid transporters via PEPT1 (Miner-Williams et al., 2014). Indeed, it has recently been shown that the ingestion of 2 grams of dileucine significantly increased cumulative myofibrillar protein synthesis over 3 hours, whereas 2 grams of free leucine did not produce a significant increase (Paulussen et al., 2021). The dileucine condition produced a significantly greater plasma dileucine AUC than leucine, whereas plasma leucine increased similarly after both conditions. The authors also report a trend for a faster time-to-peak for the dipeptide versus free leucine, consistent with more rapid absorption kinetics via peptide transport (PepT1). Consistent with these acute metabolic observations, a 10-week resistance training study reported greater improvements in lower-body strength and muscular endurance with daily supplementation of 2 g of

dileucine compared with leucine and placebo in resistance-trained males (Hagele et al., 2024). This indicates that short-chain leucine containing peptides may exert anabolic effects distinct from those of free amino acids.

A recent crossover trial examined postexercise anabolic responses to a dileucine-supplemented essential amino acid formulation (DIEAA: 2 g dileucine, 1 g leucine, 9.15 g total EAA) compared with leucine-matched BCAA and an isonitrogenous collagen hydrolysate control following a 60-min whole-body resistance exercise bout. Using exogenous leucine retention (leucine intake minus tracer-derived leucine oxidation) as a proxy for whole-body anabolism during the 6-h recovery period, DIEAA and BCAA supported greater whole-body anabolism than collagen, whereas DIEAA did not differ from BCAA (J. A. Aguilera et al., 2025). Interestingly, postprandial serum dileucine increased not only after DIEAA but also after BCAA, despite the absence of dileucine in the BCAA formulation, suggesting that dileucine may arise, at least in part, from endogenous formation in response to BCAA/leucine ingestion. In contrast to prior work reporting greater stimulation of myofibrillar protein synthesis after ingestion of dileucine compared with free leucine (Paulussen et al., 2021), this postexercise study observed no significant difference in a whole-body anabolism proxy between DIEAA and leucine-matched BCAA.

The mechanism underpinning the superior anabolic action of dileucine relative to leucine remains unclear. In fact, dileucine ingestion does not appear to differentially modulate canonical mTORC1-related signaling compared with leucine when assessed via phosphorylation of downstream targets such as p70S6K and rpS6 (Paulussen et al., 2021). In other words, both conditions exhibit evidence of anabolic signaling activation, yet these conventional signaling readouts do not explain the greater cumulative increase in muscle protein synthesis observed with dileucine. This dissociation suggests that additional regulatory processes, beyond the magnitude of phosphorylation of canonical mTORC1 targets at the sampled time points, may contribute to dileucine's anabolic effect. However, hypothesized pathways may involve uptake via a putative skeletal muscle dipeptide transporter, extracellular hydrolysis prior to standard leucine entry via LAT1 (Poncet et al., 2014), or direct detection by an unidentified extracellular sensor. The previous findings showed that dileucine ingestion causes partial hydrolysis to leucine and a

rapid rise in plasma dileucine without altering muscle intracellular concentrations (Paulussen et al., 2021). Hence, it could be speculated that the superior anabolic properties of dileucine may relate from its ability to better signal the utilization of circulating essential amino acids. Given that protein ingestion acts as a primary most anabolic stimulus for skeletal muscle within a healthy-eating pattern, a critical next step in defining the contribution of dileucine to dietary protein-mediated anabolism is determining the extent to which whole food ingestion elevates postprandial dileucine concentrations.

8. Aims and hypotheses

The purpose of this study was to determine the influence of ingesting whole protein foods on modulating the postprandial rise in plasma dileucine concentrations. Based on results from isolated protein sources (Morifuji et al., 2010; Paulussen et al., 2021), we hypothesized that whole food ingestion would elevate postprandial plasma dileucine concentrations compared to crystalline amino acid enriched mixtures or carbohydrate controls. Regarding food matrix effects, we further hypothesized that the more rapidly digested low-fat meat would elicit higher peak dileucine concentrations compared to high-fat ground meat or a plant-based meal of beans and rice. Lastly, we hypothesized that the magnitude of the plasma dileucine response would be positively associated with the stimulation of myofibrillar protein synthesis.

II. METHODS

This study is a secondary analysis of bio-banked samples obtained from two independent randomized controlled trials registered at ClinicalTrials.gov (NCT05876299 and NCT06781723). The work presented in this thesis was submitted for publication to *Molecular Nutrition & Food Research* (Wiley) and was under review at the time of writing. The primary analytical contribution of this present work was the quantification of postprandial plasma dileucine and leucine concentrations by LC-MS/MS. Other experimental data, including FSR measurements and nutritional analyses, were obtained from the parent studies.

1. Participants and ethical approval

Participants were healthy adults (n=26; males = 21 and females = 5; mean ± SD: age: 25 ± 4.3 y; BMI: 25 ± 2 $kg \cdot m^{-2}$). Table 1 details participants' characteristics. Data were collected in the clinical research laboratory at the University of Illinois Urbana–Champaign from July 2023 to April 2025. Female participants completed trials during the assumed follicular phase. All participants provided written informed consent. The study procedures were approved by the University of Illinois Urbana–Champaign Institutional Review Board (IRB #23257 and #240582), conducted in accordance with the Declaration of Helsinki.

Table 1.
Participants characteristics

Variable	Mean ± SD
n	26 (21 M / 5 F)
Age (yr)	25 ± 4.3
Body mass (kg)	75 ± 10.9
BMI ($kg \cdot m^{-2}$)	25 ± 2.3
LBM (kg)	54 ± 11.1
Body fat (%)	28 ± 7.5
Systolic blood pressure, mmHg	115 ± 12.6
Diastolic blood pressure, mmHg	68 ± 9.9
Fasting plasma glucose (mg/dl)	89.5 ± 7.3
EI (kcal/d)	1792.1 ± 463.5
Protein intake (g/d)	103.5 ± 38.8
Carbohydrate intake (g/d)	165.1 ± 56.1
Fat intake (g/d)	76.2 ± 27.6
IPAQ	3668 ± 3371.1
GSLTPAQ	43 ± 20.4

Data are mean ± SD; SD, standard deviation; BMI, Body Mass Index; LBM, Lean Body Mass; EI, Energy Intake; GSLTPAQ, Godin-Shepherd Leisure-Time Physical Activity Questionnaire; IPAQ, International Physical Activity Questionnaire.

