THE FOOD MATRIX AND ITS EFFECT ON HUMAN PROTEIN METABOLISM

BY

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DISSERTATION

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ABSTRACT

Skeletal muscle plays a vital role in human health and disease prevention. Dietary protein is a main anabolic stimulus and thus essential for the maintenance of skeletal muscle mass. Most research on the effects of dietary protein on muscle protein turnover (synthesis and breakdown) have been investigated from a reductionist viewpoint utilizing isolated nutrients. Dietary recommendations to support muscle mass are based on these studies, which have been extrapolated to generalize to the diet in its entirety. While many early studies provided the framework for future directions, it is important to recognize that diets do not consist of isolated nutrients. Evidence suggests that the food matrix, a term to describe both the nutrient and non-nutrient components of whole foods, may play an important role in the regulation of muscle protein turnover. The studies reported in this dissertation investigated aspects of the food matrix as it relates to muscle protein turnover and health. In study 1, we showed that salmon ingestion is as potent for the stimulation of muscle protein synthesis rates as the sum of its isolated nutrients. In study 2, we demonstrated that an isolated food component, di-leucine, was more effective for the stimulation of muscle protein synthesis rates when compared to leucine. In study 3, we assessed the efficacy of the co-ingestion of a microbial protease blend with isolated pea protein and demonstrated greater plasma postprandial essential and total amino acid concentrations when compared to ingestion of pea protein alone. The studies contained in this dissertation investigated aspects of the food matrix including nutrient-nutrient interactions (salmon vs. isolated nutrients), isolated food components (dileucine) and manipulation of food structure (via a microbial protease supplement) in the regulation of human muscle protein turnover and underline the necessity of a more holistic view when it comes to dietary regulation of skeletal muscle mass.

Veur mien moojer en vaajer.

Die m'ch opgeveujd höbbe door te zètte en te geluive tot alles meugelek is.

Zoonder hun aonmoedeging, steun en leefde zouw dit werk neet dinkbaar zien gewees.

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CHAPTER 1: GENERAL INTRODUCTION

Dietary protein has been extensively and has a well-established role as an essential nutrient necessary for survival due to its structural and regulatory functions. Current dietary guidelines for protein, such as the recommended dietary allowance (RDA) in the United States and the population reference intake (PRI) within the European Union are based on research investigating isolated protein sources. Nutrients are known to interact with one another when ingested as part of a whole food (Moughan, 2020). Since diets consist of whole foods and not isolated nutrients, more research is warranted on the role of the food-matrix in human metabolism of dietary protein. Experiments conducted in this dissertation sought to investigate aspects of the food matrix on the regulation of muscle protein turnover in healthy adults. The food matrix is defined as the nutrient and non-nutrient components of foods and their molecular relationships (i.e., chemical bonds) to each other. This chapter will provide an overview of key topic areas related to this dissertation.

1.1 THE ROLE OF SKELETAL MUSCLE IN HUMAN HEALTH

It is widely recognized that skeletal muscle is essential for locomotion and enables us to directly interact with the world. Healthy skeletal muscle allows for the completion of a broad spectrum of activities, from basic movements like walking or writing to more complex activities like mowing the lawn. Well-trained skeletal muscle allows for more physical vocations (e.g., construction, first responders, etc.) and high-performance sports. However, what is less widely recognized is the importance of skeletal muscle in disease. Skeletal muscle functions as a reservoir of amino acids in the absence of amino acid absorption from the gut, which is especially crucial during times of disease (Cahill Jr, 1970). For example, high amounts of skeletal muscle mass are associated with

lower mortality rates in patients with cancer, AIDS, and other chronic diseases (Wolfe, 2006). About fifty percent of body mass is comprised of skeletal muscle and is a major reservoir for glucose disposal and metabolism (Ferrara et al., 2006). Skeletal muscle serves as a 'sink' for circulating glucose and thus has critical role in the treatment and prevention of conditions of chronically elevated glucose, such as type 2 diabetes (Ferrara et al., 2006). Furthermore, there is an abundance of evidence that shows that muscle metabolism plays a key role in other pathological conditions, like obesity, cardiovascular disease, and osteoporosis (Wolfe, 2006). Given these contributions, it is clear that skeletal muscle mass plays a substantial role in the maintenance of both health and quality of life.

1.2 MUSCLE PROTEIN TURNOVER

A main determinant of skeletal muscle health is protein synthesis and breakdown. As with all animate tissues, skeletal muscle is in a constant state of protein turnover, meaning that it is continually breaking down and resynthesizing old proteins (Phillips et al., 1997). For the maintenance of total skeletal muscle mass over time, the rates of protein synthesis and breakdown need to be in equilibrium. Conversely, if there is a need to increase skeletal muscle mass, daily muscle protein turnover needs to be in a positive balance and muscle protein synthesis rates need to exceed protein breakdown rates.

The role of protein turnover in health is multifactorial. First, old proteins become damaged and dysfunctional due to interactions with reactive oxygen and nitrogen species (Powers et al., 2011), sugars (Watanabe et al., 1992), or aldehydes (Jaganjac et al., 2013). The accumulation of old and damaged proteins can contribute to a multitude of pathologies including pathogenesis of diabetes (Jaleel et al., 2010), aging (Watanabe et al., 1992), and muscular dystrophy (Haycock et

al., 1996) and thus, old and damaged proteins need to be replaced. Second, tissue demands like contractile activity and subsequent muscle damage can increase protein synthetic activity, thereby assisting in muscle plasticity by adaptation and repair (Damas et al., 2016, Phillips et al., 1997, Moore et al., 2009b). Lastly, muscle tissue is the body's largest pool of amino acids, and provides tissues in need with amino acids for protein synthesis (Biolo et al., 2002) and gluconeogenic precursors during times of starvation or increased metabolic demand (e.g. exercise) (Felig et al., 1970).

Various methods to assess muscle protein synthesis and breakdown have been deployed in the past. Stable isotope amino acid tracers have been the gold standard due to their ability to trace protein kinetics without affecting protein metabolism itself (Trommelen et al., 2019, Wilkinson et al., 2017). Deuterated water has been gaining much traction in recent years as it gave researchers the ability to assess protein synthesis without confining research subjects to the laboratory (Salvador et al., 2021). Other methods include the use of radioactive isotopes (Umpleby and Sönksen, 1987) or puromycin (Goodman and Hornberger, 2013), which due to their detrimental effects on health are currently limited to animal or single cell models. This dissertation will focus on the use of contemporary stable isotope amino acid tracers.

Experiments that used stable isotopes (^{15}N) to assess protein turnover occurred as early as 1939. These first experiments provided evidence that the living cell is a dynamic system consisting of proteins that are continually degraded and re-synthesized (Schoenheimer et al., 1939). The first quantification of the rate of protein synthesis on a whole-body level occurred ten years later by utilizing ^{15}N amino acid tracer excretion in urine (Sprinson and Rittenberg, 1949). The first turnover rates by intravenous infusion of radioactive isotopes were determined in 1967 (Waterlow, 1967). Around the same time, the accretion of amino acids into muscle protein was first studied in

animal models (Florini, 1964). Subsequently, the first reports of sarcoplasmic and myofibrillar protein-specific synthetic rates in humans were examined using [alpha-^{15}N]lysine intravenous infusion and repeated muscle biopsy sampling in 1975 (Halliday and McKeran, 1975). The primed continuous infusions of stable isotopes, which are used in the experiments in this dissertation, were first published in 1982 utilizing a [1-^{13}C]leucine tracer (Rennie et al., 1982). It is important to recognize these early efforts in understanding protein metabolism, as this work has laid the foundation for decades of stable isotope tracer research, including this dissertation.

1.3 MAIN ANABOLIC STIMULI

The two main anabolic stimuli for skeletal muscle protein turnover are food ingestion and exercise. The pop-culture icon popularizing modern-day fitness, Jack Lalanne, often remarked: "Exercise is King, nutrition is Queen – put them together and you have a kingdom." These words are truly justified when it comes to muscle protein turnover as these two work synergistically and have the capacity to increase net protein balance by increasing muscle protein synthesis while reducing muscle protein breakdown (Moore et al., 2009b, Biolo et al., 1997, Tipton et al., 2003).

Emphasis on protein synthesis

Historically, most studies have emphasized the measurement of protein synthesis over muscle protein breakdown. The measurement of muscle protein breakdown is methodologically costly and complex. One of the critical problems is the re-utilization of tracer, where it is hard to discern if the protein synthetic machinery is re-using a recently liberated amino acid from its own muscle protein pool or synthesizing *de novo* from newly delivered exogenous amino acids. Secondly, the current methodologies to assess muscle protein breakdown rates do not allow for fraction-specific

data (more on muscle fractions below). One approach that has attempted to address both limitations is 3-methylhistidine, due its incorporation in only the myofibrillar proteins and inability to be re-utilized for protein synthesis. However, use of this method is limited due to insufficient sensitivity of the method and confounding effects from the 3-methylhistidine present in smooth and cardiac muscle cells (Hansen et al., 2009, Rennie et al., 2008). Nevertheless, it has been reported that muscle protein breakdown plays a minor role in the post-exercise response to feeding when compared to muscle protein synthesis (Glynn et al., 2010). In addition, very modest insulin concentrations (to 104.2 pmol/L) are needed to maximally inhibit muscle protein breakdown (Wilkes et al., 2009). Ultimately, muscle protein synthesis and breakdown seem to be tightly regulated together and respond in a concomitant manner (Phillips et al., 1997). Although it would be ideal to measure both muscle protein synthesis and muscle protein breakdown, muscle protein synthesis is a greater determinant of total protein turnover balance (Biolo et al., 1997, Glynn et al., 2010, Phillips et al., 1997, Phillips et al., 2002).

Anabolic effect of exercise

Both endurance and resistance exercise provide a potent stimulus for mixed muscle protein synthesis. Mixed muscle protein refers to the weighted average of protein synthesis of all the individual proteins within skeletal muscle, which each have their unique turnover rates (Rooyackers et al., 1997). These sub-fractions include the myofibrillar (contractile), sarcoplasmic (cytoplasm), mitochondrial (energy production), and collagen (structural) fractions. The myofibrillar fraction is the most predominant, constituting about 50-60 % of total muscle protein (Hasten et al., 2000, Balagopal et al., 2001). Therefore, the myofibrillar fraction is not only responsible for the contractile properties of the muscle, but also plays an important role as a storage

depot of amino acids for the rest of the body. Each exercise modality places differential demands on specific muscle sub-fractions. Additionally, exercise factors such as exercise intensity, volume, and time under tension play significant roles as well in determining the protein synthetic response within each muscle fraction (Burd et al., 2012). More details about the exercise-induced effects are out of the scope of this dissertation. Nevertheless, it is important to recognize that exercise directly affects nutrient utilization and is a crucial factor in determining muscle protein turnover. As part of this dissertation, we standardized exercise or habitual activity to reduce any confounding effect.

Anabolic effect of nutrition

Dietary protein is the most potent anabolic stimulus compared to other macronutrients. In fact, dietary protein ingestion at adequate amounts stimulates muscle protein synthesis, inhibits muscle protein breakdown, and provides the building blocks needed for building muscle proteins, independent of exercise. Chronic inadequate ingestion of protein less than the Recommended Dietary Allowance (RDA) (0.8 $g \cdot kg^{-1}$) can lead to a myriad of health issues, including an increased risk of chronic disease and age-related decline of muscle mass (sarcopenia)(Wolfe, 2006, Burd et al., 2019). Conversely, some studies have shown that an increase in daily protein intake above the RDA can have beneficial effects on health and performance in the aging population, as well as in athletes or otherwise highly active individuals (Moore et al., 2015, Cermak et al., 2012). The higher requirements are thought to be attributed to the capacity of higher protein intakes to stimulate skeletal muscle protein synthesis to a greater extent. Similarly, higher protein intake of up to 1.6 $g \cdot kg^{-1} \cdot d^{-1}$, twice the RDA, is associated with greater accretion of skeletal muscle mass (Cermak et

al., 2012, Morton et al., 2018). As described earlier, enhanced skeletal muscle mass is vital for athletic performance and clinical outcomes.

Protein dose

The total amount of protein in a single meal is one of the primary factors contributing to the amplitude and duration of postprandial muscle protein synthesis rates. Numerous dose-response studies have been done utilizing isolated dietary protein (Witard et al., 2014, Moore et al., 2009a, Churchward-Venne et al., 2020, Holwerda et al., 2019b, Moore et al., 2015). For example, utilizing whey protein, it was found that 0.24 ± 0.06 and 0.40 ± 0.19 g of protein per kg body weight is sufficient to maximally stimulate muscle protein synthesis in young (18 – 37 y) and older (65 – 80 y) males, respectively (Moore et al., 2015). For an 80 kg bodyweight male this would equate to 19 g and 32 g of protein for young and old, respectively, consistent with other studies (Holwerda et al., 2019b, Witard et al., 2014).

Protein quality

Another important dietary factor for the stimulation of muscle protein synthesis is protein quality. Protein quality is defined by its digestibility and amino acid profile. In other words, protein source plays an important role. Protein digestibility is a key component in dictating how much of the food's amino acids are released into circulation and is described in detail in **section 1.6**. The dietary amino acid profile dictates the 'building blocks' available for the synthesis of new proteins. Human muscle is comprised of twenty individual amino acids, of which nine are considered indispensable. Essential, or indispensable, amino acids are considered essential because the human body cannot synthesize these compounds *de novo* to meet the metabolic demand. Therefore, these essential

amino acids need to be acquired exogenously. Thus, it has been postulated that foods that mimic the skeletal muscle amino acid profile optimize skeletal muscle protein turnover when ingested.

Another reason dietary amino acid profiles of food are important is that certain amino acids function as signaling molecules for anabolic pathways. Early work utilizing flooding doses of individual amino acids have shown that essential, but not nonessential, amino acids are able to stimulate muscle protein synthesis (Smith et al., 1998). In line, whey protein contains high amounts of essential amino acids and has the capacity to maximally stimulate muscle protein synthesis with doses as low as 20 g (Moore et al., 2009a). More specifically, whey protein's ability to stimulate muscle protein synthesis to a high extent is commonly attributed to its high leucine levels (Churchward-Venne et al., 2012). Leucine plays a key role in the regulation of the protein signaling pathway responsible for protein turnover and it has been postulated that leucine functions as a 'trigger' to skeletal muscle protein synthesis (Norton and Layman, 2006). This leucine trigger hypothesis suggests that a rapid (~60 min) postprandial plasma leucinemia dictates the muscle protein synthetic response. However, it should be noted that, given the protein dose-response studies mentioned earlier, it is likely that plasma leucine concentrations function more in a dose-response fashion rather than an all-or-nothing trigger for stimulating muscle protein synthesis. Furthermore, recent evidence has emerged (Zaromskyte et al., 2021) that many other factors in food, beyond leucine and the other essential amino acids, regulate muscle protein synthetic rates. Thus, a more holistic perspective is needed, including studies that examine the effects of whole foods on anabolic responses.

1.4 PROTEIN OXIDATION

To support skeletal muscle as a contractile unit and amino acid store, exogenous amino acids need to be incorporated within functional proteins within the skeletal muscle cell. Alternatively, amino acids not incorporated into functional proteins are oxidized for use as a substrate in energy metabolism. This higher amino acid oxidation is not preferred, as energy acquirement via other macronutrients (i.e., fats and carbohydrates) is a more energy-efficient process (Veldhorst et al., 2009). Thus, it is important to consider protein turnover in the context of amino acid oxidation as well to ensure that dietary protein gets used optimally to support skeletal muscle health.

As described above, it is clear that there is a maximal rate to which muscle tissue can accrete proteins from amino acids per meal (Moore et al., 2009a, Holwerda et al., 2019b). Seminal work in the 1980s has suggested that with suboptimal doses of protein intake, protein turnover appears to regulate most adaptive changes. In contrast, at surfeit intakes the major adaptive component is a marked increase in leucine oxidation (Motil et al., 1981, Garlick et al., 1980). More recent work has confirmed that when increasing isolated protein intake above amounts necessary to maximally stimulate muscle protein synthesis (Moore et al., 2009a) or mixed meal intake above amounts necessary to plateau whole-body net balance (Mazzulla et al., 2018), deamination and oxidation of excess amino acids occur. Thus, to most efficiently utilize nutrients contained within food, an emphasis needs to be placed on the optimal stimulation of muscle protein synthesis (i.e., high rates of protein synthesis with low rates of protein oxidation), not maximal stimulation rates (**Figure 1.1**)

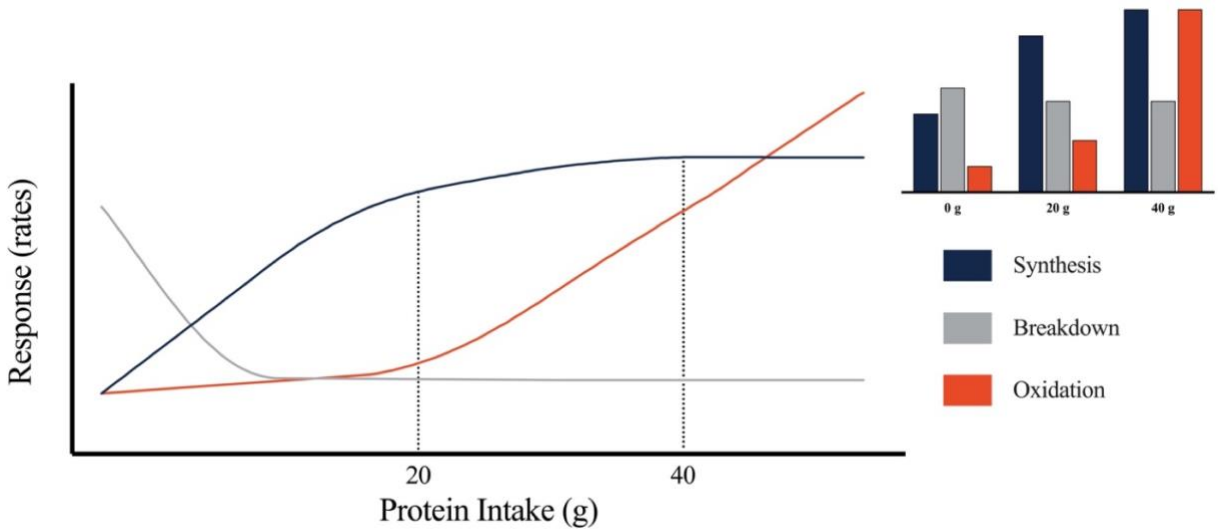

Figure 1.1. Theoretical model of post-exercise responses to protein ingestion. A lot of emphasis has been placed on finding a protein dose that can maximally stimulate muscle protein synthesis. However, as the ingested protein dose goes up (Moore et al., 2009a), leucine oxidation rates go up as well. Therefore, it is pertinent to differentiate between a maximal stimulation of muscle protein synthesis, and an optimal stimulation of muscle protein synthesis, where protein synthesis gets stimulated to a great extent, without wasting too much protein into oxidation.

1.5 WHOLE FOODS AND THE FOOD-MATRIX

The invaluable work preceding this dissertation provided the framework to understand the interactions between specific nutrients and skeletal muscle. However, the reductionist approach of investigating single nutrients (i.e., isolated protein, amino acids, leucine etc.) may lack ecological validity and more work is needed to assess whether these findings can adequately define the role of dietary protein within a complete diet on muscle mass and health.

Diets consist of foods, not isolated nutrients. Consequently, there is a need to determine the effects of protein ingestion on muscle metabolism when these proteins are contained within their original food-matrix. The USDA defines the food matrix as: “the nutrient and non-nutrient components of food and their molecular relationships (i.e., chemical bonds) to each other”. Indeed, it has been shown that the food matrix can influence nutritional outcomes in a way that cannot be explained by the mere sum of its nutrients alone (Moughan, 2020). Nutrient additivity effects have

been described (Bayley and Lewis, 1965), as well as nutrient interactions (including amino acids) (D'mello and Lewis, 1970), effects of food components other than the classical nutrients (Hill et al., 2014), and food structure effects on the kinetics of nutrient digestion and absorption (Singh et al., 2015). However, to date, few studies have investigated the effects of whole-food sources of protein on skeletal muscle protein metabolism.

The first evidence for a food-matrix effect on the postprandial muscle protein synthetic response arose in 2006 when researchers in Kevin Tipton's lab found that whole milk ingestion led to greater amino acid arteriovenous balance across the leg when compared to an isocaloric amount of fat-free milk, despite an almost two-fold difference in protein content (Elliot et al., 2006). Two years later, researchers in Robert Wolfe's found that ingesting whey protein results in greater muscle protein accrual when compared to the ingestion of its constituent essential amino acids in older adults (Katsanos et al., 2008).