2. Experimental design

In the first parent study, a randomized semi-crossover design was employed, whereby participants completed two of three experimental conditions. In the second parent study, a randomized crossover design was used, with participants completing both conditions.

3. Preliminary testing

In both parent trials, baseline testing was performed approximately one week prior to the first experimental trial in the morning following an overnight fast. Measurements included anthropometrics (height and body mass), resting blood pressure assessed using an automated device (HEM-907XL; Omron Healthcare Inc., Lake Forest, IL), body composition determined by dual-energy X-ray absorptiometry (DXA; QDR 4500A; Hologic, Marlborough, MA), resting metabolic rate assessed according to established procedures (ParvoMedics True One Metabolic System, Sandy, Utah, USA) (Jagim et al., 2023), and maximal strength assessed via 10-repetition maximum testing for the leg press and leg extension (Askow et al., 2025).

Treatment allocation in all trials was determined using computer-generated randomization with block randomization to ensure balanced distribution across conditions.

4. Experimental trials

All trials were conducted at 7am after an overnight fast of at least 10 h. Upon arrival, an intravenous catheter was inserted into an antecubital vein and an arterialized blood sample. Subsequently, a primed-constant infusion of L-[ring-^{13}C6]phenylalanine (prime: 2 μmol/kg; 0.05 μmol/kg/min) was initiated and maintained throughout the trial. A second intravenous catheter was placed in a contralateral arm for repeated blood sampling and remained patent by a 0.9% saline drip. Serial blood samples were collected before and after treatment ingestion to assess plasma dileucine and leucine concentrations, and muscle biopsies were obtained to determine myofibrillar fractional synthesis rate (FSR). Muscle samples

were freed from any blood, fat, and visible connective tissue and immediately frozen in liquid nitrogen before storage at −80°C until further analysis. Muscle biopsies were performed with a Bergström needle obtained from the *vastus lateralis* under local anesthetic using standard procedures. After collection of the resting muscle biopsy sample at $t = -30$ for both trials, the participants performed a bout of resistance exercise. The acute resistance exercise bout consisted of 4 sets of 10 repetitions at 90% of 10RM for both the leg press and leg extension exercises (Zupančič et al., 2025). Immediately after completion of the exercise bout, participants consumed one of the food conditions: high fat pork (HFP), low fat pork (LFP), carbohydrate control (CHO), complementary protein (COMP), or an isolated nutrition condition (ISO).

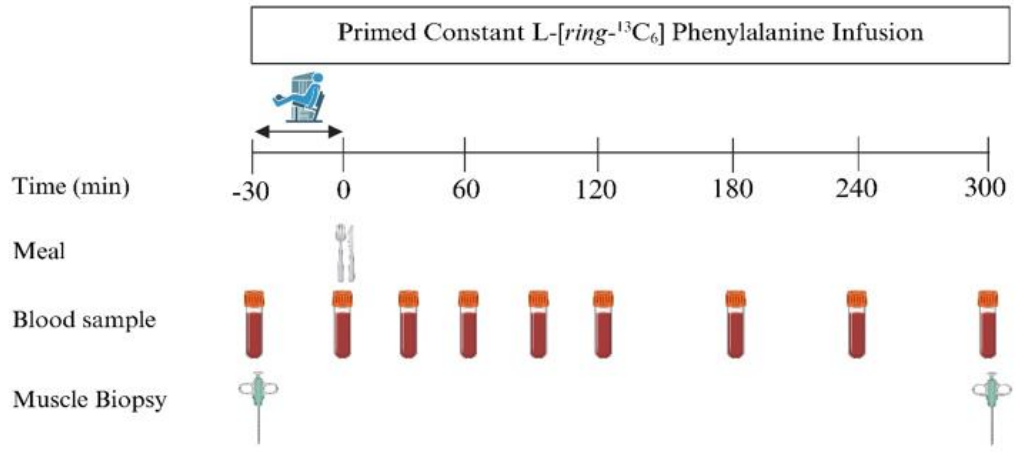

Figure 6. Schematic of the experimental design. Created in BioRender.

5. Treatments and food preparation

The treatment conditions consisted of animal-based (ground pork), plant-based whole foods (beans and rice), an isolated nutrient beverage, and a carbohydrate control. The ground pork was either high fat (HFP) or low-fat (LFP) and was oven-cooked at 200 °C until an internal temperature of 75 °C was reached. Long-grain jasmine rice (Iberia Foods, Miami, FL, USA) and canned black beans (Bush's, Knoxville, TN, USA) were used for the complementary plant-based protein condition (COMP). The carbohydrate control (CHO) was energy-matched to HFP

using a commercially available carbohydrate beverage (Gatorade®; PepsiCo, Harrison, NY, USA) prepared in 600 mL of water on the day of the trial. The isolated nutrition condition (ISO) was formulated to match the amino acid profile, carbohydrate, fat, and fiber content of the COMP condition. ISO ingredients included crystalline amino acids (Ajinomoto, Raleigh, NC, USA), maltodextrin (Nutricost, Vineyard, UT, USA), cellulose (Vital Nutrient, Middletown, CT, USA), pectin (Hoosier Hill Farm Fort Wayne, IN, USA), and soy oil (Arawana Brand, Shanghai, China). The ISO mixture was dissolved in ~250 mL of water prior to ingestion. Participants consumed all treatments as quickly and comfortably as possible. Macronutrient and amino acid composition of uncooked pork, rice, and black beans were determined using standard AOAC methods (Latimer, 2023). The energy content and macronutrient composition of the five experimental treatments are shown in **Table 2**.