More recently, studies directly assessing muscle protein synthesis after ingesting whole foods challenged, at that time, the well-established leucine trigger hypothesis. Researchers in Luc van Loon's lab compared the effects of beef vs. milk ingestion on post-exercise muscle protein synthesis and found that despite divergent plasma leucine profiles, both foods stimulated muscle protein synthesis to the same extent (Burd et al., 2015). This indicates that the ingestion of whole foods is potent enough for the stimulation of post-exercise muscle protein synthesis rates despite not facilitating a rapid rise in postprandial leucinemia. Similarly, a study performed in our lab fed healthy young adults isonitrogenous amounts of whole eggs or egg whites and found that, despite similar plasma leucine concentrations, the post-exercise muscle protein synthetic response was stimulated to a greater extent in the whole egg group (Van Vliet et al., 2017). Since both of these studies looked at the muscle protein synthetic response in the exercised state, it remains to be

determined if the discrepancy between leucinemia and muscle protein synthesis upholds in the absence of exercise. Nevertheless, perhaps more interestingly, in the latter study, the researchers found a greater stimulation of muscle protein synthesis after ingesting whole eggs, with no differences in whole-body leucine oxidation rates. This would imply that the whole egg ingestion led to a more optimal use of dietary protein as more amino acids were directed towards protein accrual rather than oxidation. This finding contrasts with previous studies investigating isolated protein sources (including egg protein), as an increase in muscle protein synthesis typically is accompanied by an increase in leucine oxidation (Mazzulla et al., 2018, Moore et al., 2009a).

1.6 PROTEIN DIGESTION AND ABSORPTION

In the past, a popular method for assessing protein quality was the *protein digestibility corrected amino acid score* (PDCAAS) (FAO., 1990). This method compares the most limiting amino acid of that food to the concentration of that amino acid in a reference pattern. One of the limitations of this method is that its score is truncated, deeming it impossible to distinguish between different higher-quality protein foods. The most recently accepted method by the FAO (Food and Agriculture Organization of the United Nations) is the dispensable indigestible amino acid score (DIAAS) (Consultation, 2011). This method uses true ileal amino acid digestibility estimates based on measuring undigested amino acids at the end of the small intestine, without them being truncated above a certain point. Thus, the DIAAS score determines what amino acids are limiting in circulation after accounting for digestion and absorption to support whole-body protein metabolism.

According to both methods referenced above, plant proteins are regarded as lower quality than most animal-based proteins. For example, according to experimental evidence from Hans

Stein's laboratory, isolated pea protein has a DIAAS score of 62, whereas milk protein has a DIAAS score of 120 (Mathai et al., 2017). This is in part due to the plant protein's lower essential amino acid content, but also due to its lower digestibility rates. It should be noted that due to the invasiveness of the method, it is not feasible to routinely perform ileal digestibility in humans, and therefore the growing pig model is often used. Given the similarity between the pig's and human's digestive tract, DIAAS scores are similar and thus not a problem (Deglaire et al., 2009). However, the method does prevent measurement of DIAAS scores after exercise, and thus post-exercise differences between plant- and animal protein digestibility remain to be determined.

Recent work utilizing DIAAS has shown that the cooking method impacts food protein quality (Hodgkinson et al., 2018). For example, the cooking method of meat (boiling, grilling, sous-vide, etc.) affects the food's structural properties and subsequent DIAAS score (Williams et al., 2006). Cooking in general increases protein digestibility by allowing greater bio-accessibility of proteolytic enzymes to its cleavage sites (Williams et al., 2006). However, this ameliorating effect seems to follow a bell curve, as severe heat treatment can negatively impact the nutritional value of amino acids (Mehta and Deeth, 2016). Together, these data are evidence of the food structure, and thus food matrix, and prove to be an important factor in modulating protein quality scores and consequently the muscle anabolic effect.

1.7 DIPEPTIDES

Protein is one of the main anabolic stimuli for muscle protein turnover. One of the essential amino acids, leucine, is of particular interest due to its capacity to 'trigger' muscle protein synthesis by activating the master regulator of protein synthesis, the signaling protein *mechanistic target of rapamycin complex 1* (mTORC1) (Norton and Layman, 2006). Studies on the importance of

leucine for skeletal muscle protein metabolism date back as early as 1975 (Buse and Reid, 1975). Ever since, a multitude of studies have investigated the effects of fortifying foods and protein drinks with additional leucine to increase the anabolic properties of those foods. Leucine seems to be an excellent candidate for fortifying foods, especially those containing suboptimal amounts of protein (Holwerda et al., 2019a).

Within whole foods, leucine not only occurs as a single peptide but also as its dipeptide dileucine. Altering the chemical positioning of this essential amino acid might have important implications in terms of its effects on human protein metabolism. Dipeptides are shown to have differential effects on protein absorption and digestion. More specifically, dipeptides are absorbed faster than single peptides. In addition to being transported into circulation by the *L-Type amino acid transporter* (LAT1), like single amino acids and peptides, dipeptides can be transported into circulation by the *Human Peptide Transporter 1* (PePT1).

1.8 SPECIFIC OBJECTIVES AND HYPOTHESES TESTED

The primary objective of the studies within this dissertation was to investigate the effects of the food-matrix on the regulation of skeletal muscle protein turnover using direct tracer incorporation methods (studies 1 and 2) or indirect indicators such as plasma amino acid concentrations as in study 3. Furthermore, these experiments assessed how changes to the molecular relationships between nutrients within the food matrix can modulate these food effects. These investigations are necessary to set forth clear and usable nutrition guidelines with real-world applications.

Study 1: food-first approach for stimulating muscle protein synthesis

Recent interest has emerged in the holistic properties of food (i.e., the effect of nutrient additivity, nutrient-nutrient interactions, and food matrix effects). Emerging evidence suggests that nutrient effects cannot be predicted based on the effects of those nutrients in isolation. In other words, food seems to be more than the sum of its nutrients. To elucidate the effects of the food matrix on human muscle protein metabolism, study 1 (chapter 2) of this dissertation was conducted. In this randomized crossover study, the effects of eating protein-rich food, salmon filet (a common food component of the Mediterranean diet), on muscle protein synthesis and whole-body protein oxidation was compared to eating the exact nutrients of that salmon filet in isolation in the form of crystalline amino acids and fish oil. Ten healthy adults volunteered for the study, and protein synthesis and oxidation were determined by primed continuous infusion of stable isotope tracers in combination with repeated blood, breath, and muscle samples. We hypothesized that eating nutrients within its original food matrix will lead to higher rates of myofibrillar protein synthesis and a reduction in whole-body protein oxidation when compared to eating those same nutrients in isolation. The findings of this study will have important implications for future nutrition guidelines, which are currently based on research utilizing isolated nutrients.

Study 2: food-borne peptides

As the speed of absorption has important implications for the effects of food on muscle protein metabolism (Boirie et al., 1997), dipeptides present a potential candidate for increasing the anabolic properties of food. The second study (Chapter 3) of this dissertation aims to assess the effects of dileucine ingestion on muscle protein turnover compared to leucine ingestion in healthy young males. In this study, participants were fed either 2g of dileucine or 2g of leucine in a double-

blind, randomized crossover fashion. After ingestion of the compound, muscle protein synthesis and breakdown were assessed through a primed continuous infusion of stable isotope tracers in combination with repeated blood sampling and muscle biopsies. We hypothesized that dileucine would lead to greater stimulation of muscle protein synthesis and lower rates of muscle protein breakdown when compared to the ingestion of an equivalent amount of leucine. The findings of this study will help gain valuable insight into the role of dipeptide ingestion on muscle protein synthesis.

Study 3: microbial proteases

The third study of this dissertation (Chapter 4) aims to assess the effects of adding a microbial protease blend to pea protein before ingestion and compare its effect to ingestion of the pea protein alone. This study was performed using a double-blind, randomized crossover design. Twenty-four healthy adults ingested either 20g of pea protein with microbial proteases or 20g of pea protein with a placebo (maltodextrin), after which amino acid concentrations were measured for 5 h postprandial. We hypothesized that there would be higher levels of aminoacidemia after ingestion of the pea protein with proteases when compared to ingestion of pea protein with a placebo. The findings of this study will add to our understanding of the effects of exogenous proteolytic enzymes in humans. Furthermore, proteases will present a viable candidate for food fortification techniques and could potentially increase the efficient use of proteins, promoting food anabolism and limiting food waste.

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CHAPTER 2: FOOD-FIRST APPROACH

Salmon ingestion stimulates post-exercise muscle protein synthesis to a similar extent as the sum of its isolated nutrients in healthy young adults: a randomized controlled trial

2.1 ABSTRACT

Healthy eating patterns consist of whole foods as opposed to single nutrients. As such, there is a need to underpin the role of eating protein within its natural whole food matrix versus isolated nutrients on the regulation of postprandial muscle protein synthesis. This study assessed the effects of eating salmon on the stimulation of post-exercise muscle protein synthesis (MPS) and whole-body leucine oxidation rates versus eating these same nutrients as an isolated mixture in healthy young adults. In a crossover design, 10 recreationally active adults (24±4 y; 5 M, 5 F) performed an acute bout of resistance exercise followed by the ingestion of salmon (SAL) (20.5 g protein and 7.5 g fat) or its matched constituents in the form of crystalline amino acids and fish oil (ISO). Blood, breath, and muscle biopsies were collected at rest and after exercise during primed continuous infusions of L-[*ring*-$^{2}H_{5}$]phenylalanine and L-[1-$^{13}C_{6}$]leucine. Postprandial leucine oxidation rates increased over time ($P < 0.001$), with a time × condition interaction ($P = 0.003$). Time-to-peak leucine oxidation occurred sooner in ISO (63 ± 25 min; 1.239 ± 0.321 nmol·kg^{-1}·min^{-1}) when compared to SAL (105 ± 20 min; 1.230 ± 0.561 nmol·kg^{-1}·min^{-1}; $P = 0.003$). Post-exercise MPS was stimulated by SAL and ISO ($P < 0.001$) with no differences between conditions ($P = 0.329$) in both the early (0-2 h: 0.077 ± 0.037 %/h SAL compared to 0.059 ± 0.047 %/h ISO) and late-phase (2-5 h: 0.045 ± 0.019 %/h SAL compared to 0.039 ± 0.026 %/h ISO) of recovery. Similarly, SAL (0.056 ± 0.022 %·h^{-1}; $P = 0.001$) and ISO (0.046 ± 0.025 %·h^{-1}; $P = 0.025$) stimulated MPS during the entire 0-5 h recovery period, with no differences between conditions

(P = 0.308). We show that the ingestion of salmon or its isolated nutrients enhances the stimulation of post-exercise MPS. Our results indicate that ingesting protein within its whole-food matrix similarly stimulates post-exercise MPS when compared to ingesting those same nutrients as an isolated mixture in healthy young adults. This trial was registered at www.clinicaltrials.gov as NCT03870165.

2.2 INTRODUCTION

Dietary protein ingestion is one of the main anabolic stimuli to increase postprandial muscle protein synthesis rates and, as such, is fundamental in the regulation of skeletal muscle mass. Most nutrition studies investigating the feeding-mediated stimulation of muscle protein synthesis rates have focused on the use of isolated protein sources (Burd et al., 2012, Tang et al., 2009, Yang et al., 2012, Witard et al., 2014, Burd et al., 2014, Van Vliet et al., 2019, Boirie et al., 1997, Holwerda et al., 2019b) or free amino acid mixtures (van Loon et al., 2000, Børsheim et al., 2002, Volpi et al., 2003, Paddon-Jones et al., 2004, Katsanos et al., 2006, Rieu et al., 2006, Churchward-Venne et al., 2012, Holwerda et al., 2019a). Healthy eating patterns, however, are based on the ingestion of whole-food protein sources as opposed to isolated nutrients and supplemental protein powders (Burd et al., 2019b). Hence, it is relevant to extend our understanding of the regulation of postprandial muscle protein synthesis rates to nutrient and protein dense foods. For example, previous research has established that whole milk enhanced leg amino acid uptake to a greater extent than the ingestion of an equivalent amount of fat-free milk, despite an equivalent amount of protein (Elliot et al., 2006). Similarly, our lab has previously shown that ingestion of whole eggs resulted in a greater stimulation of muscle protein synthesis and reduced whole body leucine oxidation rates when compared to the ingestion of an isonitrogenous amount of egg whites (Van

Vliet et al., 2017). As such, underpinning the potential anabolic properties of the food matrix (i.e., the interaction of all the nutrients in an ingested food source) will provide a better understanding of the value of whole food vs. isolated nutrient based recommendations to stimulate muscle protein synthesis rates (Burd et al., 2019a). The latter is an important marker of both hypertrophic and non-hypertrophic protein modeling; a process relevant for the maintenance of muscle mass and health (Phillips et al., 1997, Biolo et al., 2002).

Indeed, some studies suggest that the anabolic action of certain foods cannot be solely attributed to the essential amino acid (and leucine) content of the ingested food source (Burd et al., 2015, Van Vliet et al., 2017, Elliot et al., 2006, Katsanos et al., 2008) as previously believed (Tang and Phillips, 2009). Certainly, protein content is a primary predictor of the anabolic potential of a food source, but it is unknown if discrepancies between treatment conditions in either energy or macronutrient composition have influenced our previous observation on the anabolic potency of a protein food source compared to its protein in isolation (Van Vliet et al., 2017). Furthermore, whole foods also contain non-energy bioactive compounds, such as vitamins, minerals, and microRNAs (Baier et al., 2014, Baier et al., 2015, Quitete et al., 2019) that may, separately or together, potentiate the use of dietary amino acids to stimulate of muscle protein synthesis. Therefore, an investigation that matches total energy and macronutrient composition between protein food sources would more reliably characterize the influence of the food matrix on the stimulation of postprandial muscle protein synthesis rates.

The purpose of the current study was to investigate the effects of the food matrix on the stimulation of post-exercise muscle protein synthesis rates after the ingestion of salmon (SAL) (20.5 g protein and 7.5 g fat) or its matched constituents in the form of crystalline amino acids, coconut oil, and fish oil (ISO) in healthy young adults. We also examined the stimulation of whole-

body leucine oxidation rates and anabolic signaling pathways to provide additional insight into the anabolic potential of the treatment conditions. We hypothesized that eating protein within its original food matrix will lead to greater stimulation of postprandial myofibrillar protein synthesis and lower whole body leucine oxidation rates when compared to eating those same nutrients in isolation.

2.3 METHODS

Participants and ethical approval

Ten healthy, recreationally active adults (n=5 males and n=5 females; 24 ± 4 y; 25.5 ± 2.1 $kg \cdot m^{-2}$; Mean ± SD) volunteered to participate in this study. All participants were recruited and performed two infusion trials in our clinical laboratory at the University of Illinois Urbana-Champaign from June 2019 to November 2019. Female participants performed the trials during the follicular phase of the menstrual cycle to eliminate any potential differences in protein metabolic responses (Draper et al., 2018, Lariviere et al., 1994). All participants were deemed healthy based on their responses to a routine medical screening questionnaire. Volunteers had no history of participating in stable isotope amino acid tracer experiments prior to the first trial. All participants were informed about the experimental procedures, the purpose of the study, and potential risks before giving written consent. All trials conformed to standards for the use of human participants in research as outlined in the Helsinki Declaration (ClinicalTrials.gov ID NCT03870165) and were approved by the local Institutional Review Board at the University of Illinois at Urbana-Champaign (IRB # 18897).

Study design and trial preparations

A randomized, crossover design was used for this study. Two weeks before the infusion trial, participants reported to the laboratory in the morning after an overnight fast for measurement of body weight and height, body composition by dual-energy x-ray absorptiometry (DEXA; Horizon W, Hologic Inc., Marlborough, MA, USA), and muscular strength via 10-repetition maximum (RM) testing. After the preliminary testing session, participants were randomized to ingest either salmon (SAL) or isolated nutrients (ISO) in a counterbalanced fashion on their first infusion trial. For trial allocation, we used a computer-generated list of random numbers. Participants were instructed to refrain from any strenuous physical exercise and alcohol consumption for at least 72 hours prior to the infusion trials. Participants were provided an identical standardized meal for consumption the evening before both trials (25-30% of estimated energy requirement; 50% of energy from carbohydrate, 25% energy from protein and 25% energy from fat). In addition, participants were instructed to record their dietary intake for 2 days prior to the first infusion trial by using the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool (version 2016; National Cancer Institute, Bethesda, MD, USA). Subsequently, during the 2 days leading up to the second infusion trial, participants were given a copy of their dietary intake and instructed to repeat their dietary intake from the 2 days before the first infusion trial. The time between crossover trials was 7 days. A CONSORT (Consolidated Standards of Reporting Trials) flowchart of the study is presented **Figure A.1.**

Treatment conditions

Salmon filets were obtained from the same farm-raised Scottish salmon. All filets had the skin removed and were cut to 3.5 oz (99 g) portions prior to storage. The nutrient content of the salmon

filets were analyzed by proximate analysis for protein, lipid and carbohydrate concentrations according to methods set forth by the Association of Official Analytical Collaboration (**Table B.1)** (Latimer, 2016). For the isolated nutrients treatment, amino acid contents were then matched using individual crystalline amino acids (Ajinimoto, Itasca, IL, USA). Fish oil was used to match eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content, after which coconut oil was used to match total fat content between treatments. The salmon filets were frozen after harvest and thawed the night before each trial in a refrigerator at 4°C. The morning of the trial day, each salmon filet was prepared sous-vide for ~30 min until the internal temperature reached 63°C. The isolated nutrients were dissolved in 300 mL of water. Participants consumed 300 mL of water with the salmon in the SAL condition. The portion size of salmon was selected to provide an amount of protein (20 g) known to robustly stimulate muscle protein synthesis while minimizing whole body amino acid oxidation rates (Moore et al., 2009). Salmon was selected as the food source because it is a lipid and protein dense food matrix that complies with both the Healthy US-style and Mediterranean-style dietary pattern.

Infusion protocol

An overview of the infusion trial is shown in **Figure 2.1** For both trials, participants reported to the laboratory at 0600 h after an overnight fast by car or public transport. Following assessment of blood pressure and body weight, a Teflon catheter was inserted into an antecubital vein and an arterialized baseline blood sample was collected. In addition, a baseline breath sample was collected in a 10-mL additive-free Vacutainer tube. Participants subsequently (t = -210 min) received priming doses of L-[*ring*-$^{2}H_5$]phenylalanine (2.0 μmol·kg^{-1}), L-[1-$^{13}C_6$]Leucine (7.6 μmol·kg^{-1}) and $NaH^{13}CO_3$ (2.35 μmol·kg^{-1}). After administration of priming doses, a continuous

intravenous infusion was started of L-[*ring*-$^{2}H_5$]phenylalanine (0.05 μmol·kg·min^{-1}) and L-[1-$^{13}C_6$]Leucine (0.130 μmol·kg·min^{-1}) for the measurement of myofibrillar protein synthesis rates (MPS) and whole body leucine oxidation rates, respectively. A second Teflon catheter was inserted into a heated dorsal hand, or antecubital, vein of the opposing arm for repeated blood sampling and kept patent by a 0.9% saline drip. Muscle biopsies were collected before (*t* = -150 and -30 min) and after (*t* = 120 and 300 min) the ingestion of SAL or ISO. On the second trial day, no *t* = -150 biopsy was performed as post-absorptive MPS rates were determined in trial 1. Biopsies were collected under local anesthetic from the middle region of the *m. vastus lateralis* (~15 cm above the patella) with a Bergström needle modified for manual suction. Each consecutive biopsy was taken more proximal with a new incision. The post-absorptive muscle biopsy samples in trial 1 were collected from the same incision with the needle pointed to distal and proximal directions, respectively. Muscle samples were freed from any blood, fat, and visible connective tissue and immediately frozen in liquid nitrogen prior to storage at -80°C until further analysis. After collection of the resting muscle biopsy sample at *t* = -30 for both trials, the participants performed a bout of resistance exercise that consisted of 4 sets of 10 repetitions at 90% of 10-RM for both the leg press and leg extension exercises. External work (repetitions ×load) was matched between the SAL and ISO trials. Immediately after completion of the exercise bout, participants consumed 3.5 oz of salmon or the matched isolated nutrients dissolved in water. Arterialized blood samples were collected in EDTA-containing tubes before (*t* = -210, -150, -90, -60, -30, -5 min) and after treatment ingestion (*t* = 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240 and 300 min). Blood samples were centrifuged at 3000 *g* for 10 min at 4°C and the plasma was subsequently aliquoted and stored at -80°C for future analysis. Breath samples were taken at baseline and concomitantly with the postprandial blood samples. Total carbon dioxide production rates were measured with a

metabolic cart (TrueOne 2400; ParvoMedics, Salt Lake City, UT, USA) at regular intervals throughout the infusion trials. The breath samples were collected in 10 mL additive-free evacuated tubes and stored at room temperature until subsequent determination of $^{13}CO_2$ enrichment by isotope ratio mass spectrometry (IDmicro Breath; Compact Science Systems Ltd., Newcastle, UK).