Table 2. Nutritional content of ISO, COMP, CHO, HFP and LFP

	ISO	COMP	CHO	HFP	LFP
Amount (g)	143	251	76.6	109.6	90.2
Crude Protein (g)	20	20	/	20	20
Carbohydrates (g)	114	114	73.3	/	/
Crude Fiber (g)	2.1	2.1			
Crude Fat (g)	0.83	0.83	/	20.6	4.4
Energy (kcal / kJ)	548/ 2293	548/ 2293	266/1113	266 / 1113	120 / 502
Amino acid content (g)					
EAAs					
Histidine	0.50 (63%)	0.50 (63%)	/	0.73	0.74
Isoleucine	0.94 (58%)	0.94 (58%)	/	0.99	0.92
Leucine	1.62 (52%)	1.62 (52%)	/	1.54	1.46
Lysine	1.05 (44%)	1.05 (44%)	/	1.75	1.68
Methionine	0.35 (44%)	0.35 (44%)	/	0.52	0.51
Phenylalanine	1.11 (55%)	1.11 (55%)	/	0.79	0.74
Threonine	0.73 (61%)	0.73 (61%)	/	0.87	0.85
Tryptophan	0.14 (42%)	0.14 (42%)	/	0.27	0.25
Valine	1.14 (55%)	1.14 (55%)	/	1.03	0.96
∑ EAAs	7.57	7.57	/	8.53	8.14
NEAAs					
Alanine	0.93	0.93	/	1.08	0.99
Arginine	1.25	1.25	/	1.24	1.12
Aspartic Acid	2.00	2.00	/	1.79	1.72
Cysteine	0.32	0.32	/	0.19	0.18
Glutamic Acid	3.29	3.29	/	2.56	2.64
Glycine	0.79	0.79	/	1.08	0.86
Hydroxylysine	0.03	0.03	/	0	0
Hydroxyproline	0.00	0.00	/	0.19	0.1
Lanthionine	0.00	0.00	/	0	0
Ornithine	0.00	0.00	/	0.05	0.04
Proline	0.83	0.83	/	0.85	0.72
Serine	0.96	0.96	/	0.64	0.64
Taurine	0.24	0.24	/	0.06	0.04
Tyrosine	0.55	0.55	/	0.88	0.88
∑NEAAs	11.21	11.21	/	10.66	10.01
Leucine by total AA (%)	8.64	8.64	/	8.06	8.07
∑ BCAAs	3.70	3.70	/	3.58	3.35
∑Total	18.78	18.78	/	19.19	18.15

AA, amino acids; CHO, carbohydrate beverage; COMP, complementary pairing of rice and black beans; BCAAs, branch chain amino acids; EAAs, essential amino acids; HFP, high-fat ground pork; ISO, isolated mixture of crystallized amino acids, maltodextrin, cellulose, pectin, and soy oil; LFP, low-fat ground pork; NEAAs, non-essential amino acids.

6. Plasma and muscle analyses

Plasma samples were thawed, and 2 µL aliquots were deproteinized with 193 µL of a ddH_2O:acetonitrile:isopropyl alcohol mixture (2:3:3, v/v/v) containing 5 µL of DL-p-chlorophenylalanine (0.01 $mg \cdot mL^{-1}$) as an internal standard. Samples were centrifuged at 20,817 g for 10 min at 4 °C, after which 100 µL of supernatant was transferred to a new tube, dried under vacuum, and reconstituted in 100 µL of 0.1% formic acid for analysis. Plasma amino acid concentrations (limit of quantification: 2–2000 $ng \cdot mL^{-1}$) were determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a 5500 QTRAP mass spectrometer (SCIEX, Framingham, MA, USA) coupled to a 1290 Infinity II LC system (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a Hypersil GOLD C18 column (2.1 × 150 mm, 1.9 µm; Thermo Fisher Scientific, Waltham, MA, USA) using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a flow rate of 0.45 $mL \cdot min^{-1}$. The autosampler and column temperatures were maintained at 4 °C and 50 °C, respectively, with an injection volume of 0.5 µL. Mass spectra were acquired in positive electrospray ionization mode, and calibration curves were generated over a concentration range of 0.05–5 $\mu g \cdot mL^{-1}$. Data acquisition and analysis were performed using Analyst software (version 1.7).

Myofibrillar protein-enriched fractions were extracted from ~50 mg of wet muscle using an ice-cold lysis buffer (Pierce® RIPA Buffer; ThermoFisher Scientific, Waltham, MA, USA; 10 $\mu L \cdot mg^{-1}$). Myofibrillar proteins were isolated by differential centrifugation and analyzed for ^{13}C phenylalanine enrichments as described previously (Salvador et al., 2020).

7. Reference Dileucine Data

For retrospective comparison, we used data from the dileucine arm of a prior independent trial (NCT03952884) involving ten healthy men (Paulussen et al., 2021). In this referenced study, participants consumed 2 g of dileucine and plasma concentrations were assessed over 180 min. Due to inter-study variations in analytical platforms that influence absolute quantification, we normalized the dataset by analyzing only the incremental area under the curve (iAUC). This

approach allowed for a reference assessment of total exposure relative to the whole-food conditions while mitigating potential baseline or calibration offsets between assays.

8. Calculations

Net incremental area under the curve (iAUC) for plasma dileucine and leucine concentrations was assessed over 0-60, 0-180 and 0-300 min of the postprandial period using the trapezoidal method, with baseline values defined at $t = 0$ min. Peak concentration (C*max*) were calculated for all plasma concentration time curves. Fractional synthesis rates (FSR; $\%\cdot h^{-1}$), collated from the parent studies, used for correlation analyses between dileucine and leucine C*max* were calculated using standard precursor–product equations, whereby the change in myofibrillar protein–bound L-[ring-$^{13}C_6$]phenylalanine enrichment was divided by the average enrichment of the plasma precursor pool over time, as previously described (Trommelen et al., 2023).

9. Statistical analyses

All data are presented as mean ± standard deviation (SD) and/or mean difference (95% confidence interval [CI]). Differences in blood variables and myofibrillar synthesis rates were analyzed using linear mixed-effects models with time and condition as fixed factors and the participant-specific random intercept. Plasma dileucine and leucine iAUC were calculated for the postprandial period, and differences were determined using linear mixed-effects models with the condition as a fixed factor and the participant-specific random intercept. Bonferroni's post hoc test was conducted when a significant main effect or interaction was identified. Associations between changes in myofibrillar protein synthesis rates and plasma dileucine C*max* were examined using Pearson's correlation coefficients. The level of statistical significance was set at $P < 0.05$ for all analyses. Normality was assessed via visual inspection of normal Q-Q plots and skewness/kurtosis values. Data were $\log_{10}$-transformed prior to correlation analysis to improve normality and reduce heteroscedasticity. Statistical analyses were performed on the transformed values. Missing data were accommodated within the linear mixed-effects modeling

framework. All analyses were performed using IBM SPSS Statistics for Windows (Version 29.0.2.0; IBM Corporation, Armonk, NY, USA).

III. RESULTS

1. Plasma dileucine concentrations

Plasma dileucine concentrations were significantly increased above baseline from 90-120 min only in the LFP condition (P<0.001; **Figure 7A**). At these time points, dileucine concentrations in LFP were also significantly higher than in all other conditions (all $P < 0.05$). From 180 to 300 min, plasma dileucine concentrations returned to baseline and did not differ between conditions (all $P > 0.05$). Postprandial iAUC over 300 min was significantly greater in LFP compared to CHO ($P = 0.038$), with no differences observed between LFP, ISO, COMP, or HFP conditions (all $P > 0.05$; **Figure 7B**). Peak dileucine concentrations (C*max*) was significantly higher in LFP compared with ISO ($P = 0.029$) and CHO ($P = 0.016$), but did not differ from HFP ($P = 0.190$) or COMP ($P = 0.074$; **Table 3**).

The retrospective comparison with the dileucine condition (Paulussen et al., 2021) showed a significantly greater early postprandial iAUC (0–60 min) compared with all other conditions (all $P < 0.05$; **Figure 7C**). Over the 0-180 min period, however, dileucine was comparable to LFP and HFP ($P = 1.000$**),** with both dileucine and LFP demonstrating greater total net exposure than COMP or CHO ($P<0.05$; **Figure 7D**).

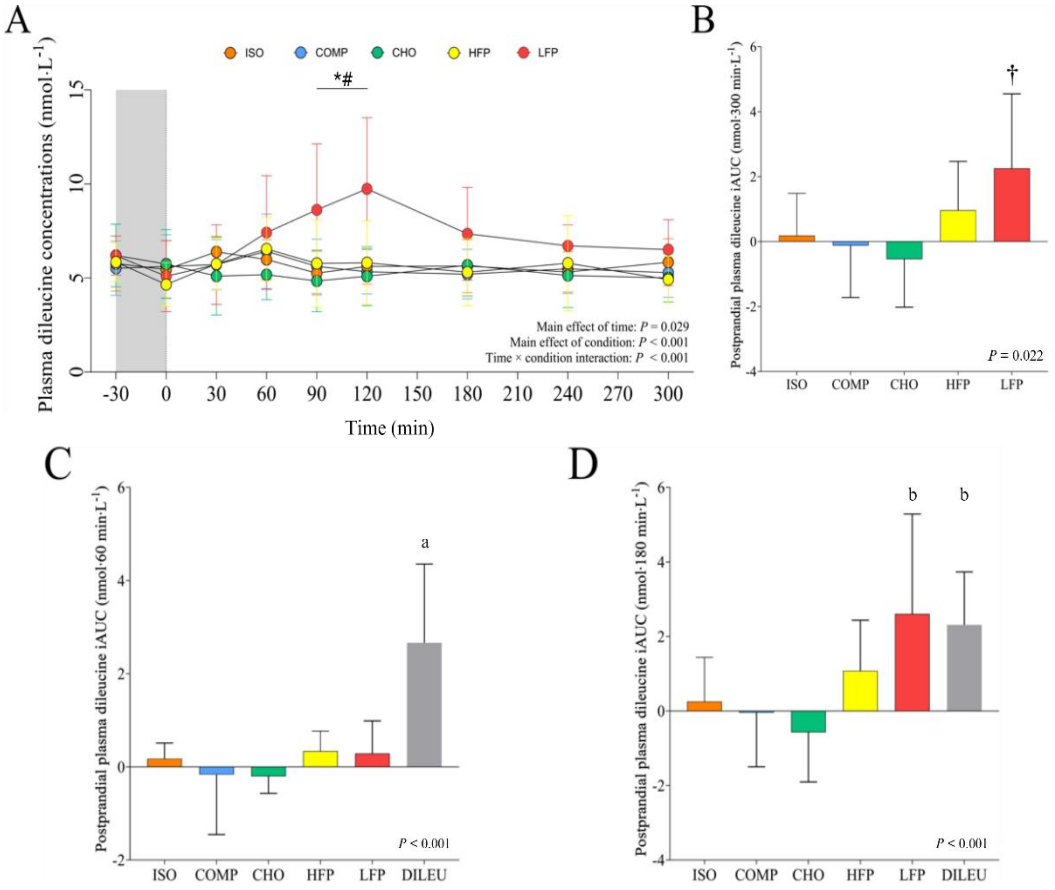

Figure 7. Plasma dileucine concentrations (nmol·L-1) before and after the ingestion of ISO, COMP, CHO, HFP, or LFP (A). Data are shown as means ± SD. iAUC plasma dileucine (B) concentrations at baseline to 300 min postprandial of ISO, COMP, CHO, HFP, and LFP. iAUC plasma dileucine (C) concentrations at baseline to 60 min early postprandial of ISO, COMP, CHO, HFP, LFP, and DILEU. iAUC plasma dileucine (D) concentrations at baseline to 180 min late postprandial of ISO, COMP, CHO, HFP, LFP, and DILEU. *Denotes significantly difference from basal (t = 0; P < 0.05). #Denotes a significant difference from LFP and other conditions (P < 0.05). †Denotes a significant difference between LFP and CHO (P < 0.05). [a] DILEU significantly (P < 0.05) different from other conditions. [b] Denotes significant differences from CHO and COMP. CHO, carbohydrate beverage; COMP, complementary pairing of rice and black beans; DILEU, dileucine beverage; HFP, high-fat ground pork; ISO, isolated mixture of crystallized amino acids, maltodextrin, cellulose, pectin, and soy oil; LFP, low-fat ground pork; SD, standard deviation.

Table 3. Postprandial plasma concentration maximums (C*max*)

	C*max*				
Variable	**ISO**	**COMP**	**CHO**	**HFP**	**LFP**
Dileucine ($nmol \cdot L^{-1}$)	6.7±0.9	7.07±1.5	6.4±1.7	7.5±2.1	10.3±3.5[bc]
Leucine ($\mu mol \cdot L^{-1}$)	185.7±23.6[a]	147±19.3	139±16.6	171.5±25.7	223.3±42.3*

Data are mean ± SD; *Significant (P < 0.05) difference between LFP, COMP, CHO, and HFP; [a] Significant (P < 0.05) difference between ISO and CHO; [b] Significant (P < 0.05) difference between LFP and ISO; [c] Significant (P < 0.05) difference between LFP and CHO; CHO, carbohydrate beverage; COMP, complementary pairing of rice and black beans; HFP, high-fat ground pork; ISO, isolated mixture of crystallized amino acids, maltodextrin, cellulose, pectin, and soy oil; LFP, low-fat ground pork; SD, standard deviation.