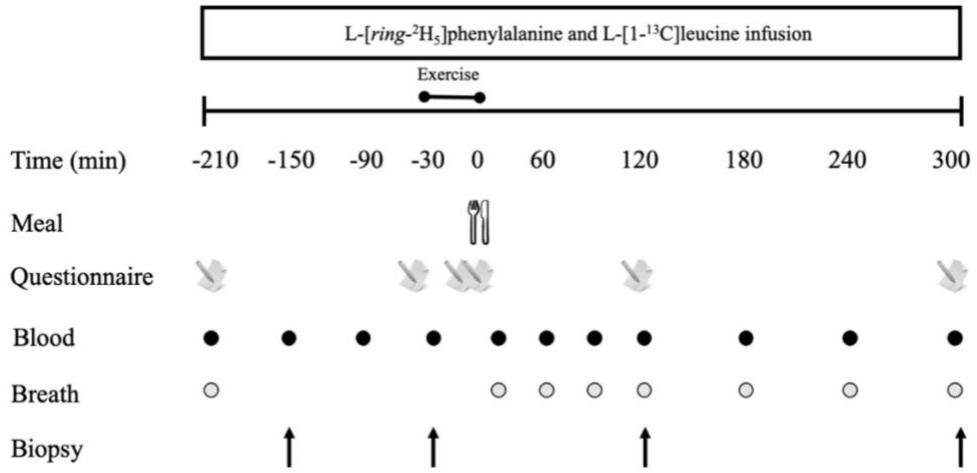

Figure 2.1. An overview of the experimental trials. Participants consumed 99 g of salmon filet (20.5 g protein, 7.5 g fat) or an isolated nutrients drink matched for macronutrient content.

Questionnaires

A rating of perceived exertion scale (RPE; Borg scale, 6-20) was used to monitor the participant's subjective perception of effort during the exercise bout (Borg, 1998), as well as a Feeling Scale (FS) as a measurement of affect (Hardy and Rejeski, 1989), a Felt Arousal Scale (FAS) to measure perceived activation level (Svebak and Murgatroyd, 1985), and a 4-item Visual Analog Scale (VAS) to measure emotion (Lee et al., 1991). All questionnaires were combined on one sheet of paper and totaled 7 short questions. The questionnaires were filled in at multiple time points (t = -210, -30, -5, 0, 120 and 300) to assess affective state throughout the trial day (**Figure 1**).

Plasma hormones analyses

Plasma insulin concentrations were determined using commercially available enzyme-linked immunosorbent assays (ELISA, Alpco Diagnostics; Salem, NH, USA). To confirm female participants performed both experimental days during the follicular phase of their menstrual cycle, plasma estradiol and progesterone were determined using commercially available ELISAs (Alpco Diagnostics; Salem, NH, USA)

Plasma metabolite analyses

Plasma glucose concentrations were analyzed by an automated biochemical analyzer (2900 Stat Plus, YSI Life Sciences, Yellow Springs, OH). Plasma amino acid concentrations and enrichments were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS; Thermo Altis Triple Quadrupole, Thermo Fisher Scientific, Waltham, MA, USA) The amino acid standard solution (AAS18, Sigma, USA), containing 2.5 μmol·mL^{-1} each of l-alanine, l-arginine, l-aspartic acid, l-glutamic acid, glycine, l-histidine, l-isoleucine, l-leucine, l-lysine·HCl, l-methionine, l-phenylalanine, l-proline, l-serine, l-threonine, l-tyrosine, and l-valine, and 1.25 μmoL·mL^{-1}l-cystine and a custom mixture containing 2.5 μmol·mL^{-1}each of l-tryptophane, l-glutamile, l-asparagine, l-citrulline, l-cysteine were used for the calibration curve. Plasma samples (50 μL) were deproteinized with methanol (930 μL), centrifuged after supernatant evaporation in vacuum and resuspended in 1 mL of 0.1% formic acid in water before instrument injection. Twenty microliters of internal standard (DL-p-chlorophenylalanine, 0.01 mg·mL^{-1}) was added to each sample and standard solution. Samples were analyzed by Thermo Altis Triple Quadrupole LC/MS/MS system. Software TraceFinder 4.1 was used for data acquisition and analysis. The LC separation was performed on a Thermo Accucore Vanquish C18+ column (2.1 × 100 mm, 1.5 μm)

with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetontrile) and the flow rate was 0.2 $mL \cdot min^{-1}$. The linear gradient was as follows: 0 to 0.5 min, 0% B; 0.5 to 3.5 min, 60% B; 3.5 to 5.5 min, 100% B; 5.5 to 7.5 min, 0% B. The autosampler and HPLC column chamber were set at 10°C and 50°C, respectively. The injection volume was 1 µL. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage of 3500 V. Selected reaction monitoring used for the amino acid quantitation.

Plasma fatty acid composition was analyzed by a GC-MS system (Agilent Inc, CA, USA) consisting of an Agilent 7890B gas chromatograph and an Agilent 5977A MSD. Plasma samples were extracted as follows: each sample (150 µL plasma) was extracted with 0.5 mL of methanol:chloroform (1:2 v/v) and centrifuged at 20817 g for 10 min at 4 °C. Extract was divided into 2 equal parts each for free and total fatty acid analysis. 15 µL of C23:0 (1 $mg \cdot mL^{-1}$) was added as an internal standard to each part and organic phase was evaporated under vacuum. Total fatty acid analysis was conducted by hydrolyzing dried extract with 500 µL of 3 N methanolic HCl containing 2 $g \cdot L^{-1}$ BTH for 1 h at 85°C. After cooling to room temperature, 500 µL of hexane + BTH (2 $g \cdot L^{-1}$) was added, shaken for 30 s and, subsequently, the organic phase was collected and injected into the GC/MS system. Separation was performed on a HP-5MS (60 m × 0.32 mm I.D. and 250 µm film thickness) capillary column (Agilent J&W, CA, USA). The inlet temperature was 220°C, MSD interface temperature 230°C and the ion source temperature adjusted to 230°C. An aliquot of 1 µL was injected in a split less mode (20 $mL \cdot min^{-1}$ at 1.5 min). The helium carrier gas was kept at a constant flow rate of 2 $mL \cdot min^{-1}$. The temperature program was: 2 min at 150°C followed by a temperature increase of 5°C·min^{-1} to 300°C for 3 min. The mass spectrometer was operated in positive electron impact (EI) mode at 69.9 eV ionization energy at m/z 33-800 scan range. Target peaks were evaluated using Mass Hunter Quantitative Analysis B.08.00 (Agilent

Inc., USA) software. Standard curves were generated for 200 – 0.07 $\mu g \cdot mL^{-1}$ range. Standards were prepared according to the same steps as actual samples. Fatty acid standards were obtained from Cayman Chemical (USA).

Muscle analyses

Myofibrillar protein-enriched fractions were extracted from ~50 mg of wet muscle using an ice-cold lysis buffer (Pierce® RIPA Buffer; Thermo Fisher Scientific, Waltham, MA, USA; 10 $\mu L \cdot mg^{-1}$;) supplemented with protease and phosphatase inhibitor tablets (cOmplete and PhosSTOP; Roche Applied Science, Mannhein, Germany). Myofibrillar proteins were isolated by differential centrifugation as described previously (Salvador et al., 2019). The remaining muscle homogenate was stored at −80°C for subsequent Western blot analysis. The isolated myofibrillar protein fractions were hydrolyzed overnight in 6 M HCl at 110°C. The resultant free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before analysis by 5500 QTRAP (Sciex, Redwood City, CA, USA) LC-MS/MS (Van Vliet et al., 2017). The myofibrillar protein-bound L-[*ring*-$^2H_5$]phenylalanine enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 166.0 → 103.0 and 171.0 → 106.0 for unlabeled and labelled L-[*ring*-$^2H_5$]phenylalanine, respectively. Analyst V1.6.2 (Sciex, Framingham, MA, USA) was used for data acquisition and analysis.

Western blotting

A portion of the whole-muscle homogenates isolated during the myofibrillar extractions were used for western blotting analysis. Total protein concentrations were determined using BCA assays

(Pierce® BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein from each sample were mixed with loading buffer and diluted to the same concentration using homogenization buffer. Subsequently, samples were denatured at 95°C for 5 minutes, separated on 7.5% or 10% (w/v) polyacrylamide gels, and then electrophoretically transferred to polyvinylidene fluoride membranes (PVDF; MilliporeSigma, St. Louis, MO, USA). Membranes were blocked with non-fat milk or bovine serum albumin diluted in Tris-buffered saline with Tween solution for 1 h at room temperature before overnight incubation in primary antibodies at 4°C. Proteins of interest were detected with primary antibodies as follows: rabbit anti-α-tubulin (1:1000; Abcam Cat# ab4074, RRID:AB_2288001), rabbit anti- phospho-Ribosomal protein S6 kinase β 1 (p70S6K; Thr389) (1:500; Cell Signaling Technology Cat# 9205, RRID:AB_330944), rabbit anti-p70S6K (1:500; Cell Signaling Technology Cat# 9202, RRID:AB_331676), rabbit anti-phospho-Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; Thr37/46) (1:1000; Cell Signaling Technology Cat# 9459, RRID:AB_330985), rabbit anti-4E-BP1 (1:1000; Cell Signaling Technology Cat# 9452, RRID:AB_331692), rabbit anti-phospho-Eukaryotic elongation factor 2 (eEF2; Thr56) (1:1000; Cell Signaling Technology Cat# 2331, RRID:AB_10015204), rabbit anti-eEF2 (1:1000, Cell Signaling Technology Cat# 2332, RRID:AB_10693546),), rabbit IgG anti-phospho-Ribosomal protein S6 (rpS6; Ser240/244) (1:1000, Cell Signaling Technology Cat# 5364, RRID:AB_10694233) and rabbit IgG anti-rpS6 (1:1000, Cell Signaling Technology Cat# 2217, RRID:AB_331355). After primary incubation, blots were exposed to horseradish-peroxidase-conjugated horse anti-mouse IgG (1:2000; Cell Signaling Technology Cat# 7076, RRID:AB_330924) or goat anti-rabbit IgG (1:2000-1:10,000; Abcam Cat# ab6721, RRID:AB_955447) and detected using the ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA) and the ChemiDoc XRS+ Imaging System (Bio-Rad

Laboratories, Hercules, CA, USA). Bands were quantified using ImageJ software (Schneider et al., 2012) and normalized to α-tubulin as the internal control.

Calculations

Whole body leucine oxidation rates were calculated from the appearance of the ^{13}C-label in the expired carbon dioxide by using the a-[^{13}C]ketoisocaproate reciprocal pool model with fractional bicarbonate retention factors of 0.70 and 0.83 for postabsorptive and postprandial states, respectively (Hoerr et al., 1989). Myofibrillar protein fractional synthesis rates (FSR) were calculated using the standard precursor product method by dividing the increment in L-[*ring*-$^{2}H_5$]phenylalanine enrichment into myofibrillar proteins by the enrichment of a surrogate precursor pool (i.e., plasma) over time as described previously (Salvador et al., 2019).

Statistical analyses

All data are presented as mean ± standard deviation (SD). *A priori* power analysis of the human experiment was conducted using GPOWER version 3.1.9.2 (Faul et al., 2007). Based on previous research (Van Vliet et al., 2017, Burd et al., 2015), our power analysis showed that a sample size of 10 participants was sufficient to detect differences in postprandial muscle protein synthesis between conditions when using a repeated measures statistical test ($P < 0.05$, 80% power, $f = 0.45$). Differences in blood metabolite concentrations, temporal muscle protein synthesis rates, and intramuscular signaling were analyzed using linear fixed effects models with time and group as fixed factors. Bonferroni's post hoc test was used when significant main effects were identified. The leucine oxidation, plasma amino acid availability, plasma leucine, branched-chain amino acids, essential amino acids, total amino acids, and insulin concentrations were calculated as an

area under the curve above baseline values. Differences in the area under the curve measures were tested with a Student's t-test. The level of statistical significance was set at $P < 0.05$ for all analyses. All analyses were performed using IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA).

2.4 RESULTS

Participant and exercise characteristics

Participants' characteristics and dietary intake are presented in **Table 2.1**. There was no significant difference in the resistance exercise volume load for leg press in the ISO (14877 ± 3712 kg) and SAL trials (14859 ± 3733 kg; $P = 0.343$). Similarly, leg extension exercise did not differ between the ISO (7206±1179 kg) and SAL (7224±1176 kg; $P = 0.343$) trials.

RPE and affective responses

4-point VAS scores were as follows: fatigue increased over time ($P < 0.001$), with no differences between conditions ($P = 0.573$); participants reported being more tense and jittery after the exercise session (both $P < 0.001$), with no differences between conditions ($P = 0.378$ and $P = 0.700$, respectively); participants reported being less energetic over time ($P = 0.026$), with no differences between conditions ($P = 0.224$). RPE increased after the exercise sessions ($P < 0.001$), with no differences between conditions ($P = 0.960$). FS did not change over time ($P = 0.822$), with no differences between conditions ($P = 0.556$). FAS increased after exercise ($P < 0.001$), with no differences between conditions ($P = 0.333$).

Table 2.1. Participant characteristics

Variable	
Age (y)	24 ± 4
Weight (kg)	80.51 ± 9.04
BMI ($kg \cdot m^{-2}$)	25.52 ± 2.08
Systolic Blood Pressure (mmHg)	120 ± 14
Diastolic Blood Pressure (mmHg)	74 ± 12
Body Fat (%)	27.7 ± 6.5
Lean Body Mass (kg)	54.42 ± 7.22
Fasting Glucose ($mg \cdot dL^{-1}$)	79.79 ± 6.33
Energy Intake ($kcal \cdot day^{-1}$)	2301 ± 729
Protein Intake ($g \cdot d^{-1}$)	115 ± 37
Carbohydrate Intake ($g \cdot d^{-1}$)	241 ± 119
Fat Intake ($g \cdot d^{-1}$)	98 ± 25

Data are presented as Mean ± SD

Blood analyses

Plasma insulin concentrations increased over time ($P < 0.001$) and was significantly higher in ISO when compared to SAL condition ($P = 0.038$). The net area under the curve (AUC) for postprandial plasma insulin concentrations was significantly higher in ISO when compared to SAL condition ($P = 0.042$; **Figure 2.2A**). Plasma glucose concentrations increased after exercise ($P = 0.022$) and immediately returned to baseline values with no differences between groups ($P = 0.477$; **Figure 2.2B).** Baseline plasma progesterone (SAL: 0.66 ± 0.31 $ng \cdot mL^{-1}$ vs. ISO: 0.74 ± 0.47 $ng \cdot mL^{-1}$; $P = 0.371$) and estradiol concentrations (SAL: 50.36 ± 90.62 $pg \cdot mL^{-1}$ vs. ISO: 20.09 ± 23.91 $pg \cdot mL^{-1}$; $P = 0.295$) did not differ between conditions in female participants. All female baseline plasma progesterone values were <2 $ng \cdot mL^{-1}$ and all female baseline plasma estradiol values ranged

between 20 – 200 pg·mL^{-1}, indicating compliance of study participation within the follicular phase in both experimental conditions (Allen et al., 2016).

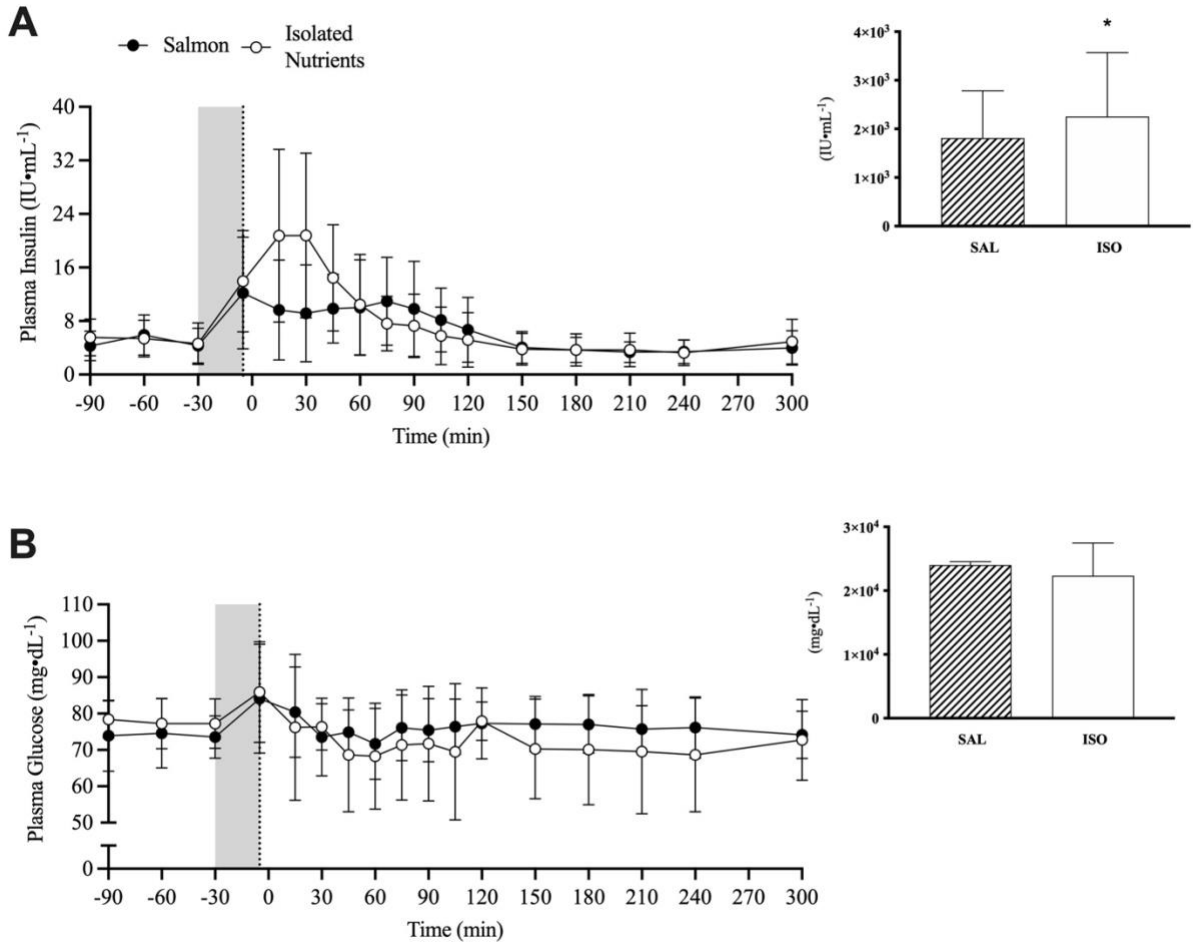

Figure 2.2. Plasma insulin (A) and glucose (B) concentrations during the post-absorptive and postprandial period in young adults (n = 10). Insets show the postprandial area under the curve (AUC). Ingestion of salmon or isolated nutrients drink is denoted by dashed line. Grey highlighted area represents exercise bout. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors. A Bonferroni post hoc test was used when significant differences were identified. * Indicates significant ($P < 0.05$) difference between groups. Insulin: time effect, $P < 0.001$; condition effect: $P = 0.038$; AUC: $P = 0.042$. Glucose: time effect, $P = 0.022$.