2. Plasma leucine concentrations

A significant time × condition interaction was observed for plasma leucine ($P < 0.001$). ISO elicited a rapid peak at 30 min ($P = 0.016$; **Figure 8A**), while LFP and HFP resulted in delayed elevations from 60-180 min and 90-120 min, respectively. LFP maintained a significantly higher plasma leucine concentrations than all other conditions during the 60-180 min postprandial window (P<0.05). In contrast, plasma leucine values fell below baseline in CHO, COMP, and ISO conditions ($P < 0.05$; **Figure 8A**). As such, LFP and HFP demonstrated greater 0-300 min total net exposure (iAUC) than other conditions (P<0.05). Peak leucine concentration (C*max*) was significantly higher in the LFP condition compared to COMP, CHO, and HFP (all $P < 0.05$), but did not differ from ISO ($P = 0.153$; **Table 3**).

In a retrospective analysis, early postprandial leucine exposure (iAUC 0–60 min) was significantly greater in the DILEU condition compared to all other conditions (all $P < 0.001$; **Figure 8C**). In addition, early net exposure (iAUC 0-60 min) in ISO and LFP was significantly greater than in CHO (both $P < 0.001$; **Figure 8C**). Over the 180 min period, LFP and DILEU resulted in the highest total leucine exposure (iAUC), significantly exceeding ISO, COMP, and CHO (all $P < 0.05$; **Figure 8D**). HFP was statistically similar to LFP and DILEU, similarly exceeding CHO ($P < 0.001$) and COMP ($P = 0.022$; **Figure 8D**).

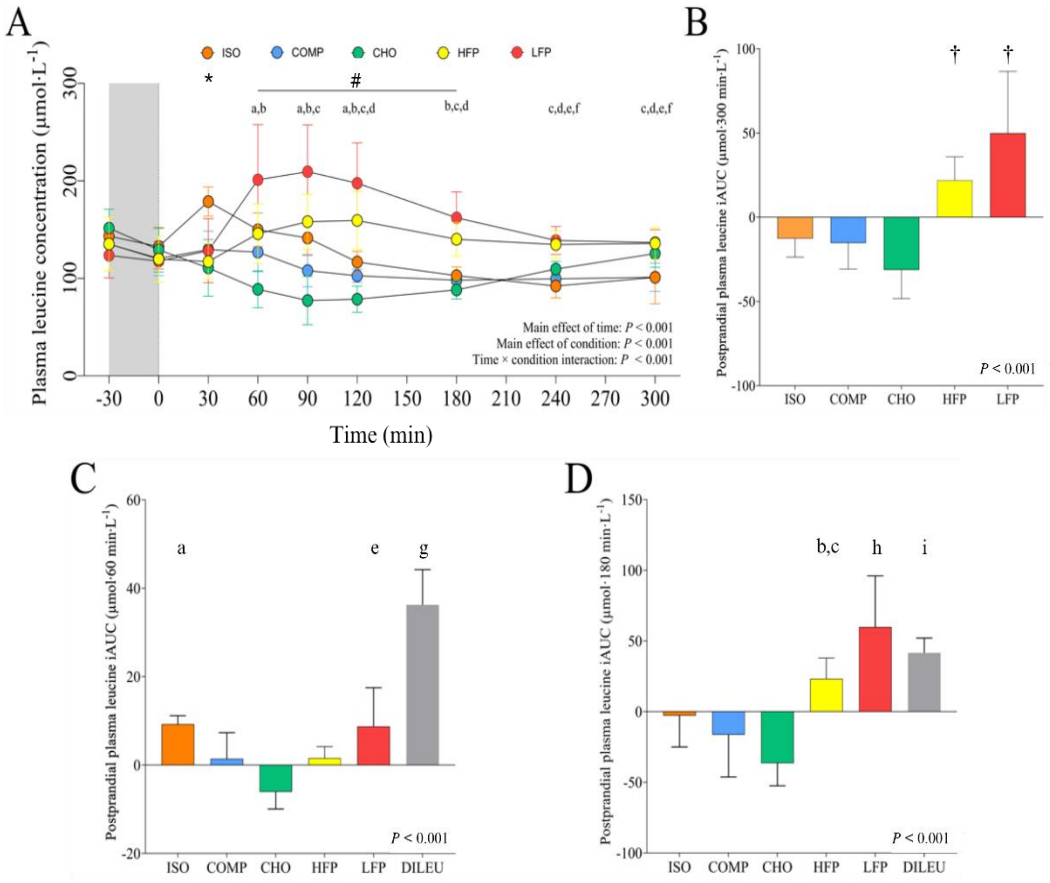

Figure 8. Plasma leucine concentrations (µmol·L-1) before and after the ingestion of ISO, COMP, CHO, HFP, or LFP (A). Data are shown as means ± SD. iAUC plasma leucine (B) concentrations at baseline to 300 min postprandial of ISO, COMP, CHO, HFP, and LFP. iAUC plasma leucine (C) concentrations at baseline to 60 min early postprandial of ISO, COMP, CHO, HFP, LFP, and DILEU. iAUC plasma leucine (D) concentrations at baseline to 180 min late postprandial of ISO, COMP, CHO, HFP, LFP, and DILEU. *Denotes significant difference from ISO and other conditions ($P < 0.05$). #Denotes significant difference from LFP and other conditions ($P < 0.05$). [a]Denotes significant difference from ISO to CHO. [b]Denotes significant difference from HFP and CHO ($P < 0.05$). [c]Denotes significant difference from HFP and COMP ($P < 0.05$). [d]Denotes significant difference from HFP and ISO ($P < 0.05$). [e]Denotes significant difference from LFP and ISO ($P < 0.05$). [f]Denotes significant difference from LFP and COMP ($P < 0.05$). [†]Significant difference from ISO, COMP and CHO ($P < 0.05$). [g]Denotes significant difference from DILEU and other conditions ($P < 0.05$). [h]Denotes significant difference from LFP and ISO, COMP and CHO ($P < 0.05$). [i]Denotes significant difference from DILEU and ISO, COMP and CHO ($P < 0.05$). CHO, carbohydrate beverage; COMP, complementary pairing of rice and black beans; DILEU, dileucine beverage; HFP, high-fat ground pork; ISO, isolated mixture of crystallized amino acids, maltodextrin, cellulose, pectin, and soy oil; LFP, low-fat ground pork; SD, standard deviation

3. Myofibrillar fractional synthetic rate

Myofibrillar FSR showed a significant main effect of time ($P < 0.001$), a significant main effect of condition ($P < 0.001$), and a significant time × condition interaction ($P = 0.009$). At basal, myofibrillar FSR did not differ between conditions (all $P > 0.05$). Over time, myofibrillar FSR increased significantly from basal to 300 min in the COMP ($P = 0.011$), HFP ($P = 0.003$), ISO ($P = 0.021$), and LFP ($P < 0.001$) conditions, whereas no significant increase was observed in CHO ($P = 0.078$). Postprandial myofibrillar FSR was significantly greater in LFP than in CHO ($P < 0.001$), COMP ($P < 0.001$), ISO ($P < 0.001$), and HFP ($P = 0.011$). No significant differences in postprandial myofibrillar FSR were observed among CHO, COMP, ISO, and HFP (all $P > 0.05$).