Plasma amino acid concentrations and enrichments

Plasma phenylalanine concentrations increased over time ($P < 0.001$*),* with no differences between the ISO and SAL conditions ($P = 0.096$*;* **Figure 2.3A**). Time to peak phenylalanine concentrations was faster in ISO (53 ± 27 min; 184.0 ± 41.5 μmol·L^{-1}) compared to SAL (95 ± 40 min; 157.8 ± 50.5 μmol·L^{-1}; $P = 0.009$). Plasma leucine concentrations increased after food ingestion ($P < 0.001$), with no main effect of condition ($P = 0.826$**),** but differed between treatments from $t = 15$ to 45 min and $t = 105$ and 120 min (time x condition interaction, $P < 0.001$; **Figure 2.3B**). Time to peak plasma leucine concentrations was faster in ISO (47 ± 23 min; 555.2 ± 128.4 μmol·L^{-1}) compared to SAL (97 ± 34 min; 475.2 ± 145.5 μmol·L^{-1}; $P < 0.001$) condition. Postprandial plasma essential amino acid concentrations increased over time ($P < 0.001$), with no main effect of condition ($P = 0.122$), but differed between treatments from $t = 15$ to 45 min (time x condition interaction, $P = 0.024$; **Figure 3C**). Time to peak plasma essential amino acid concentrations was faster in ISO (45 ± 23 min; 3092.5 ± 1034.4 μmol·L^{-1}) compared to SAL (96 ± 37 min; 3854.7 ± 1223.2 μmol·L^{-1}; $P < 0.001$).

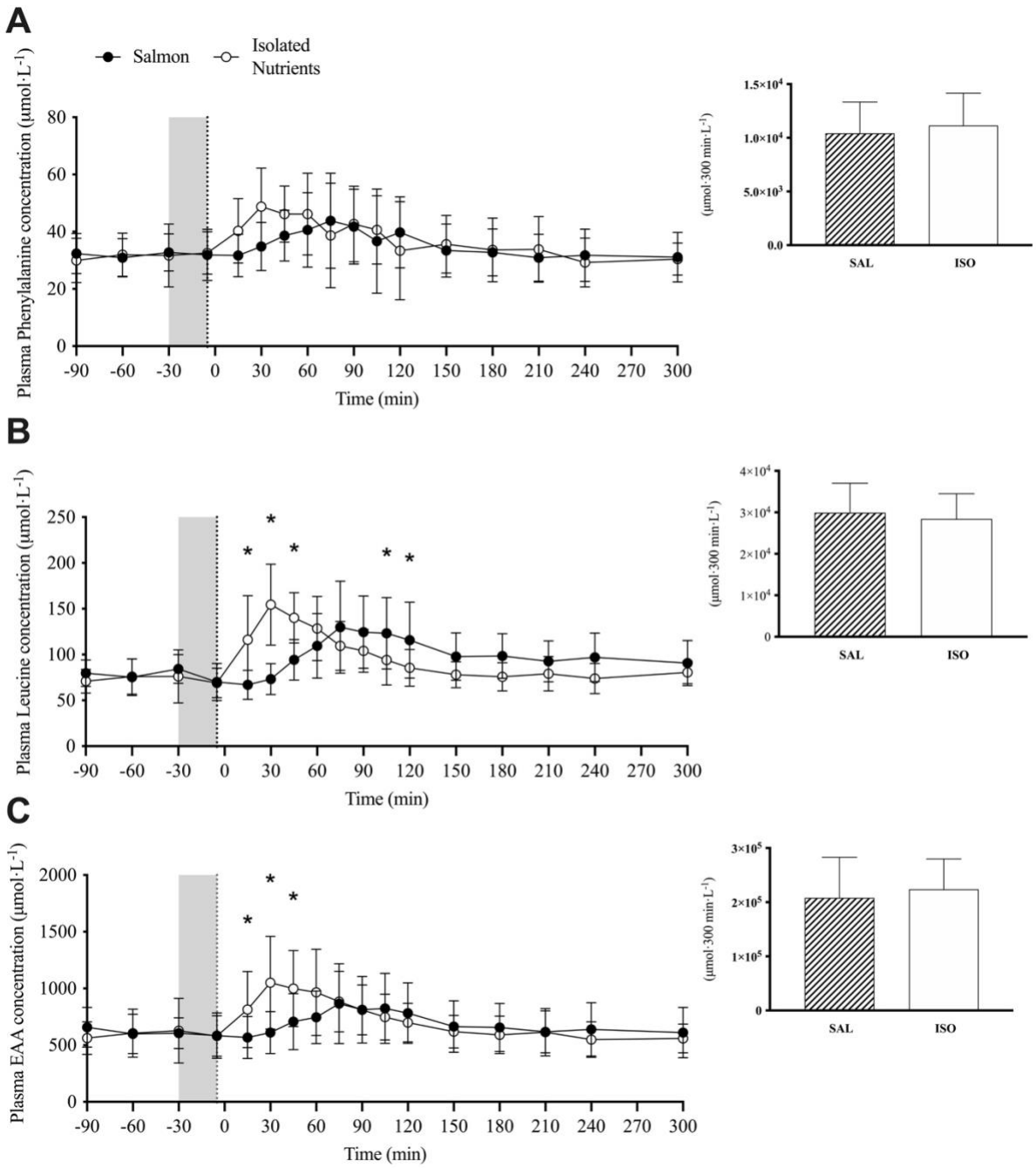

Figure 2.3. Plasma Phenylalanine (A), leucine (B), and essential amino acid (EAA; C) concentrations during the post-absorptive and postprandial period in young adults (n=10). Insets show the postprandial area under the curve (AUC). Ingestion of salmon or isolated nutrients drink is denoted by dashed line. Grey highlighted area represents exercise bout. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors. A Bonferroni post hoc test was used when significant differences were identified. * Indicates significant ($P < 0.05$) difference between groups. Phenylalanine: time effect, $P < 0.001$. Leucine: time effect, $P < 0.001$; time × condition, $P < 0.001$. EAA: time effect, $P < 0.001$; time × condition, $P = 0.024$.

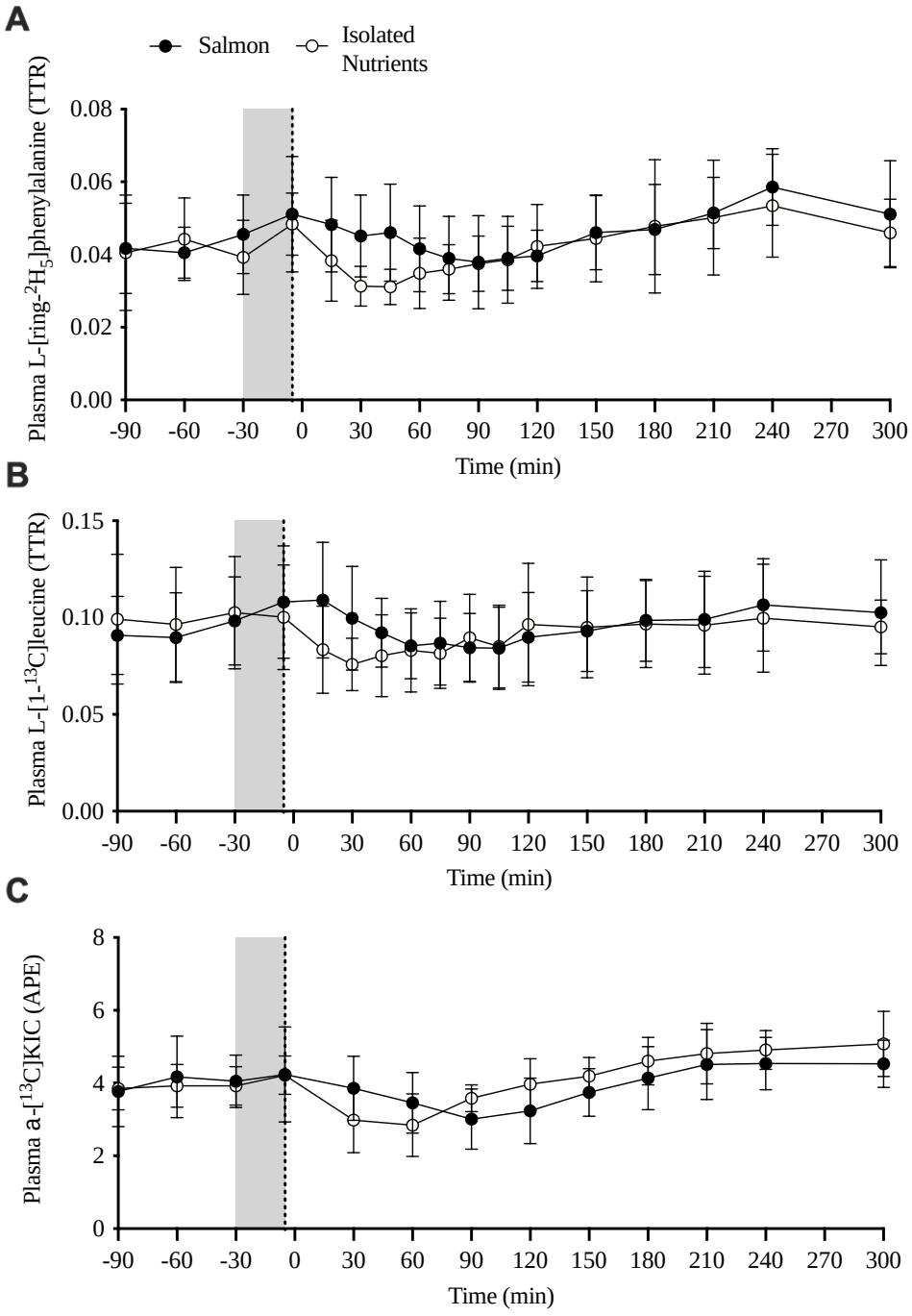

Figure 2.4. Plasma L-[ring-$^{2}H_5$]phenylalanine (A), L-[1-$^{13}C_6$]Leucine (B) and a-[^{13}C]ketoisocaproate (KIC) (C) enrichments in the basal-state and after ingestion of salmon or isolated nutrients drink in young adults (n=10). All enrichments are expressed as tracer·tracee^{-1}. Ingestion of salmon or isolated nutrients drink is denoted by dashed line. Grey highlighted area represents exercise bout. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors. A Bonferroni post hoc test was used when significant differences were identified. L-[ring-$^{2}H_5$]phenylalanine: time effect, $P < 0.001$; condition effect: $P = 0.001$. α-[^{13}C]ketoisocaproate: time effect, $P < 0.001$. TTR, tracer-to-tracee ratio; APE, atom percent excess.

Plasma L-[ring-$^{2}H_5$]phenylalanine enrichments decreased 75 min after food ingestion (P < 0.001) and returned to baseline 105 min after food ingestion, with higher enrichments in SAL when compared to ISO (P = 0.001; **Figure 2.4A**). Plasma L-[1-^{13}C]leucine enrichments remained stable over time (P = 0.178), with no differences between condition (P = 0.268; **Figure 2.4B**). Plasma α-[^{13}C]ketoisocaproate enrichments decreased 60 min after food ingestion (time effect: P < 0.001) and returned back to baseline 150 min after food ingestion, with no differences between condition (P = 0.281; **Figure 2.4C**).

Plasma lipid analyses

Plasma total fatty acids concentrations did not change over time (P = 0.421), with no differences between conditions (P = 0.996; **Figure 2.5A**). Plasma total EPA increased over time (P = 0.001), with higher plasma total EPA concentrations in SAL condition overall (P < 0.001). Similarly, postprandial plasma total EPA AUC was greater in SAL when compared to ISO (P = 0.010; **Figure 2.5B**). Plasma total DHA did not change over time (P = 0.154), with no effect of condition (P = 0.084). Postprandial plasma total DHA AUC was similar in both conditions (P = 0.076; **Figure 2.5C**).

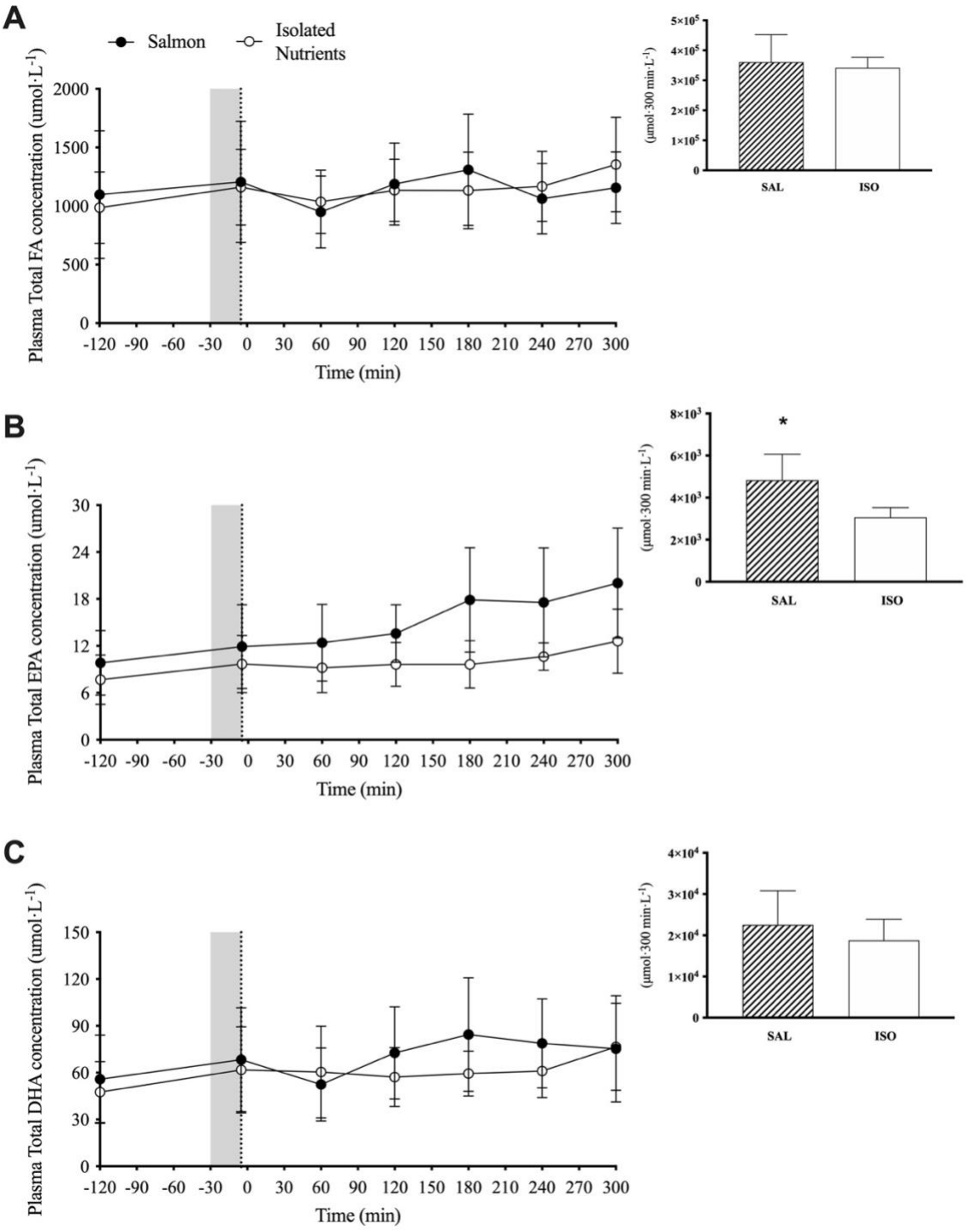

Figure 2.5. Plasma total fatty acids (FA; A), total eicosapentaenoic acid (EPA; B) and total docosahexaenoic acid (DHA; C) in the basal-state and after ingestion of salmon or isolated nutrients drink in young adults (n=10). Insets show the postprandial area under the curve (AUC). Ingestion of salmon or isolated nutrients drink is denoted by a dotted line. Grey highlighted area represents exercise bout. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors. A Bonferroni post hoc test was used when significant differences were identified. * Indicates significant ($P < 0.05$) difference between conditions. Total EPA: time effect, $P = 0.001$; condition effect, $P < 0.001$. Total DHA: condition effect, $P = 0.084$; AUC, $P = 0.076$.

Intramuscular signaling

Phosphorylation of p70S6K did not change over time (P = 0.786), with no differences between condition (P = 0.076). Phosphorylation of 4E-BP1 did not change over time (P = 0.290), nor was there a significant difference between conditions (P = 0.126). Phosphorylation of eEF2 did not change over time (P = 0.701), with no differences between condition (P = 0.781). Phosphorylation of rpS6 increased over time (P = 0.037), with no differences between condition (P = 0.267).

Myofibrillar protein synthesis

The post-exercise myofibrillar protein synthetic rates were stimulated over time (P < 0.001) in both nutrition conditions early (0-2 h; SAL: 0.077 ± 0.037 %·h^{-1}, ISO: 0.059 ± 0.047 %·h^{-1}; P < 0.001) and remained elevated during the later (2-5h; SAL: 0.045 ± 0.019 %·h^{-1}, ISO: 0.039 ± 0.026 %·h^{-1}; P = 0.003) recovery phase, with no effect of condition (P = 0.329). Similarly, SAL (0.056 ± 0.022 %·h^{-1}; P = 0.001) and ISO (0.046 ± 0.025 %·h^{-1}; P = 0.025) stimulated myofibrillar protein synthesis rates during the entire 0-5 h recovery period, with no differences between conditions (P = 0.308; **Figure 2.6**).

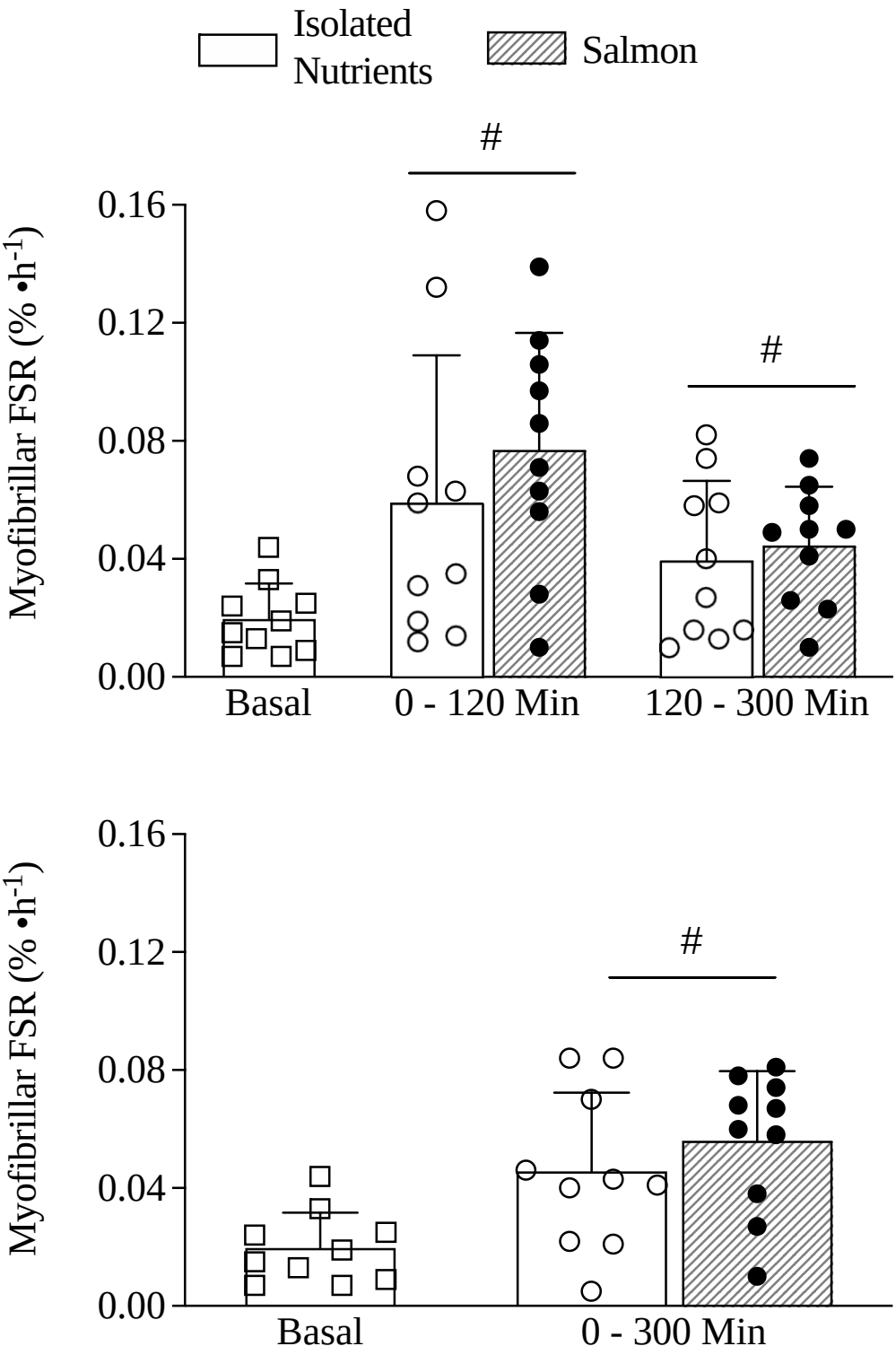

Figure 2.6. Myofibrillar fractional synthetic rate (FSR) temporal (A) and cumulative (B) at rest and after the ingestion of salmon or isolated nutrients drink in young adults (n=10). Data were analyzed using a linear fixed effects model. Values are presented as mean ± SD. # indicates significantly ($P < 0.05$) different from rest. Time effect, $P < 0.001$.

Whole-body leucine oxidation

Whole-body leucine oxidation rates increased over time ($P < 0.001$), with no differences between conditions ($P = 0.702$), and displayed a significant time x condition interaction ($P = 0.003$). Whole-body leucine oxidation rates were elevated from baseline at $t = 30$ ($P < 0.001$) and returned to basal at $t = 150$ min ($P = 0.376$) in ISO. Conversely, whole body leucine oxidation rates were elevated at $t = 60$ min ($P = 0.002$) and returned to basal at $t = 150$ min ($P = 0.061$) in the SAL condition. Time to peak leucine oxidation rates occurred sooner in ISO (63 ± 25 min; 1.239 ± 0.321 nmol·kg^{-1}·min^{-1}) when compared to the SAL condition (105 ± 20 min; 1.230 ± 0.561 nmol·kg^{-1}·min^{-1}; $P = 0.003$; **Figure 2.7**).

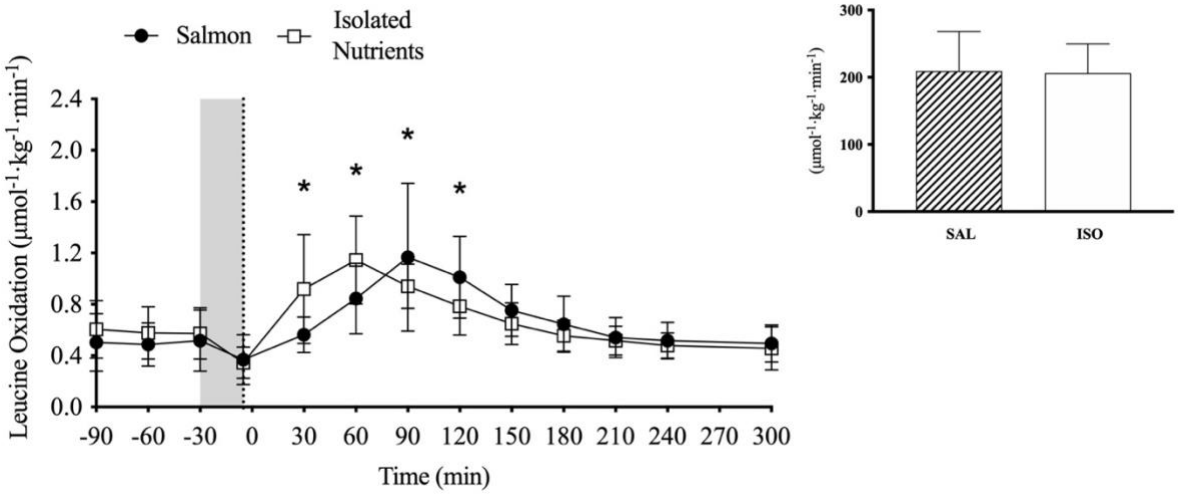

Figure 2.7. Leucine oxidation rate at rest and after the ingestion of salmon or isolated nutrients drink in young adults (n=10). Insets show the postprandial area under the curve (AUC). Ingestion of salmon or isolated nutrients drink is denoted by a dotted line. Grey highlighted area represents exercise bout. Data were analyzed using a linear fixed effects model. Values are presented as mean ± SD. A Bonferroni post hoc test was used when significant differences were identified. * Indicates significant ($P < 0.05$) difference between conditions. Time effect: $P < 0.001$; time × condition, $P = 0.003$.

2.5 DISCUSSION

The anabolic potential of protein foods has largely been attributed to their amino acid composition, and subsequent digestibility, to robustly elevate plasma amino acid availability and muscle protein synthesis rates (Gwin et al., 2020). Food matrix effects, however, have also been implicated as a potential mediator of the post-exercise muscle protein synthetic response (Burd et al., 2019a). However, no study to date has sought to directly investigate the anabolic properties of consuming protein within its food matrix vs. the sum of its parts. This study demonstrated that the ingestion of salmon resulted in a more prolonged postprandial increase in plasma leucine concentrations, which lead to a delayed stimulation of whole-body leucine oxidation rates, when compared to the ingestion of isolated nutrients during the immediate recovery period after resistance exercise. However, both the temporal (0-2 or 2-5 h) and cumulative (0-5 h) stimulation of post-exercise myofibrillar protein synthesis rates were not statistically different between the two conditions.

Current protein recommendations aimed at robustly stimulating muscle protein synthesis rates have been primarily derived from the ingestion of isolated proteins with little to no consideration of the anabolic properties of other nonprotein nutritive factors that are commonly contained in protein rich foods (Vliet et al., 2018). Certainly, the ingestion of crystalline amino acids and isolated protein supplements (e.g., whey) have been shown effective at stimulating post-exercise muscle protein synthesis rates (Biolo et al., 1997, Katsanos et al., 2008). However, these 'fast' forms of amino acid ingestion result in a rapid, and transient, increase in blood amino acid profiles during the postprandial/postexercise period. Importantly, in addition to non-nutritive factors, it has been demonstrated that the pattern of aminoacidemia is more prolonged with the ingestion of nutrient and protein dense foods *vs.* isolated nutrients (Burd et al., 2015, Van Vliet et al., 2017, Hermans et al., 2022, Weijzen et al., 2022). In line with this, our work demonstrated that

the ISO condition resulted more rapid time to peak in plasma leucine and essential amino acid concentrations when compared to the SAL condition (**Figure 3**). This differential pattern of peak aminoacidemia resulted in a greater early stimulation of leucine oxidation rates in the ISO vs. SAL condition, but similar net AUC during the 0-5 h postprandial period (**Figure 7**).

Despite this differential pattern of aminoacidemia and leucine oxidation rates, we did not observe a differential stimulation of post-exercise myofibrillar protein synthesis rates during the early (0-2 h), later (2-5 h), or cumulative (0-5 h) periods of recovery. Such results are consistent with the notion that the anabolic trigger related to blood leucine concentrations on muscle anabolism was likely more pertinent to the original whey (a higher leucine protein source) vs. soy comparison (Tang et al., 2009) as opposed to an absolute rule of thumb when compared to a wider variety of food protein comparisons (Pinckaers et al., 2021, Churchward-Venne et al., 2015, Hamarsland et al., 2019, Burd et al., 2015, Van Vliet et al., 2017, Hermans et al., 2022, Hermans et al., 2021).

The ingestion of whole protein foods provides other nutrients beyond amino acids alone. In this particular case, salmon is an optional protein food that can be incorporated into both US-style and Mediterranean-style dietary patterns. The health benefits of salmon ingestion are often attributed to its high concentrations of omega-3 fatty acids (Jensen et al., 2012, Dewailly et al., 2007). Here, we observed that the postprandial plasma concentrations of EPA were higher and DHA concentrations tended to be higher when consuming salmon in its whole-food form when compared to ingesting its isolated nutrients (despite matched EPA and DHA content). These results are in line with recent findings that intact salmon ingestion leads to higher postprandial plasma EPA and DHA concentrations when compared to minced salmon or defatted salmon + oil ingestion (Nasef et al., 2021). It is well established that chronic consumption of omega-3 fatty

acids, including EPA and DHA, have benefits to whole body (Wall et al., 2010, Bernasconi et al., 2021, Mocking et al., 2016) and potentially muscle health (Ferguson et al., 2021). However, there is currently not enough evidence to suggest that eating salmon is more effective than free form at supporting the previously established health benefits of omega-3 supplementation, a point for future studies.

Despite increases in muscle protein synthesis rates, we demonstrated a lack of phosphorylation of several muscle signaling targets downstream of mTORC1; this pathway underpins the regulation of exercise- and feeding-mediated protein translation initiation and elongation (Fujita et al., 2007, Drummond et al., 2009, Fry et al., 2011). Specifically, with the exception of a significant postprandial phosphorylation of rpS6 after exercise in both conditions, we saw no changes in phosphorylation of p70S6K, 4E-BP1 and eEF2. The lack of phosphorylation of these particular signaling proteins is surprising given the robust exercise- and feeding-induced stimulation of muscle protein synthesis rates in the current study. However, we are not the first to report a dissociation between signaling and muscle protein synthesis rates. Our results are in line with increasing evidence that the extent of phosphorylation of several anabolic signaling proteins measured via Western Blotting does not necessarily dictate changes in muscle protein synthesis rates (Moore et al., 2009, Staples et al., 2011). Nevertheless, *in vivo* human evaluation of molecular regulators serves only as translation of preclinical findings and is not mechanistic in nature; as such, observations should be interpreted accordingly. Additionally, it is possible that alternative mechanisms to mTORC1-associated signaling were driving the changes observed in muscle protein synthesis. For example, it has been reported that the rapamycin-insensitive AKT/GSK-3β/eIF2B signaling pathway is associated with changes in muscle protein synthesis (Mirzoev et al., 2016). Activity of eIF2B, the downstream target of AKT, is shown to be highly sensitive to

amino acid concentrations and has proven to be a key step for the control of protein synthesis by regulation of translation initiation (Wang and Proud, 2008). Notwithstanding, our results show that the phosphorylation status of primary downstream targets of mTORC1 involved in protein synthesis do not elucidate the mechanistic underpinnings of food-matrix related changes in the exercise-mediated postprandial muscle protein synthetic response.

The effect of different food sources on the stimulation of muscle protein synthesis rates is multi-factorial and thus it could be speculated that the current study minimized those food differences to such an extent where differences in protein synthesis were undetectable. Despite a lack of statistical significance, there was a 23% (Cohen's d = 0.44, moderate difference) higher cumulative muscle protein synthetic response with SAL (95% CI [0.042, 0.070]) compared to ISO (95% CI [0.030, 0.061]). This non-significant observation suggests lower effect sizes when investigating the delicate intricacy of food matrix interactions when compared to previous studies investigating isolated proteins. Therefore, it is possible that larger sample sizes are needed to appropriately discern physiological influence. We advise that future studies investigating the effects of whole foods on skeletal muscle protein synthesis take a potentially lower effect size for whole-food investigations into account during *a priori* power analysis to achieve appropriate statistical power.

In conclusion, we show that the ingestion of salmon or its isolated nutrients enhances the stimulation of post-exercise muscle protein synthesis rates, with a similar net increase in leucine oxidation in healthy young adults. The ingestion of salmon resulted in a delayed stimulation of leucine oxidation, lower postprandial insulin concentrations, and larger plasma EPA concentrations when compared to free nutrients ingestion.

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CHAPTER 3: FOOD-BORNE PEPTIDES

Dileucine ingestion is more effective than leucine in stimulating muscle protein turnover in young males: a double blind randomized controlled trial

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3.1 ABSTRACT

Leucine is regarded as an anabolic trigger for the mTORC1 pathway and the stimulation of muscle protein synthesis rates. More recently, there has been an interest in underpinning the relevance of BCAA-containing dipeptides and their intact absorption into circulation to regulate muscle anabolic responses. We investigated the effects of dileucine and leucine ingestion on postprandial muscle protein turnover. Ten healthy young men (age: 23±3 y) consumed either 2 g of leucine (LEU) or 2 g of dileucine (DILEU) in a randomized crossover design. The participants underwent repeated blood and muscle biopsy sampling during primed continuous infusions of L-[*ring*-$^{13}C_6$]phenylalanine and L-[^{15}N]phenylalanine to determine myofibrillar protein synthesis (MPS) and mixed muscle protein breakdown rates (MPB), respectively. LEU and DILEU similarly increased plasma leucine net area under the curve (AUC; P = 0.396). DILEU increased plasma

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dileucine AUC to a greater extent than LEU (P = 0.013). Phosphorylation of Akt (P = 0.002), rpS6 (P <0.001) and p70S6K (P < 0.001) increased over time in both LEU and DILEU conditions. Phosphorylation of 4E-BP1 (P = 0.229) and eEF2 (P = 0.999) did not change over time irrespective of condition. Cumulative (0-180 min) MPS increased in DILEU (0.075±0.032 %·hour^{-1}), but not in LEU (0.047±0.029 %·hour^{-1}; P=0.023). MPB did not differ between LEU (0.043±0.030 %·h^{-1}) and DILEU conditions (0.051±0.027 %·hour^{-1}; P = 0.659). Our results showed that dileucine ingestion elevated plasma dileucine concentrations and muscle protein turnover by stimulating MPS in young men.

Keywords

Dipeptide; muscle protein synthesis; muscle protein breakdown; anabolism

New & noteworthy

The role of dipeptides as anabolic agents remains unresolved in humans. We show that the ingestion of 2 g dileucine increased plasma dileucine concentrations and resulted in an enhancement of muscle protein turnover by stimulating an increase in muscle protein synthesis rates in healthy young males. The ingestion of 2 g leucine, however, did not stimulate an increase in muscle protein turnover. Our work provides the first insights into the effects of dipeptides on human protein metabolism.

3.2 INTRODUCTION

Most high-quality food proteins that strongly stimulate postprandial muscle protein synthesis rates are high in leucine by total amino acid content (Tang et al., 2009). Mechanistically, leucine is

regarded as an anabolic trigger for the mTORC1 pathway and translation initiation, allowing for the assembly of the structural machinery for protein synthesis at the ribosomal level (Norton and Layman, 2006). Additionally, the insulinotropic properties of leucine may have an inhibitory effect on muscle protein breakdown rates (MPB) with aging (Wilkes et al., 2009), albeit at modest postprandial insulin concentrations (Fukagawa et al., 1985). Hence, leucine is often a focal point of nutrition research (van Loon, 2012) and may be useful as a food fortification technique in order to enhance the use of dietary amino acids for postprandial muscle protein accretion (Koopman et al., 2006, Norton et al., 2009, Tipton et al., 2009).

Beyond single amino acids, there has been an interest in underpinning the role of food-borne peptides (e.g., di- or tripeptides) and their intact absorption into circulation in regulating human metabolic responses. These efforts are intended to ultimately establish their potential as components of anabolic feeding formulations (Miner-Williams et al., 2014). Absorption of di- and tripeptides at the enterocyte is substantial. These polypeptides are transported across the intestinal endothelium by the PepT1 H^+/peptide co-transporter (Ganapathy and Leibach, 1983, Fei et al., 1994). Consequently, previous research has shown that constituent amino acids are absorbed faster when digested as dipeptides versus free amino acids (Hellier et al., 1972). In addition, there is evidence that suggests the potential of di- and tripeptides to avoid cytosolic hydrolysis, thereby allowing for intact transport across the basolateral membrane (Miner-Williams et al., 2014). In support, it has also been demonstrated that plasma leucine and branched-chain amino acid (BCAA)-containing dipeptide, such as leucine-leucine, concentrations rapidly increased above baseline after the ingestion of protein hydrolysates when compared to intact non-hydrolyzed protein in young adults (Morifuji et al., 2010). What is less understood, however, is whether the oral delivery of dipeptides can be used as a more effective anabolic trigger to phosphorylate

mTORC1 signaling targets (e.g., p70S6K and 4E-BP1) and the subsequent regulation of human muscle protein turnover (both synthesis and breakdown rates).

Therefore, the primary aim of this study was to assess the effects of dileucine and leucine ingestion on the modulation of muscle protein synthesis and protein breakdown rates in healthy young adults. We hypothesized that dileucine ingestion would result in a greater stimulation of muscle protein turnover by augmenting muscle protein synthesis rates when compared to the ingestion of leucine. Secondary aims of this study were to evaluate plasma and intramuscular leucine and dileucine concentrations and phosphorylation of down-stream mTORC1 signaling proteins. We further hypothesized that dileucine ingestion would result in greater plasma and intramuscular dileucine concentrations and phosphorylation of down-stream mTORC1 signaling readouts. An additional, exploratory analysis included evaluation of various commercially available intact protein sources for their di- and tri-peptide content.

3.3 METHODS

Participants and ethical approval

Ten healthy, recreationally active men (23 ± 3 y; 24.2 ± 1.8 $kg \cdot m^{-2}$; Mean ± SD) volunteered to participate in this study. All participants were recruited and performed two infusion trials in our clinical laboratory at the University of Illinois at Urbana-Champaign from June 2019 to November 2019. All participants were deemed healthy based on their response to a routine medical screening questionnaire. Volunteers had no history of participating in past stable isotope amino acid tracer experiments prior to the first trial. Participants' characteristics are presented in **Table 3.1**. All participants were informed about the experimental procedures, the purpose of the study, and potential risks before giving written consent. The human trials conformed to all standards for the

use of human participants in research as outlined in the Helsinki Declaration and approved by the local Institutional Review Board at the University of Illinois at Urbana-Champaign (IRB # 18897). This study was registered at clinicaltrials.gov, NCT03952884.

Table 3.1. Participant characteristics

Variable	
Age (y)	23 ± 3
Weight (kg)	77.10 ± 9.73
BMI ($kg \cdot m^2$)	24.19 ± 1.91
Systolic BP (mmHg)	118 ± 11
Diastolic BP (mmHg)	72 ± 11
Fat (%)	22.8 ± 3.6
Lean Body Mass (kg)	55.04 ± 6.30
Fasting Glucose ($mg \cdot dL^{-1}$)	81.1 ± 5.4
Energy Intake ($kCal \cdot day^{-1}$)	1961 ± 738
Protein Intake ($g \cdot d^{-1}$)	92 ± 43
Carbohydrate Intake ($g \cdot d^{-1}$)	209 ± 87
Fat Intake ($g \cdot d^{-1}$)	86 ± 26

Data are presented as Mean ± SD

Study design

A double-blind, randomized, crossover design was used for this study. Two weeks before the infusion trial, participants reported to the laboratory for measurement of body weight and height, as well as body composition by dual-energy x-ray absorptiometry (DEXA; Horizon W, Hologic Inc., Marlborough, MA, USA). After the pre-testing session, participants were randomized to ingest either leucine or dileucine in a counterbalanced fashion on their first infusion trial. For

allocation of the participants, an independent researcher used a computer-generated list of random numbers. The same researcher prepared the interventional drink for consumption on the day of the trial. Both interventions were odorless and tasteless powders and indistinguishable by both researcher and participant. Participants were instructed to refrain from any strenuous physical exercise and alcohol consumption for at least 72 hours prior to the infusion trials. Participants were provided an identical standardized dinner the evening before both trials (25-30 % of estimated energy requirement; 50 % of energy of carbohydrate, 25 % energy of protein and 25 % energy of fat). In addition, participants were instructed to maintain the same dietary intake for two days prior to each infusion trial, which was confirmed by an online dietary recall system (ASA24 version 2016; National Cancer Institute, Rockville, MD, USA). A CONSORT (Consolidated Standards of Reporting Trials) flowchart of the study is presented in **Figure A.2.**

Treatment conditions

The dileucine monohydrate (RAMPS™) and leucine were obtained from Ingenious Ingredients, L.P., Lewisville, TX, USA. The content and purity of dileucine was confirmed via reverse phase HPLC with diode-array detection by an independent third-party laboratory (Heartland Assays, Ames, IA). Leucine and dileucine were administered in 2 g aliquots and dissolved in 300 mL of water on the morning of each infusion trial. The dose of leucine was selected to represent the amount of leucine contained in a recommended serving of a high-quality food protein (e.g., 3 oz meat). Moreover, based on previous ingested protein dose-response studies, this amount of leucine should, theoretically, not oversaturate the postprandial muscle protein synthetic response (Moore et al., 2009). This will allow for improved anabolic sensitivity to be detected between conditions. The dileucine dose was chosen to match the leucine dosage.