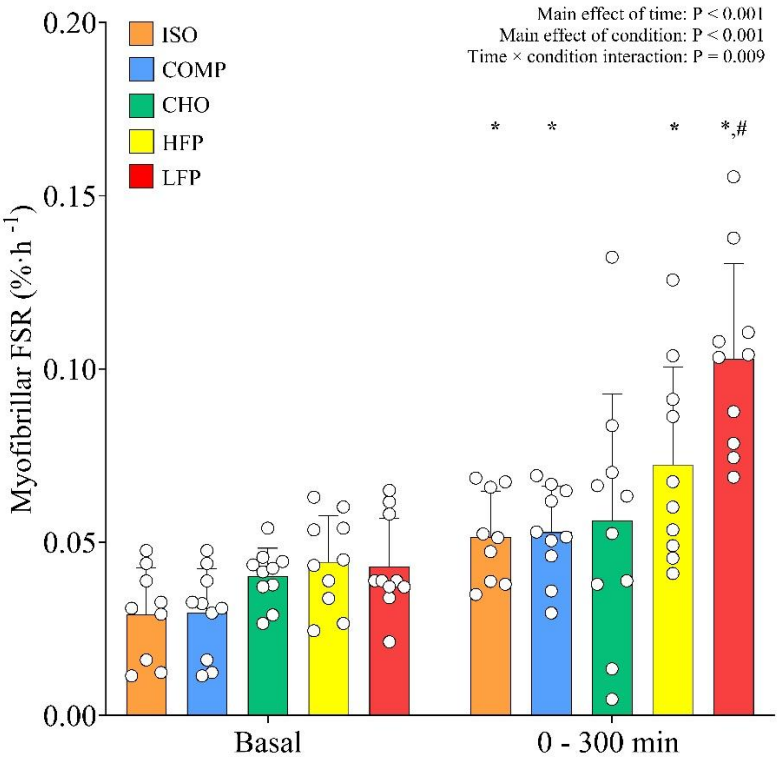

Figure 9. Myofibrillar fractional synthesis rates (FSR, %·h⁻¹) at basal and over the 0–300 min postprandial period following ingestion of ISO, COMP, CHO, HFP, or LFP. Data are expressed as means ± SD; individual data points are overlaid. A significant main effect of time ($P < 0.001$), a significant main effect of condition ($P < 0.001$), and a significant time × condition interaction ($P = 0.009$) was observed. *Denotes a significant increase from basal to the 0-300 min postprandial period within the respective condition ($P < 0.05$). #Denotes a significant difference between LFP and all other conditions during the 0-300 min postprandial period ($P < 0.05$, Bonferroni-corrected). CHO, carbohydrate beverage; COMP, complementary pairing of rice and black beans; FSR, fractional synthetic rate; HFP, high-fat ground pork; ISO, isolated mixture of crystallized amino acids, maltodextrin, cellulose, pectin, and soy oil; LFP, low-fat ground pork; SD, standard deviation.

4. Associations between peak plasma concentrations (C*max*) and muscle protein synthesis

A significant moderate positive correlation was observed between peak plasma dileucine concentrations (C*max*) and the change in myofibrillar protein synthesis rates from baseline (ΔMPS) across protein conditions (r = 0.436; *P* = 0.037; **Figure 10A**). However, leucine C*max* demonstrated a negligible/weak association with ΔMPS (r = 0.265; *P* = 0.102; **Figure 10B**).

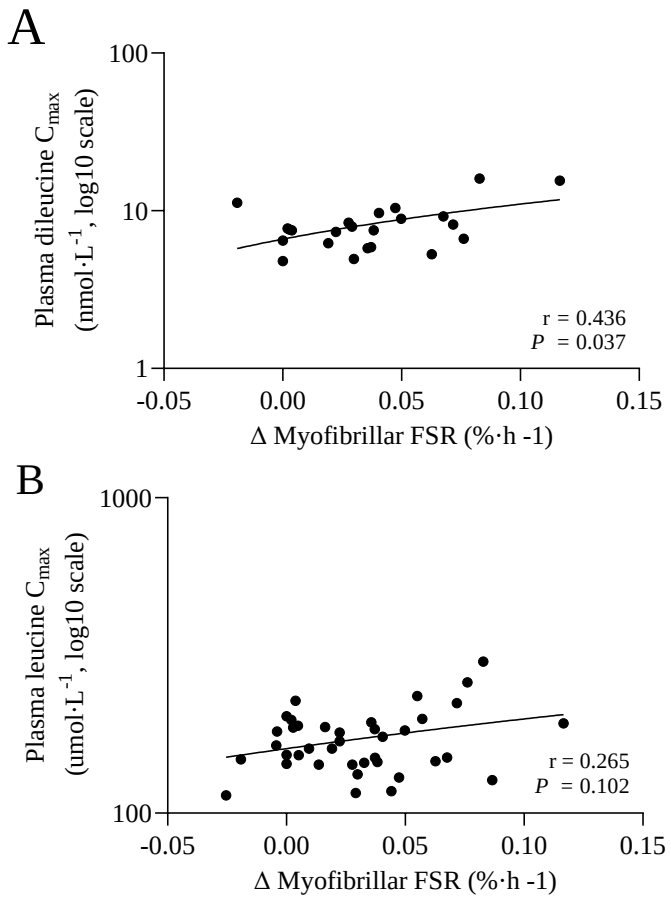

Figure 10. Relationship between peak plasma leucine, dileucine, and the change in myofibrillar protein synthesis. (A) Correlation between peak plasma dileucine C*max* (μmol·L, log10 scale) and the change (Δ) in myofibrillar fractional synthesis rates (FSR, %·h-1) from baseline to 300 min (r = 0.43, P = 0.0347). (B) Association between peak plasma leucine C*max* (μmol·L, log10 scale; B) and Δ myofibrillar FSR (%·h-1) from baseline to 300 min. (r = 0.265, P = 0.102). Each point represents an individual participant.

IV. DISCUSSION

This is the first study to examine how ingesting animal and plant whole foods influence plasma dileucine concentrations and its association with post-exercise myofibrillar protein synthesis rates in healthy young adults. We demonstrated that ingestion of moderate amounts of protein from whole-food sources generally did not result in a robust elevation of postprandial dileucine concentrations, except in the case of low-fat pork. The latter also resulted in a higher magnitude of postprandial leucinemia and postprandial FSR than the other conditions. Despite the relatively low plasma concentrations of dileucine compared to leucine, the peak plasma dileucine (C*max*) was positively correlated with the postprandial rise in myofibrillar protein synthesis rates (ΔMPS), whereas the leucine C*max* was not. Our results suggest that the postprandial increase in plasma dileucine after ingesting whole foods may be limited by the food matrix and digestion kinetics, at least during the post-exercise state.

The present findings extend prior work showing that ingestion of isolated proteins, such as whey or soy, increased postprandial plasma concentrations of BCAA-containing dipeptides, like dileucine, regardless of whether the protein is hydrolyzed or intact (Morifuji et al., 2010). Notably, while protein hydrolysates yielded higher concentrations of Val-Leu and Ile–Leu compared to intact proteins, the postprandial rise in Leu-Leu concentrations were similar between the two hydrolyzed and non-hydrolyzed conditions. With respect to protein source, whey elicited greater dileucine concentrations than soy. In contrast, the current whole-food data, indicate that the whole food matrix is modulating the postprandial rise in dipeptides concentrations. Unlike isolated protein sources, proteins are entrapped within cellular structures and co-ingested with other macronutrients in whole foods. In plant sources (e.g., COMP), the proteins are encapsulated within fibrous cell walls (cellulose), whereas intact animal food sources the proteins are bound within connective tissue. As such, depending on the specific ingested food matrix, this will differentially impact gastrointestinal transit time and the subsequent hydrolysis of di-peptides by the luminal and brush border peptidases before they can be absorbed intact via PepT1. Thus, it is likely that the vast majority of dileucine within whole protein foods is cleaved into free leucine prior to entering the systemic circulation.

This framework is particularly helpful for interpreting the contrast between the plant and the animal conditions. The weaker postprandial leucinemia observed in COMP, and to a lesser extent in ISO, likely reflects multiple constraints on systemic amino acid availability. Plant-based whole foods generally exhibit lower ileal digestibility than purified protein ingredients and many animal-derived foods, in part because proteins remain embedded within a fibrous matrix and co-exist with antinutritional factors that limit enzyme access and amino acid release (Hodgkinson et al., 2025). This is likely particularly relevant for COMP, which was provided as a whole-food plant meal and contained a large carbohydrate load. Carbohydrate co-ingestion with a moderate protein dose can blunt the early postprandial rise in plasma amino acids and leucine, without necessarily reducing the overall incorporation of dietary protein-derived amino acids into muscle protein (Hamer et al., 2013). Moreover, dietary protein digestion and absorption can be impaired during acute post-exercise recovery (van Wijck et al., 2013), which may further disadvantage structurally complex plant meals relative to free-amino-acid conditions such as ISO. Together, these factors provide a plausible explanation for why COMP failed to generate a marked postprandial leucine response, whereas ISO produced only a rapid and transient leucinemia and neither condition matched the more favorable leucinemic profile of the animal-based treatments.

Importantly, the present FSR results reinforce that distinction. Although COMP, ISO, HFP, and LFP all increased myofibrillar FSR above basal values, only LFP was significantly greater than all non-animal conditions at 300 min, whereas CHO did not significantly increase FSR over time. Thus, despite providing amino acids, neither COMP nor ISO elicited a postprandial anabolic response that clearly exceeded the non-protein exercise control over the 0-5 h recovery period. By contrast, LFP produced the most favorable combination of postprandial plasma leucine and dileucine concentration, and myofibrillar FSR, indicating that simply improving amino acid composition is insufficient when the broader digestive and absorptive context remains unfavorable. This interpretation aligns with recent work showing that whole-food omnivorous meals can stimulate greater postprandial MPS than isocaloric and isonitrogenous vegan meals, again emphasizing the importance of whole-food matrix characteristics for postprandial anabolic potential (Pinckaers et al., 2024).

In this context, the divergent dileucine responses between low-fat and high-fat ground pork offer critical insights. Indeed, the grinding of meat will ultimately disrupt the food matrix, or the connective tissue network, to favor more rapid dietary protein digestion and amino acid absorption kinetics (Pennings et al., 2013). However, co-ingesting dietary fat is known to slow gastric emptying and delay protein digestion and absorption, thereby altering the temporal pattern of dietary protein derived amino acid and peptide appearance rates into circulation (Frost et al., 2003; Gentilcore et al., 2006). Although total protein and leucine content were matched, the higher lipid content of HFP likely delayed the liberation and intestinal transport of short-chain peptides like dileucine, resulting in an attenuated plasma dileucine response compared to LFP. In contrast, the lower fat content of LFP may have facilitated faster gastric transit and proteolysis (Zupančič et al., 2025). This resulted in a transient increase (~90-120 min) in dileucine concentrations compared to the other treatment conditions. This time-course of elevation after LFP contrasts with our previous investigation using an isolated dileucine supplement, which produced a rapid yet short-lived peak in plasma dileucine between 15-60 min (**Figure 7**) (Paulussen et al., 2021). However, the similarity in overall dileucine exposure between the more readily digested LFP and free dileucine conditions supports the notion that a portion of food-derived dileucine can surpass gastrointestinal hydrolysis and appear in circulation, albeit with a delayed temporal profile after whole-food intake.

The divergent leucine and dileucine responses observed across conditions are mechanistically informative. ISO elicited the most rapid rise in plasma leucine, which is expected given that this condition provided crystalline amino acids and therefore largely bypassed the digestive constraints imposed by intact food matrices. By contrast, plasma dileucine did not increase significantly in ISO. A similar dissociation was observed in HFP, where leucine increased postprandially but dileucine did not. Together, these findings suggest that circulating dileucine is not simply a function of leucine availability per se. Rather, its appearance likely depends on the presence of preformed dietary peptides, their resistance to gastrointestinal hydrolysis, and their subsequent transfer into the systemic circulation. This interpretation is consistent with evidence that di- and tripeptides appear in human plasma after protein ingestion and that their abundance is shaped

not only by transport properties, but also by peptide stability and dietary source (Morifuji et al., 2010; Rohm et al., 2019).