Infusion protocol

For both trials, participants reported to the laboratory at 0700 h after an overnight fast by car or public transport. After re-assessment of blood pressure and body weight, a Teflon catheter was inserted into an antecubital vein and an arterialized baseline blood sample was collected. Participants subsequently (*t* = -180 min) received priming doses of L-[*ring*-$^{13}C_6$]phenylalanine (2.0 µmol·kg^{-1}) and L-[^{15}N]phenylalanine (2.0 µmol·kg^{-1}), which was followed by continuous intravenous infusions of L-[*ring*-$^{13}C_6$]phenylalanine (0.05 µmol·kg·min^{-1}) and L-[^{15}N]phenylalanine (0.05 µmol·kg·min^{-1}) for the measurement of myofibrillar protein synthesis rates (MPS) and mixed muscle protein breakdown (MPB) rates, respectively. A second Teflon catheter was inserted into a heated dorsal hand vein of the opposing arm for repeated blood sampling and kept patent by a 0.9% saline drip. The L-[*ring*-$^{13}C_6$]phenylalanine infusion was constant for the duration of the trial. The L-[^{15}N]phenylalanine infusion was terminated at *t* = 0. Muscle biopsies were collected before (*t* = 0) and after the ingestion of leucine or dileucine at t = 30, 60 and 180 min. Biopsies were collected under local anesthesia from the middle region of the *m. vastus lateralis* (15 cm above the patella) with a Bergström needle that was modified for manual suction. Each consecutive biopsy was taken more proximal from a new incision. Biospies at *t* = 30 and *t* = 60 were taken from the same incision site. Muscle samples were freed from any blood, fat, and visible connective tissue and immediately frozen in liquid nitrogen prior to storage at -80 °C until further analysis. Blood samples were collected in EDTA-containing tubes before (*t* = -180, -120, -60 and 0 min) and after treatment ingestion (*t* = 15, 30, 45, 60, 90, 120 and 180 min). Blood samples were centrifuged at 3000*g* for 10 min at 4°C and the plasma was subsequently aliquoted and stored at -20°C or -80°C for future analysis. An overview of the infusion trial is shown in **Figure 3.1.**

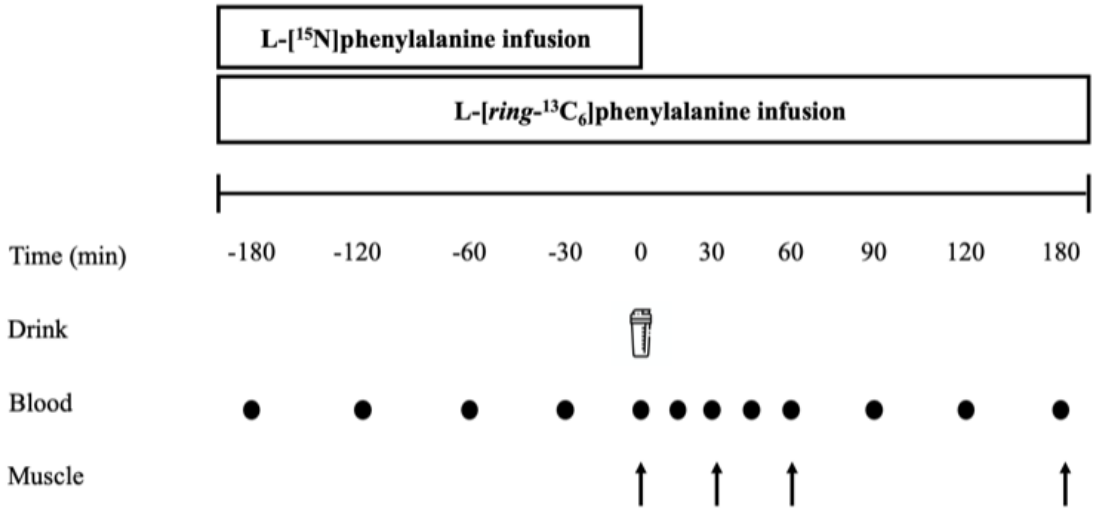

Figure 3.1. Schematic of the infusion trial. Participants consumed 2 g of leucine or dileucine in a randomly assigned order (n=10).

Plasma analyses

Plasma glucose concentrations were analyzed by an automated biochemical analyzer (2900 Stat Plus, YSI Life Sciences, Yellow Springs, OH). Plasma insulin concentrations were determined using commercially available enzyme-linked immunosorbent assays (Alpco Diagnostics; Salem, NH). Plasma amino acid concentrations and enrichments were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS; Thermo Altis Triple Quadrupole, Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Salvador et al., 2019).

Muscle analyses

Myofibrillar protein-enriched fractions were extracted from ~50 mg of wet muscle using an ice-cold lysis buffer (Pierce® RIPA Buffer, Thermo Fisher Scientific, Waltham, MA, USA; 10

μL·mg^{-1};) supplemented with protease and phosphatase inhibitor tablets (cOmplete and PhosSTOP, Roche Applied Science, Mannhein, Germany). Myofibrillar proteins were isolated by differential centrifugation as described previously (Salvador et al., 2019). The remaining muscle homogenate was stored at −80°C for subsequent Western blot analyses. The isolated myofibrillar protein fractions were hydrolyzed overnight in 6 M HCL at 110°C. The resultant free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before analysis by 5500 QTRAP (Sciex, Redwood City, CA, USA) LC-MS/MS (Van Vliet et al., 2017). The myofibrillar protein-bound L-[*ring*-$^{13}C_6$]phenylalanine enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 166.0 → 103.0 and 172.0 → 109.0 for unlabeled and labelled L-[*ring*-$^{13}C_6$]phenylalanine, respectively. Analyst V1.6.2 (Sciex, Framingham, MA, USA) was used for data acquisition and analysis.

Muscle intracellular amino acids (IC) were isolated from a separate piece of wet muscle (~30 mg) by homogenizing on ice using a Teflon pestle with 500 μL of 0.6 M perchloric acid. Samples were centrifuged at 4500 RPM at 4 °C for 5 min and the supernatant containing the IC was collected. This process was repeated twice by adding 500 μL 0.6 M perchloric acid, centrifuging and collecting the supernatant. The intracellular amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Subsequently, the ICs were re-suspended in 60% methanol and centrifuged before analysis by 5500 QTRAP (Sciex, Framingham, MA, USA) LC-MS/MS. Muscle IC L-[*ring*-$^{13}C_6$]phenylalanine enrichments were determined by MRM at *m/z* 166.0 → 103.0 and 172.0 → 109.0 for unlabeled and labelled L-[ring-$^{13}C_6$]phenylalanine, respectively. The L-[^{15}N]phenylalanine enrichments were determined by MRM at *m/z* 166 → 103.0 and 167 →

104.0 for unlabeled and labelled L-[^{15}N]phenylalanine, respectively. Analyst V1.6.2 (Sciex, Framingham, MA, USA) was used for data acquisition and analysis. Muscle IC leucine and dileucine concentrations were determined by LC-MS/MS (Thermo Altis Triple Quadrupole, Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Salvador et al., 2019). Muscle β-Hydroxy-β-methylbutyrate (HMB), α-ketoisocaporoic acid (α-KIC), and α-hydroxyisocaporoic acid (HICA) were determined from a separate piece of ~100 mg of wet muscle by gas chromatography/mass spectrometry (GC/MS) using a modified procedure previously described (Nissen et al., 1990, Nissen et al., 1982). Briefly, muscle tissue was transferred into a 12 x 75 mm glass tube with internal standard (d_6-HMB and α-ketocaporoic acid, KC). In addition, 0.5 mL of ultrapure water and 100 μL of formic acid was added to the tube, and the contents were homogenized by using tissue homogenizer (Lab Gen7®, Vernon Hills, IL) for 1 min at 24,000 rev./min. Methyl-τ-butyl ether (3 mL) was add to each sample, vortexed and shaken for 10 min at 500 rpm. The samples were then centrifuged at 1800 rcf at 4°C for 15 min. The organic layer was transferred to a new glass vial, dried and derivatized for analysis analyzed by GC/MS (GC model 7890B, Mass Selective Detector (MSD) model 5977A, Agilent Technologies, Santa Clara, CA).

Western blotting

A portion of the whole muscle homogenates isolated during the myofibrillar extractions were used for western blotting analysis. Total protein concentrations were determined using BCA assays (Pierce® BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein from each sample were mixed with loading buffer (2X Laemmli, Bio-Rad, Hercules, Ca, USA), diluted to a final concentration of 3.68 $\mu g \cdot \mu L^{-1}$ with lysis buffer (described above) and denatured at 95°C for 5 minutes. 50 μg of protein (13.59 μL) was separated on 7.5%, 10% or 15%

(w/v) polyacrylamide gels, and then electrophoretically transferred to polyvinylidene fluoride membranes (PVDF, MilliporeSigma, St. Louis, MO, USA). Membranes were blocked with 3 or 5% non-fat milk or bovine serum albumin (Fisher BioReagents, Waltham, MA, USA) diluted in Tris-buffered saline (Alfa Aesar, Ward Hill, MA, USA) with Tween solution (Fisher BioReagents, Waltham, MA, USA) for 1 h at room temperature before overnight incubation in primary antibodies at 4°C. Proteins of interest were detected with primary antibodies as follows: rabbit anti-α-tubulin (1:1000; Abcam Cat# ab4074, RRID:AB_2288001), rabbit anti- phospho-p70S6K (Thr389) (1:500; Cell Signaling Technology Cat# 9205, RRID:AB_330944), rabbit anti-p70S6K (1:500; Cell Signaling Technology Cat# 9202, RRID:AB_331676), rabbit anti-phospho-4E-BP1 (Thr37/46) (1:1000; Cell Signaling Technology Cat# 9459, RRID:AB_330985), rabbit anti-4E-BP1 (1:1000; Cell Signaling Technology Cat# 9452, RRID:AB_331692), rabbit anti-phospho-eEF2 (Thr56) (1:1000; Cell Signaling Technology Cat# 2331, RRID:AB_10015204), rabbit anti-eEF2 (1:1000, Cell Signaling Technology Cat# 2332, RRID:AB_10693546),), rabbit IgG anti-phospho-S6 Ribosomal Protein (Ser240/244) (1:1000, Cell Signaling Technology Cat# 5364, RRID:AB_10694233) and rabbit IgG anti-S6 Ribosomal Protein (1:1000, Cell Signaling Technology Cat# 2217, RRID:AB_331355), rabbit IgG anti-phospho-Akt (Ser473) (1:2000; Cell Signaling Technology Cat# 4060, RRID:AB_2315049), Rabbit anti-Akt (1:1000; Cell Signaling Technology Cat# 9272, RRID:AB_329827). After primary incubation, blots were exposed to horseradish-peroxidase-conjugated horse anti-mouse IgG (1:2000; Cell Signaling Technology Cat# 7076, RRID:AB_330924) or goat anti-rabbit IgG (1:2000-1:10,000; Abcam Cat# ab6721, RRID:AB_955447) and detected using the ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA) and the ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories, Hercules,

CA, USA). Bands were quantified using ImageJ software (Schneider et al., 2012) and normalized to α-tubulin as the internal control.

Calculations

Myofibrillar protein fractional synthesis rates (FSR) were calculated using the standard precursor product method by dividing the increment in enrichment in the product (i.e. myofibrillar protein-bound L-[ring-$^{13}C_6$]phenylalanine) by the enrichment of the precursor pool (i.e., plasma or intracellular) over time as described previously (Salvador et al., 2019). Mixed muscle protein fractional breakdown rates (FBR) were calculated based on the exponential decay of the plasma and intracellular L-[^{15}N]phenylalanine enrichment from a plateau enrichment using equations and assumptions discussed previously (Zhang et al., 1996, Phillips et al., 1997, Staples et al., 2011).

Food protein analysis

Analysis of dileucine (N-L-Leucyl-L-leucine) and trileucine (N-L-Leucyl-L-leucine-L-leucine) content in different animal and plant proteins were analyzed as previously described by Morifuji et al. [1]. Briefly, the isolated protein sources were diluted with deionized water and protease preparations of *Bacillus sp.* (Sigma-Aldrich) and *Aspergillus oryzae* (Sigma-Aldrich) were added to each protein solution and incubated at 50 °C for 6 h. The enzymatic reaction was stopped by heating the samples 80 °C for 10 min.

EZ:faast® amino acid analysis kits (Phenomenex, Torrance, CA) were used for dileucine and trileucine analysis by LC-MS/MS and electrospray ionization . The procedure used 50 µl of the protein solution for dileucine and trileucine determination and consisted of solid phase extraction of sample with internal standards (IS) by a sorbent tip attached to a syringe with an

eluting solvent (a 3:2 mixture of sodium hydroxide with 77% n-propanol, and 23% 3-picoline). The peptides were then derivatized by adding a mixture of 17.4% propyl chloroformate, 11% isooctane, and 71.6% chloroform. The resulting mixture was vortexed and allowed to sit at room temperature for 1 min, followed by liquid-liquid extraction with isooctane. Subsequently, 50 µl of the organic layer was removed, dried under nitrogen gas, and suspended in the HPLC run solvents before being injected into the LC-MS/MS. Chromatographic separation of the derivatized peptides were conducted on an EZ:faast amino acid analysis-mass spectrometry column (250 × 2.0 mm i.d., 4 µm) using a Agilent 6460 triple quadrupole LC-MS/MS system (Santa Clara, CA). 10 mM ammonium formate in water with 0.2% formic acid (mobile phase A) and 10 mM ammonium formate in methanol with 0.2% formic acid (mobile phase B) were used as solvent system with gradient conditions of 68% B at 0 min to 83% B over 13 min with a flow rate of 0.25 ml/min. Amino acids and (IS) data were collected using the Dynamic Multiple Reaction Monitoring mode using Mass Hunter acquisition software (Agilent, Santa Clara, CA) and quantitated using the Mass Hunter Quantitation based on best fit standard curves. The assay LOD for dileucine and trileucine was 0.04 nmol/mL. The MRM ions for dileucine and trileucine are listed in **Table 3.2**.

Table 3.2. Multiple Reaction Monitoring Ions used for the food protein analysis

Compound Name	Precursor Ion	Product Ion	Frag	Collision Energy	Cell Accelerator Voltage	Polarity
L-leucine (IS)	267.0	178.1	50	6	7	Positive
L-leucine (IS)	267.0	136.0	50	14	7	Positive
Dileucine	373.2	172.1	114	13	7	Positive
Dileucine	373.2	313.2	114	1	7	Positive
Trileucine	486.3	172.2	100	25	7	Positive
Trileucine	486.3	313.2	100	5	7	Positive

Frag: Fragmentor; Dileucine: N-L-Leucyl-L-Leucine; Trileucine: N-L-Leucyl-L-Leucine-L-Leucine

Statistical analyses

All data are presented as mean ± standard deviation (SD). A priori power analysis of the human experiment was conducted using GPOWER version 3.1.9.2 (Faul et al., 2007). Based on previous research (Van Vliet et al., 2017, Burd et al., 2015), our power analysis showed that a sample size of 9 participants was sufficient to detect differences in postprandial muscle protein synthesis between conditions when using a 2-sided statistical test ($P < 0.05$, 80% power, f = 0.7). Considering a potential dropout rate of 10%, we enrolled 10 participants. All data were checked for normality before analysis. Differences in blood metabolite concentrations, temporal muscle protein synthesis rates, and intramuscular signaling were tested using linear fixed effects models with time and group as fixed factors. Bonferroni post hoc test was used when significant differences were identified. Muscle protein breakdown rates and plasma metabolite net exposure (AUC) were analyzed using a paired samples t-test. Linear regression lines were fitted to plasma enrichments to assess the existence of any deviation in enrichment indicated by lines with a significant positive or negative slope. The level of statistical significance was set at $P < 0.05$ for all analyses. All analyses were performed using IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA).

3.4 RESULTS

Blood analyses

Plasma glucose concentrations remained stable across time ($P = 0.429$), irrespective of condition ($P = 0.978$). Plasma insulin concentrations transiently increased after drink ingestion (Main effect of time: $P = 0.017$) with no differences between conditions ($P = 0.983$).

Plasma leucine concentrations increased after LEU and DILEU ingestion (Main effect of time: $P < 0.001$; **Figure 3.2A**). Plasma leucine concentrations reached peak values at 45 min and remained elevated throughout the experiment with no differences between group ($P = 0.906$). The net area under the curve (AUC) for plasma leucine concentrations were similar between conditions ($P = 0.346$). Plasma dileucine concentrations transiently increased after treatment ingestion regardless of condition (Main effect of time: $P < 0.001$). Postprandial plasma dileucine concentrations were greater in DILEU when compared to the LEU condition ($P = 0.016$). The net AUC for plasma dileucine concentrations was greater in the DILEU condition when compared to the LEU condition ($P = 0.013$). Plasma essential amino acid concentrations increased after treatment ingestion ($P < 0.001$; **Figure 3.2C**). The net plasma EAA AUC was similar between DILEU and LEU conditions ($P = 0.273$).

Linear regression analysis showed that the slope of both post-absorptive plasma L-[^{15}N]phenylalanine ($P = 0.570$) and L-[ring$^{13}C_6$]phenylalanine ($P = 0.413$) enrichments were not significantly different from zero, indicating a steady-state during the post-absorptive period (**Figure 3**). The slope of postprandial plasma L-[ring$^{13}C_6$]phenylalanine enrichments were also not significantly different from zero ($P = 0.056$). Plasma L-[ring$^{13}C_6$]phenylalanine enrichments did not differ between LEU and DILEU conditions ($P = 0.336$) throughout the trials. Following termination of the L-[^{15}N]phenylalanine infusion, enrichments were reduced ($P < 0.001$) to a similar extent in both LEU and DILEU conditions ($P = 0.315$) .

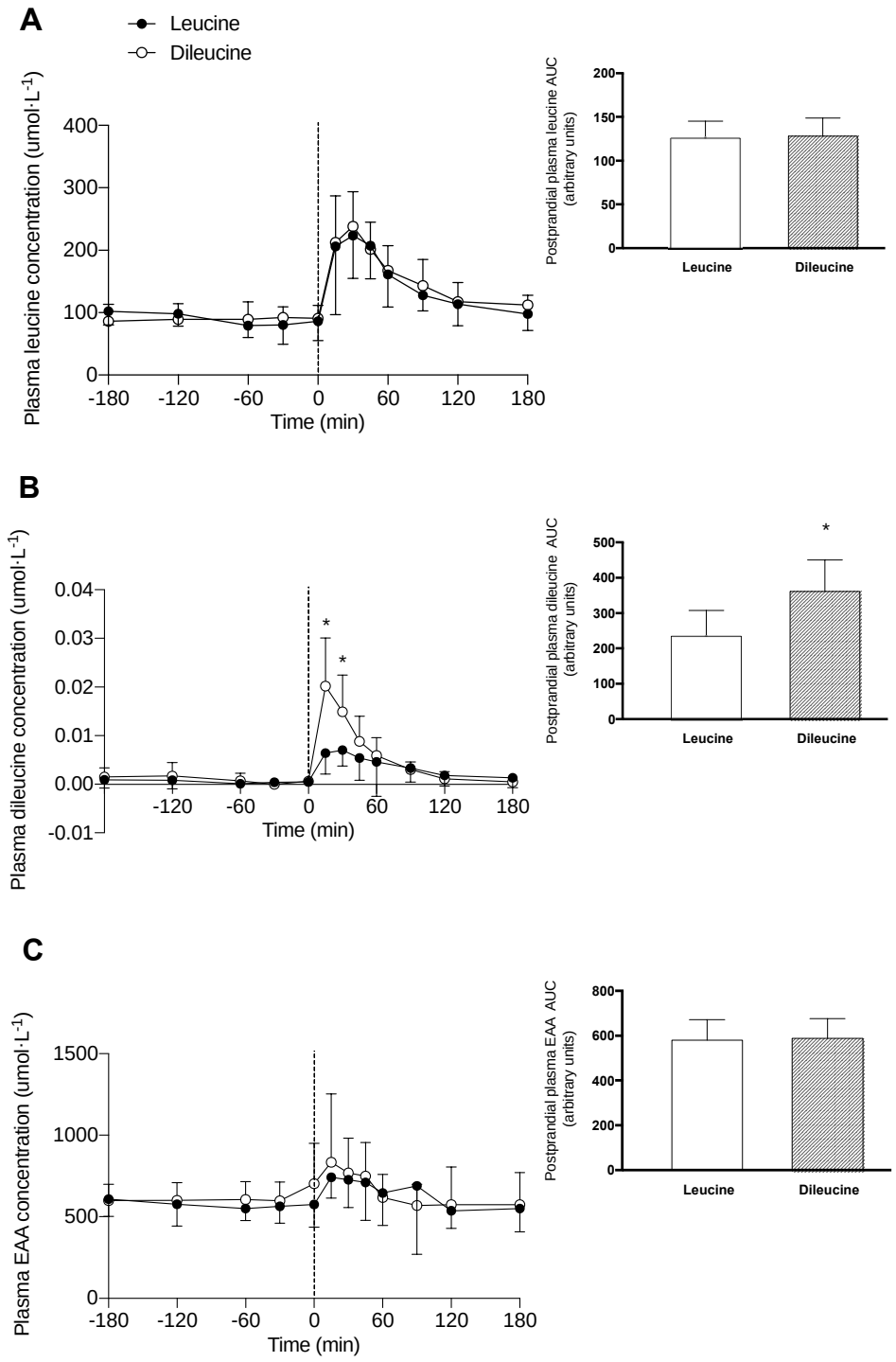

Figure 3.2. Plasma leucine (A), dileucine (B) and essential amino acid (EAA; C) concentrations during the post-absorptive and postprandial period in young men (n=10). Insets show the postprandial area under the curve (AUC). Ingestion of the leucine or dileucine drink is denoted by dashed line. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors. A Bonferroni post hoc test was used when significant differences were identified. * Indicates significant ($P < 0.05$) difference between conditions. Leucine: time effect, $P < 0.001$. Dileucine: time × treatment, $P < 0.001$; time effect, $P < 0.001$; treatment effect, $P = 0.016$; AUC: $P = 0.013$. EAA: time effect, $P < 0.001$.