It has previously been shown that ingesting 2 g of dileucine elevated plasma dileucine concentrations more than an equivalent dose of free leucine, despite similar increases in plasma leucine in healthy young adults. This result was also coupled to greater myofibrillar protein synthesis rates in dileucine vs. the leucine condition (Paulussen et al., 2021). A novel observation in the present study is that we observed a positive association between peak plasma dileucine (C*max)* and the change in myofibrillar protein synthesis rates (ΔMPS) across protein conditions (**Figure 10A**), but not with leucine C*max*. C*max* was selected as the primary metric of interest rather than the iAUC, as peak plasma amino acid concentration has been identified as the strongest predictor of postprandial muscle protein fractional synthetic rate across a wide variety of protein and amino acid formats (Church et al., 2020). However, the association between dileucine C*max* and ΔMPS is modest (r=0.436) and, coupled with the relatively low plasma concentrations of dipeptides, this should be interpreted as hypothesis-generating rather than causality. Particularly, the moderate strength of this correlation indicates that C*max* alone explains only a modest proportion of the variance (19%) in ΔMPS, and consistent with the notion that the regulation of MPS after whole foods is nuanced and cannot be predicted on a single anabolic factor (Zaromskyte et al., 2021). It is interesting, however, to speculate that dileucine C*max* might simply be a surrogate marker for improved dietary protein digestion and absorption kinetics as opposed to the notion that dileucine itself is the exclusive mechanistic driver of increased MPS, particularly with whole food ingestion.

The current study also has limitations. First, because no intrinsically labeled dietary protein or labeled dileucine tracer was used, the origin of circulating dileucine cannot be definitively assigned to the ingested meal. The present data therefore describe postprandial plasma appearance only and cannot distinguish exogenous from endogenous sources. Future studies should address this limitation by using intrinsically labeled protein sources, for example ^{13}C-leucine-labeled dietary protein, to determine whether the dileucine detected in plasma is directly derived from the ingested protein. A second limitation relates to analytical specificity. Plasma dileucine was quantified by LC-MS/MS, but without definitive structural

confirmation it remains possible that compounds with similar mass-to-charge characteristics may have contributed to the measured signal. Thus, although the detected signal was assigned to dileucine, we cannot completely exclude the possibility of interference from other structurally related peptides. Future work could strengthen compound identification by combining targeted LC-MS/MS with additional chromatographic separation, authentic standards, or complementary structural approaches.

In conclusion, the ingestion of moderate doses of whole-food protein sources does not appear to substantially elevate postprandial plasma dileucine concentrations in the post-exercise state, except for low-fat pork that elicited a detectable increase. The superior leucinemic and anabolic response to LFP compared to COMP, ISO, and HFP, supports the view that food-matrix characteristics strongly influence postprandial amino acid availability and anabolic potential. The association between peak plasma dileucine and ΔMPS raises the possibility that circulating dileucine may mark a more favorable digestive-absorptive profile, but the present data do not establish dileucine as a causal anabolic mediator. Furthermore, a retrospective comparison with free dileucine ingestion suggested distinct kinetic differences, with whole-food animal protein demonstrating a delayed postprandial rise in the plasma dileucine response. Further work is warranted to clarify the mechanisms governing dileucine formation and absorption, and its contribution to post-exercise muscle protein metabolic responses.

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APPENDIX

1. Standard operating procedures for plasma isotopic enrichment and amino acids quantification

Items needed:

1. Solvent "A" (water:ACN:IPA; 2:3:3 *v/v*; 190 µL per sample)
2. 5 uL of plasma sample
3. 0.1% formic acid in H_2O (100 µL per sample)
4. CPA standard (5 µL per sample; 10 µg/µL concentration)
5. 2 Eppendorfs for each sample with number and record file on Box
6. Autosampler vials with inserts labelled with sample codes

Procedures

1. Turn the pump and the SpeedVac on to be ready when you finish the extraction
2. Before adding the samples, make sure they are thawed and vortexed
3. Add 5 µL of sample to labelled Eppendorfs
4. Add 190 µL of water:ACN:IPA (2:3:3 *v/v*)
5. Add 5 µL of CPA standard
6. Vortex sample for 10 seconds
7. Centrifuge for 10 min at 4°C and max RCF
8. Remove 100 µL of supernatant to new Eppendorf (the rest will be precipitated protein) and discard the pellet
    a. Dry in the SpeedVac (temperature = 45°C; heat time = 2 hr; run time = 8 hr; Auto Run)
9. Confirm that samples are completely dried
    a. If the samples are wet, re-run SpeedVac at 45°C with 1-2 hours heat time and 4 hours run time
10. Re-suspend with 100 µL of 0.1% formic acid in H_2O
11. Vortex for 10 seconds
12. Transfer 50 µL supernatant to autosampler vials
13. Submit for LC-MS/MS analysis

ABSTRACT

Dileucine, a leucine-containing dipeptide, may enhance anabolic signaling more than leucine alone. This study examined postprandial plasma dileucine and leucine concentrations after ingesting animal- and plant- whole foods and their association with muscle protein synthesis rates (MPS) in humans. Secondary analysis was performed on plasma samples collected from two randomized controlled trials totaling 26 healthy adults (25 ± 4.3 y). Participants consumed low-fat pork (LFP), high-fat ground pork (HFP), complementary rice and beans (COMP), an iso-nitrogenous mixed nutrient beverage (ISO), or a carbohydrate control (CHO). Plasma concentrations and MPS was measured. Plasma dileucine increased significantly only after LFP ingestion ($P < 0.05$). Plasma leucine increased in all conditions except COMP and CHO, with LFP demonstrating the highest concentrations ($P < 0.05$). Postprandial myofibrillar FSR was significantly greater in LFP than in all other conditions ($P < 0.05$). A modest correlation was observed between peak plasma dileucine concentration (C*max*) and the change in MPS (ΔMPS; r = 0.436, $P = 0.037$), whereas leucine C*max* showed no significant association (r = 0.265, $P = 0.102$). Whole-food protein sources generally do not elevate plasma dileucine, except for LFP. While dileucine C_{max} correlated with ΔMPS, the modest strength of this relationship suggests that dileucine is likely one of several postprandial factors influencing MPS beyond free leucine.

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