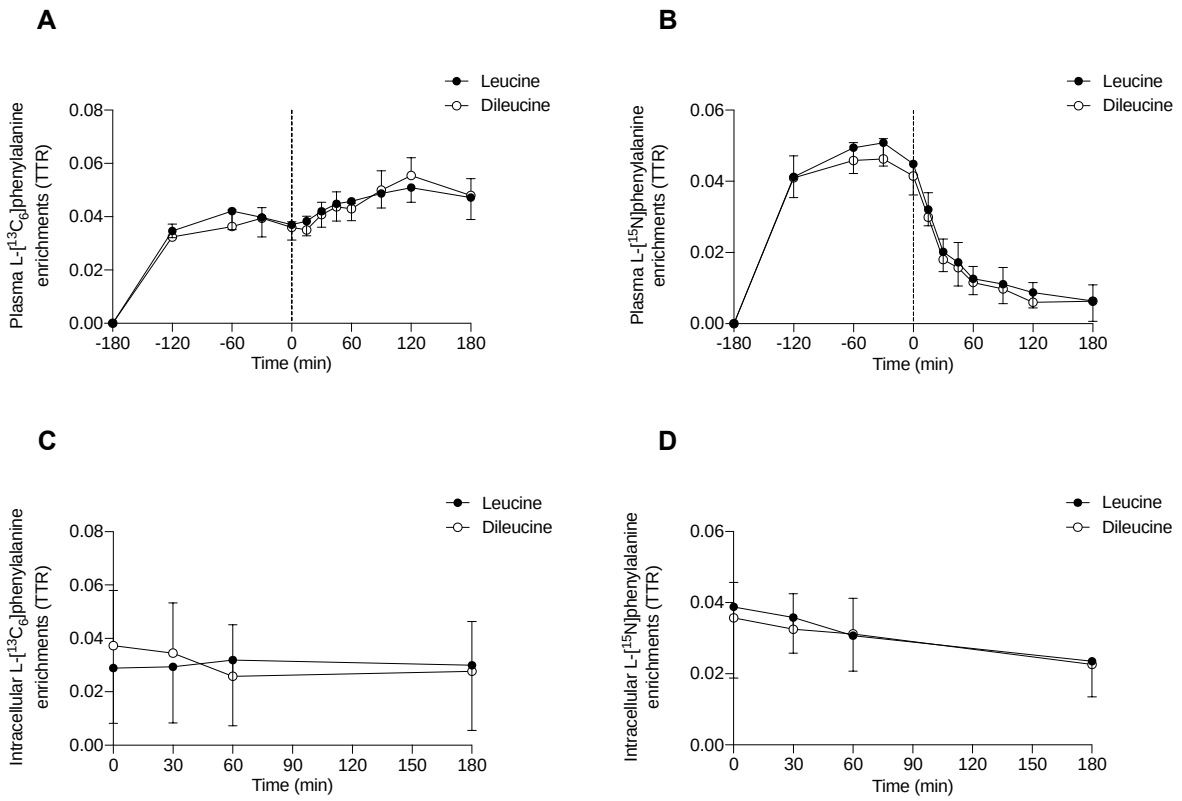

Figure 3.3. Plasma L-[$^{13}C_6$]phenylalanine (A), L-[^{15}N]phenylalanine (B) enrichments and muscle intracellular (IC) L-[$^{13}C_6$]phenylalanine (C), L-[^{15}N]phenylalanine (D) enrichments in the basal-state and after ingestion of leucine or dileucine in young men (n=10). All enrichments are expressed as tracer·tracee^{-1}. Ingestion of leucine or dileucine drink is denoted by a dotted line. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors. A Bonferroni post hoc test was used when significant differences were identified. Data were analyzed by a mixed linear model. Plasma L-[15N]phenylalanine: time effect, P < 0.001.

Western blotting

Phosphorylation of Akt increased ~2-fold above baseline at 60 min (P = 0.002) and decreased to basal levels at 180 min with no differences between conditions (P = 0.862, **Figure 3.4B**). Phosphorylation of p70S6K increased ~2-fold above baseline at 60 min (P < 0.001) and remained elevated throughout the experiment with no differences between conditions (P = 0.069, **Figure 3.4C**). Phosphorylation of 4E-BP1 did not change over time regardless of condition (P = 0.229, **Figure 3.4D**). Phosphorylation of eEF2 did not decrease over time (P = 0.999) with no differences between conditions (P = 0.948, **Figure 3.4E**). There was a robust increase in the phosphorylation

of rpS6 at 60 min ($P < 0.001$), which returned to basal values at 180 min with no differences between conditions ($P = 0.468$, **Figure 3.4F**).

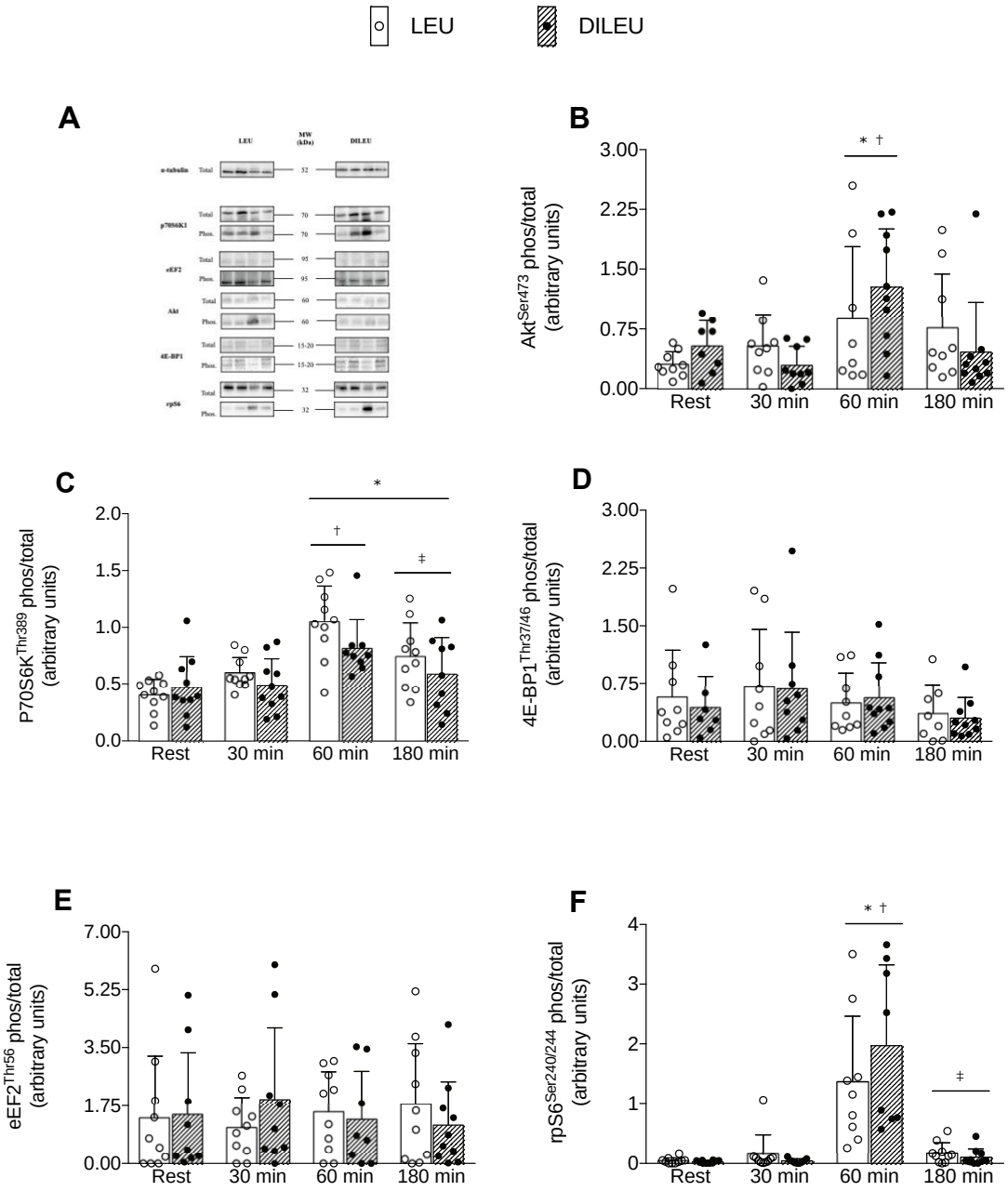

Figure 3.4. Phosphorylated-to-total protein ratio of Akt (B), p70S6K (C), 4E-BP1 (D), eEF2 (E), rpS6 (F) at rest and after the ingestion of leucine (LEU: closed circles) or dileucine (DILEU: open circles) in young men (n = 10 per condition). Representative blots are shown for each protein (A). Data were analyzed using a linear fixed effects model with time and group as fixed factors. A Bonferroni post hoc test was used when significant differences were identified. Values are expressed as mean ± SD. * indicates significantly ($P < 0.05$) different from rest. † Indicates significantly ($P < 0.05$) from t = 30 min. ‡ indicates significantly ($P < 0.05$) from t = 120 min. p70S6K: time effect, $P < 0.001$; time × condition, $P = 0.305$. Akt: time effect, $P = 0.002$; time × condition, $P = 0.182$. rpS6: time effect, $P < 0.001$; time × condition, $P = 0.241$.

Muscle analyses

Muscle IC leucine concentrations increased after treatment ingestion irrespective of condition (Main effect of time: P = 0.005; **Table 3.3. Figure 3.5A**). Muscle IC dileucine concentrations did not change over time irrespective of condition (P = 0.988, **Table 3.3, Figure 3.5B**). Muscle α-ketoisocaproic acid concentrations increased after LEU and DILEU ingestion (P = 0.016; Table 2), with no differences between conditions (P = 0.457). Muscle β-hydroxy-β-methylbutyric acid did not change after LEU and DILEU ingestion (P = 0.676; **Table 3.3**). Muscle α-hydroxyisocaporoic acid concentrations increased after LEU and DILEU ingestion (P = 0.004) with no differences between conditions (P = 0.729, **Table 3.3**). Muscle IC L-[*ring*-$^{13}C_6$]phenylalanine enrichments did not differ between the LEU or DILEU conditions in the postabsorptive (P = 0.272) or postprandial states (P = 0.825). Similarly, muscle IC L-[^{15}N]phenylalanine enrichments did not differ between the conditions (P = 0.426).

Cumulative (0-180 min) myofibrillar protein synthesis rates increased in the DILEU condition (0.075±0.032 %·hour^{-1}; P = 0.023), but not the LEU condition (0.047±0.029 %·hour^{-1}; P = 0.949 **Figure 3.6A**). There were no differences in the temporal analysis of myofibrillar protein synthesis rates between conditions at 0-30 min (LEU: 0.078±0.178 or DILEU: 0.011±0.174 %·hour^{-1}; P = 0.486), 30-60 min (LEU: 0.050±0.139 or DILEU: 0.126±0.166 %·hour^{-1}; P = 0.342), and 0-60 min (LEU: 0.001±0.001 or DILEU: 0.001±0.002 %·hour^{-1}P=0.940). Mixed muscle protein breakdown rates did not differ between the LEU (0.043±0.030 %·hour^{-1}; P = 0.659) and DILEU conditions (0.051±0.027 %·hour^{-1}; **Figure 3.6B).**

Table 3.3. Muscle metabolites after leucine (LEU) or dileucine (DILEU) ingestion in young men (*n*=10).

	0 min	30 min	60 min	180 min	AUC
LEU					
Leucine (ng·mL^{-1})	1276 (286)	1462 (376)	1618 (285)	1289 (373)	1450 (251)
Dileucine (ng·mL^{-1})	3.68 (0.36)	3.50 (0.14)	3.60 (0.27)	3.49 (0.45)	3.56 (0.22)
α-KIC (nmol·g^{-1})	13.06 (7.25)	24.58 (11.13)	25.11 (16.23)	17.75 (10.30)	22.96 (11.87)
HMB (nmol·g^{-1})	4.63 (0.73)	3.76 (1.22)	4.16 (1.05)	5.20 (1.19)	4.15 (0.67)
HICA (nmol·g^{-1})	0.16 (0.08)	0.27 (0.15)	0.42 (0.13)	0.39 (0.17)	0.40 (0.11)
DILEU					
Leucine (ng·mL^{-1})	1266 (383)	1611 (513)	1673 (307)	1315 (361)	1502 (306)
Dileucine (ng·mL^{-1})	3.58 (0.26)	3.48 (0.17)	3.61 (0.29)	3.57 (0.28)	3.54 (0.21)
α-KIC (nmol·g^{-1})	13.05 (6.50)	18.28 (9.44)	29.26 (12.03)	22.49 (16.40)	25.48 (12.24)
HMB (nmol·g^{-1})	4.68 (1.75)	6.95 (6.18)	4.16 (0.83)	4.61 (1.17)	4.46 (1.13)
HICA (nmol·g^{-1})	0.27 (0.17)	0.24 (0.09)	0.38 (0.15)	0.40 (0.20)	0.36 (0.09)

Mean (SD); α-KIC: α-ketoisocaproic acid; HMB: β-hydroxy-β-methylbutyric acid; HICA: α-hydroxyisocaporoic acid. Leucine: time effect, *P*=0.005. α-KIC: time effect, *P*=0.016. HICA: time effect, *P*=0.004.

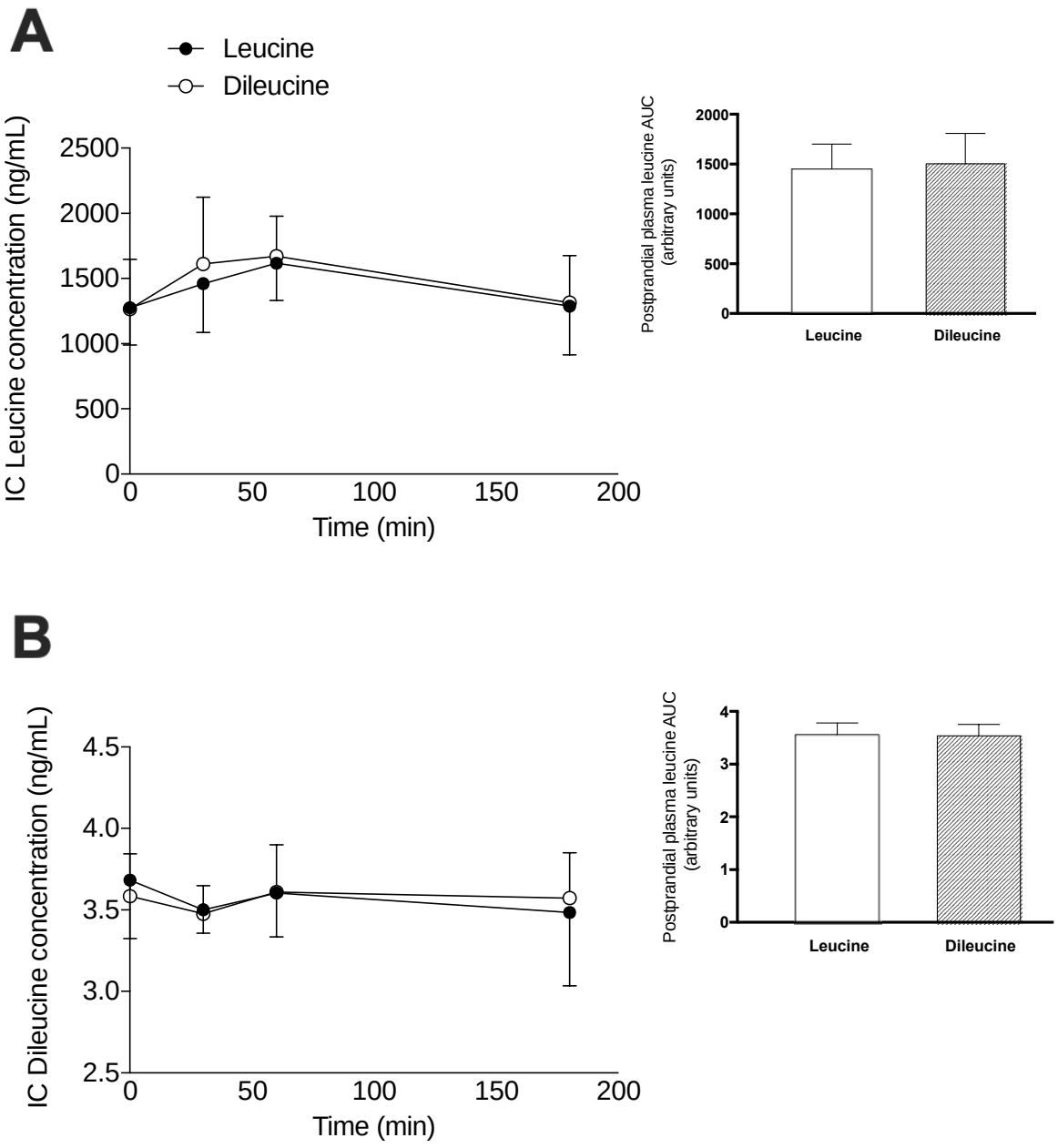

Figure 3.5. Intracellular leucine (A) and dileucine (B) concentrations during the postprandial period in young men (n=10). Insets show the postprandial area under the curve (AUC). Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors. Leucine: time effect, P = 0.005.

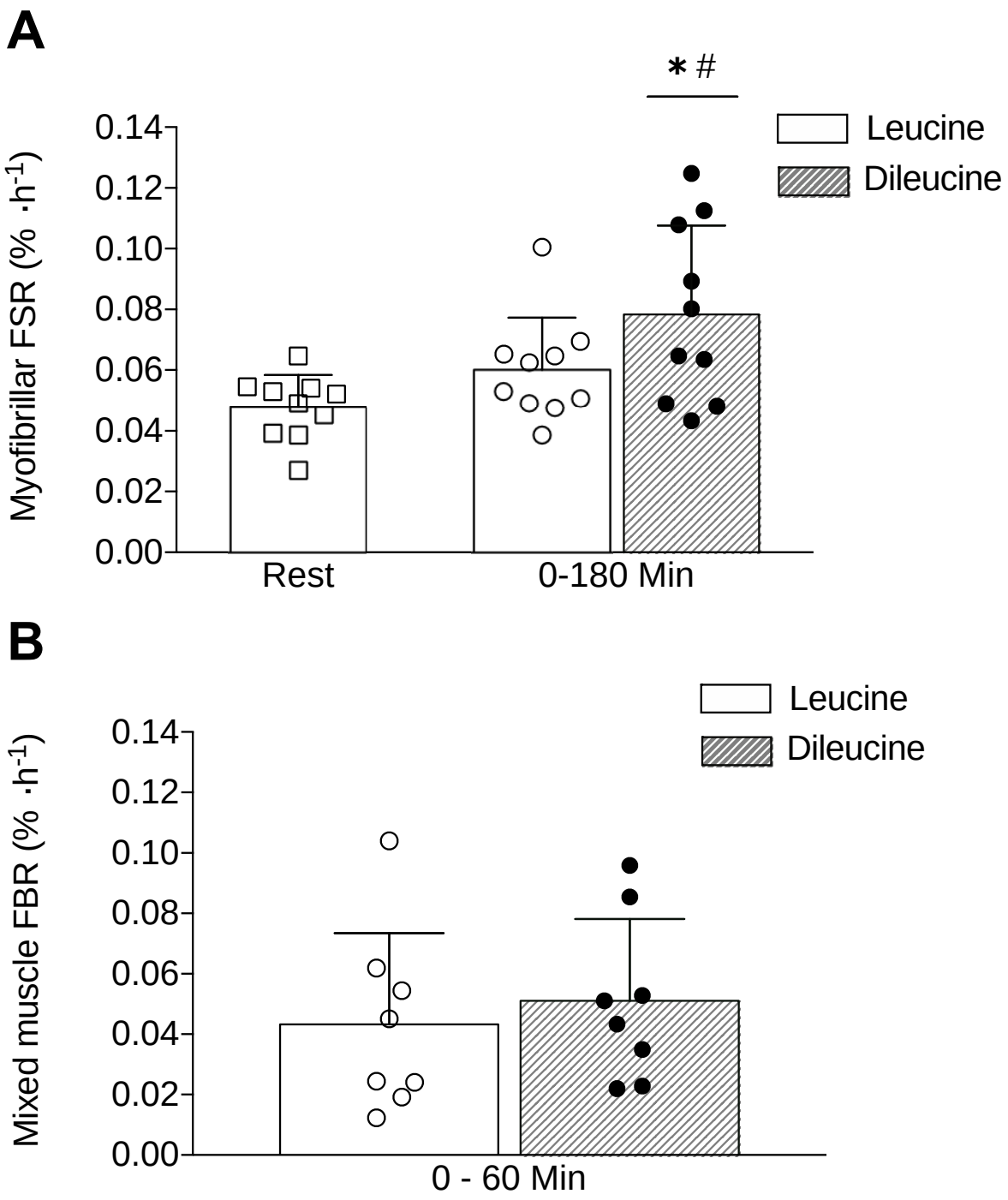

Figure 3.6. Myofibrillar fractional synthetic rate (FSR; A) and mixed muscle fractional breakdown rate (FBR; B) at rest and after the ingestion of a leucine (LEU: closed circles) or dileucine (DILEU: open circles) in young men (A; n=10) and (B; n=8). Absolute FSR values are based on the plasma free precursor. Data were analyzed using a linear fixed effects model (FSR) or a paired samples t-test (FBR). Values are presented as mean ± SD. * indicates significantly ($P < 0.05$) different from rest. # indicates significant ($P < 0.05$) difference between treatment.

Table 3.4. The di- and trileucine content of various commercially available isolated protein sources
n.d.: non detectable

Protein Source	Manufacturer	Protein Content (% Food as is)	Dileucine (g per 100g protein)	Trileucine (g per 100g protein)
Animal protein				
Collagen Peptides	Verisol B (Gelita)	93.4%	0.02	n.d.
Hydrolyzed Whey Protein Isolate	Thermax (Glanbia Nutritionals)	88.2%	1.77	0.02
Whey Protein Isolate	WHP 9015 (Milk Specialties Global)	87.3%	2.93	0.03
Micellar Casein	Nutrasol 695 (Glanbia Nutritionals)	85.4%	1.71	0.04
Micellar Casein	Idaho Milk Products	84.7%	1.14	0.01
Egg White Protein	Rempro 8000 SF (Rembrandt Foods)	79.4%	0.69	n.d.
Whey Protein Concentrate	Avonlac 282 (Glanbia Nutritionals)	78.4%	1.60	0.01
Plant Protein				
Soy Protein Isolate	Bulk Supplements	90.0%	0.72	n.d.
Mung Bean Protein	NNB Nutrition	86.5%	1.56	0.01
Rice Protein	Oryzatein SGBN (Axiom Foods)	83.4%	0.81	n.d.
Rice Protein	Oryzatein Silk 80 (Axiom Foods)	82.0%	0.30	n.d.
Pea Protein Concentrate	VegOtein P80 (Axiom Foods)	76.8%	1.19	n.d.
Pumpkin Seed Protein	NNB Nutrition-	75.8%	0.58	n.d.
Watermelon Seed Protein	NNB Nutrition	75.6%	0.60	n.d.
Hemp Protein	Cannetein plus (Axiom Foods)	69.1%	0.28	n.d.
Sacha Inchi Protein	Incatein (Axiom Foods)	63.1%	0.18	n.d.
Sunflower Seed Protein	NNB Nutrition	61.0%	0.45	n.d.

Di- and trileucine content in plant and animal proteins

Dileucine was detected in all animal and plant protein sources, with dairy based proteins and mung bean protein having the highest content of dileucine **(Table 3.4).** Trileucine is present in dairy proteins, but not in egg or collagen. Of the plant proteins, only mung bean contains Trileucine (**Table 3.4**).

3.5 DISCUSSION

Our study is the first to report the effects of dipeptide ingestion on the subsequent regulation of muscle protein turnover in healthy young men. Specifically, our results show that the ingestion of dileucine is more effective at stimulating an increase in muscle protein synthesis rates when compared to the ingestion of an equivalent amount of leucine. There were no detectable differences in muscle protein breakdown rates between the two conditions, hence the anabolic action of dileucine is primarily mediated via stimulation of muscle protein synthesis rates in healthy young adults.

Leucine is generally considered to be an "anabolic trigger" for mTORC1 related signaling (D'Souza et al., 2014) and the subsequent stimulation of postprandial muscle protein synthesis rates (Tang et al., 2009). Specifically, protein dose-dependent increases in the stimulation of postprandial muscle protein synthesis rates occur in response to increases in plasma leucine concentrations that ultimately results in an anabolic plateau at a maximum protein dose (Moore et al., 2009, Witard et al., 2014, Holwerda et al., 2019). Here, we show that plasma leucine concentrations, and net exposure to leucine (AUC), were similarly increased between the LEU and DILEU conditions (**Figure 2A**). These findings provide evidence that oral delivery of dileucine resulted in partial hydrolysis before absorption into circulation. However, we show the absorption

of intact dileucine occurred, as indicated by the rapid rise in plasma dileucine concentrations, and greater dileucine AUC, when compared to the LEU condition (**Figure 2**). Moreover, past efforts have shown that the ingestion of dipeptides occurs at faster rates when compared to free amino acids in humans (Hellier et al., 1972). In support, we demonstrated a trend (P = 0.074) for faster time to peak plasma concentrations of dileucine (17±5 min) vs. leucine (30±13 min) in the DILEU and LEU condition, respectively. Despite this, we showed no group differences in intramuscular dileucine and leucine concentrations, nor its metabolites α-KIC, HMB or HICA (**Table 2**). Overall, our findings indicate that dileucine ingestion may be a more effective anabolic trigger for the stimulation of postprandial muscle protein synthesis rates when compared to the ingestion of free leucine alone. Of course, future work would be required in order to conceptualize the effectiveness of dileucine vs. leucine against the background of co-ingesting with a full complement of essential and non-essential amino acids (i.e., enrichment of a clinical or performance nutrition feeding formula or a diet containing recommending amounts of dietary protein).

In this study, we directly measured muscle protein breakdown rates and demonstrated no difference between the LEU and DILEU conditions. Past efforts have shown that orally ingested leucine (~2.5 g) did not have anti-proteolytic effects on measured leg protein breakdown (LPB) under artificial conditions of a hyperinsulinemic-euglycemic clamp in middle-aged women (van Vliet et al., 2018) . Hence, it is not possible to decipher the direct role of leucine per se from this study on the modulation of LPB as the clamp procedure resulted in sustained plasma insulin availability of ~50 μIU/mL (van Vliet et al., 2018), thereby already maximizing the insulin-mediated suppression of LPB (Greenhaff et al., 2008, Wilkes et al., 2009). Moreover, it has been shown that β-hydroxy-β-methylbutyrate (HMB), a metabolite of leucine, reduced LPB in healthy

young adults (Wilkinson et al., 2013). However, there were no measurements of LPB in the leucine condition in the Wilkinson et. al. paper (Wilkinson et al., 2013). Likewise, due to the utilization of the isotopic decay method, we were unable to determine muscle protein breakdown rates in the basal state, as this would require a separate resting-only trial. Therefore, it is not possible to comment on whether the ingestion of leucine or dileucine suppressed MPB from the basal state.

With regards to insulin and MPB in our study, we showed that both leucine and dileucine resulted in a ~2-fold rise in plasma insulin concentrations to 7.03 ± 3.23 $\mu IU \cdot mL^{-1}$, a result that is consistent with the notion that leucine and dileucine have potent insulin secretion properties (Morifuji et al., 2010). However, this plasma insulin value (~7 $\mu IU \cdot mL^{-1}$) is generally considered to be less than the amount required to induce maximal inhibition on MPB (~30 $\mu IU \cdot mL^{-1}$) in the presence of aminoacidemia (Staples et al., 2011, Greenhaff et al., 2008). Past efforts have shown that basal mixed MPB rates typically range between 0.10 – 0.15 $\% \cdot hour^{-1}$ in healthy young volunteers (Phillips et al., 1997, Zhang et al., 1996, Kumar et al., 2009, Staples et al., 2011). As such, it could be speculated that MPB was reduced in the present study to some extent in response to the LEU and DILEU conditions as MPB averaged 0.055±0.043 $\% \cdot hour^{-1}$.

In this study, we applied repeated biopsies (3 within 60 min) immediately after leucine and dileucine ingestion. This was partly aimed at obtaining greater temporal resolution when examining mTORC1-related signaling events. We showed that the ingestion of 2 g dileucine and equal amounts of leucine increased plasma insulin concentrations, intramuscular leucine concentrations, and similarly phosphorylated Akt. This is in line with previous findings that showed Akt phosphorylation is highly sensitive to changes in plasma insulin and amino acid concentrations (Greenhaff et al., 2008). We showed that downstream targets of mTORC1 (i.e., p70S6K and rpS6) were robustly and similarly phosphorylated between the LEU and DILEU

conditions throughout the experiment. The phosphorylated state of 4E-BP1 was not influenced by treatment ingestion, which is consistent with the notion that this signaling protein is less responsive to nutrition when compared to p70S6K in healthy adults (Fujita et al., 2007). Similarly, eEF2 phosphorylation remained stable throughout the experiment, regardless of treatment ingestion. This is in contrast with previous work that demonstrated the ingestion of a mixed nutrient beverage induced a decrease in phosphorylation of eEF2 at 1 h of the postprandial period (Fujita et al., 2007), which suggests that in the absence of other EAAs and nutrients, leucine/dileucine alone is insufficient to reduce eEF2 phosphorylation.

Indeed, minimal amounts of ingested leucine (<1 g) are required to robustly phosphorylate most downstream mTORC1 targets (Mitchell et al., 2016). This response is saturable and often divergent from dynamic measurements of muscle protein synthesis rates (Greenhaff et al., 2008, Moore et al., 2009). Thus, our results suggest that a mechanism independent of phosphorylation of downstream mTORC1 targets underpinned the greater cumulative stimulation of muscle protein synthesis rates in the DILEU condition. For example, it is possible that other mTORC1 regulatory events, such as mTORC1 translocation to the lysosome (Abou Sawan et al., 2018) occurred in the DILEU condition, and were not captured by our standard Western Blot readouts. Alternatively, inherent variability within human models to underpin mechanisms via western blot (a secondary outcome) in response to nutritional manipulations may also be a factor. We acknowledge that a lack of mechanism is perhaps a limitation of the current study. Our results, however, provide a framework for future studies to further pursue the potential mechanistic basis underpinning the anabolic action of dileucine on muscle. One potential starting point is examining whether hormones from the gastrointestinal tract, such as glucagon-like peptide (GLP-1)(Hong et al.,

2019), provide an outside-in anabolic signaling impact on the stimulation of muscle protein synthesis rates.

Interestingly, we did not observe a stimulation of muscle protein synthesis rates based on the temporal analysis (i.e., 0-30 min, 30-60 min or 0-60 min), regardless of the precursor pool used, in the LEU or DILEU conditions. This finding is consistent with our past efforts where we show that minute to hourly assessments of FSR are analytically challenging given the variability in responses between participants during short tracer incorporation intervals as explained elsewhere (Van Vliet et al., 2019). The lack of cumulative MPS response with ~2 g of free leucine ingestion, despite anabolic signaling phosphorylation, is similar to past efforts that showed in absence of the other EAAs the anabolic properties of leucine is reduced (van Vliet et al., 2018). From a methodological standpoint, it is practically challenging to isolate the transfer RNA (tRNA) charged with L-[ring$^{13}C_6$]phenylalanine (Davis et al., 1999). As a result, it is common to use surrogate precursor pools, such as the plasma free and/or intracellular free L-[ring$^{13}C_6$]phenylalanine enrichments, to estimate the lower or upper limits of the 'true' muscle protein synthetic response (Rennie et al., 1994). In this paper, we used both surrogate pools to calculate muscle protein FSR, which results in different absolute FSR values, but the same answer to our research question (i.e., dileucine > leucine condition). Experimentally, it is generally more common to have a greater number of plasma samples vs. muscle samples collected throughout the experimental infusion protocol. Hence, if disruptions in steady-state enrichments are expected than the plasma free pool is commonly used as these disruptions can be better taken into account since workers have more repeated blood sampling vs. muscle sampling (**Figure 3.1**). However, it is also important to take into consideration that the intracellular free pool is often better protected from disruptions in plasma free enrichments, at least based on muscle biopsies spaced >2-3 hours apart

(Burd et al., 2011). Regardless, the overarching themes of most muscle protein synthesis measurements in humans is the FSR value represents an estimate of the 'true' muscle protein FSR given that surrogate precursor pools are used in the calculation. Furthermore, close temporal muscle biopsy sampling (e.g., 0-60 min), in our hands, commonly results in more (visually) variable results in intracellular enrichments (**Figure 3.3**), related to differences in amino acid transport kinetics diluting or increasing the intracellular enrichment.

From an applied standpoint and to provide context into the dileucine and tri-leucine content in isolated protein foods, we analyzed various commercially available animal and plant-derived protein sources (**Table 3.3**). To our knowledge, the dileucine and tri-leucine content of various protein foods has not been determined. It is believed that the leucine proportion by total amino acid content of protein foods is a predictor for the degree of stimulation of postprandial muscle protein synthesis rates. This is supported by the observation that the most anabolic protein feedings tend to contain 2-3 g of leucine (or ~20-25 g of high-quality isolated protein) for healthy adults (Volpi et al., 2013, Moore et al., 2015). Notably, the amount of leucine required to stimulate muscle protein synthesis rates after ingestion of whole protein foods may be lower, or closer to 2 g leucine, than isolated proteins (Van Vliet et al., 2017). In terms of the dileucine and tri-leucine content, isolated dairy proteins were observed to be the highest in dileucine content per 100 g of product among the animal sources, whereas mung bean and pea protein contained the highest amount of dileucine among all plant proteins. Additionally, trileucine was either not present or only in trace amounts in the various assessed isolated animal and plant-derived protein sources. Such results suggest that dileucine is more likely to be consumed in a dietary pattern when compared to tri-leucine. It is important to recognize, however, that our current work does not confirm the role of food-borne dipeptides in the regulation of postprandial muscle protein synthesis

rates or suggest that ample amounts of dietary protein should be consumed in an effort to achieve 2 g of dileucine in a meal. As a final point, we do not view this work as promoting the use of dipeptides as meal replacements. Specifically, the ingestion of single amino acids (i.e., leucine), and likely dipeptides (i.e., dileucine), has the potential to limit intramuscular amino acid availability and shorten the duration of the postprandial muscle protein synthetic response when compared to eating a complete profile of amino acids from protein foods (Churchward-Venne et al., 2012). Beyond providing all the amino acids required to build functional proteins, whole protein foods also have the benefit of contributing to nutrient density and diet quality. Instead, our work provides a reductionist perspective on the role of dileucine vs. leucine on the regulation of human muscle protein turnover.

In conclusion, these results provide the first insights into the regulation of muscle protein turnover by dipeptide ingestion. Specifically, dileucine ingestion elevated intact plasma dileucine concentrations and was more effective than leucine in stimulating muscle protein turnover by increasing muscle protein synthesis rates in young males. The greater anabolic action of dileucine was not related to intramuscular metabolite concentrations or greater activation of downstream mTORC1 related-signaling, as determined by static snapshots of protein phosphorylation. Further, this work provides the mechanistic basis for future work to assess how food-borne dipeptides may play a role in the regulation of postprandial muscle protein synthesis rates. Additionally, dileucine may prove a potential option for food-fortification in anabolic resistant populations (e.g., sarcopenia) given the anabolic potency of dileucine when compared to leucine consumed at the same dose (2 g).

3.6 ACKNOWLEDGMENTS

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Conflict of interests

RJ and MP are inventors of the international patent application WO 2019/20463 and have not been involved in the data collection or analysis. All other authors declare no competing interests.

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CHAPTER 4: MICROBIAL PROTEASES

Microbial protease co-ingestion augments pea protein derived plasma amino acid concentrations in healthy adults

4.1 ABSTRACT

Digestibility plays a key role in determining the bioavailability and thus quality of a dietary protein source. Microbial proteases provide a novel strategy for improving protein digestion and subsequent postprandial blood amino acid availability. This study assessed the effect of co-ingesting a microbial protease blend with pea protein on postprandial plasma amino acid concentrations versus pea protein alone in healthy young adults. In a double-blind randomized crossover fashion, 24 healthy adults (27 ± 4 y; 12F, 12M) ingested either 25 g of pea protein isolate (20 g protein and 2.2 g fat) with a triple microbial protease blend (P3) or 25 g of pea protein isolate with a maltodextrin placebo (PLA). Blood samples were collected at baseline and after protein ingestion over 5 h. Questionnaires were recorded throughout to assess GI symptoms and appetite. P3 ingestion decreased (time effect: $P < 0.001$) glucose concentrations to a lesser extent when compared to PLA (condition effect: $P < 0.001$), with no differences (condition effect: $P = 0.362$) in plasma insulin concentrations (time effect: $P < 0.001$). P3 ingestion resulted in greater self-reported feelings of hunger when compared to PLA ($P = 0.049$). Postprandial plasma leucine and branched-chain amino acid concentrations increased over time ($P < 0.001$), with no differences between conditions ($P = 0.602$ and 0.724, respectively). The early (0-2 h) postprandial net area under the curve (AUC) of plasma essential amino acid concentrations were significantly greater in P3 when compared to PLA ($P = 0.038$). Plasma total amino acid concentrations increased over time ($P < 0.001$) with greater concentrations in P3 when compared to PLA ($P = 0.003$). In

accordance, the early (0-2h) plasma total amino acids AUC was greater in P3 when compared to PLA (P = 0.033). Our results showed microbial protease co-ingestion with pea protein elevated plasma EAA and total amino acid concentrations when compared to ingesting pea protein alone.

4.2 INTRODUCTION

The ingestion of protein elevates postprandial amino acid concentrations and stimulates muscle protein synthesis (Rennie et al., 1982). Of particular interest for the stimulation of postprandial muscle protein synthesis rates are the branched-chain (BCAA) and essential amino acids (EAA); these amino acids are known to be particularly potent at stimulating muscle protein synthesis and cannot be synthesized endogenously (Volpi et al., 2003, Gwin et al., 2020, Churchward-Venne et al., 2012, Kobayashi et al., 2006).

Reduced digestibility of protein is rate-limiting for the stimulation of muscle and whole-body protein synthesis rates (Koopman et al., 2009, Dangin et al., 2001). Protein source (e.g., plant vs. animal derived) is one factor that has been shown to influence protein digestibility. Specifically, previous studies have shown reduced digestibility of plant based protein sources (e.g., soy, pea or wheat (Yang et al., 2012, Guillin et al., 2021, Gorissen et al., 2016)) when compared to animal based protein sources (e.g., whey, beef, milk) (Burd et al., 2015, Tang et al., 2009). In addition, decreased protein digestibility is more prevalent in certain anabolic resistant populations, such as older adults with sarcopenia (Paulussen et al., 2021). For example, advancing age is associated with greater digestive discomfort and distress, decreased digestive enzyme activity such as pepsin in the stomach, and a decreased output of pancreatic enzymes (Feldman et al., 1996, Ishibashi et al., 1991). A common strategy to overcome age-related reduced digestibility is increasing the amount of protein ingested to more robustly stimulate postprandial muscle protein synthesis rates

(Pinckaers et al., 2021). However, while such a strategy may elicit a more robust muscle protein synthetic response, it may not always be practical (or feasible) to ingest such large quantities of a certain food. For example, the consumption of larger quantities of food in an effort to increase protein intake would markedly increase the total caloric intake as well as increase the likelihood of gastrointestinal (GI) distress, especially when consuming foods with a lower protein density such as those originating from plants.

Recently, microbial proteases have been shown as a safe (Anderson, 2013) and effective strategy to enhance blood amino acid availability after protein ingestion by improving digestive enzyme activity (Oben et al., 2008, Jadhav et al., 2021, Townsend et al., 2020). A previous in-vitro study suggests that protease supplementation leads to enhanced rates of gastric emptying and digestion and absorption of pea, whey, and collagen protein (Jadhav et al., 2021). To date, two human *in-vivo* studies have investigated amino acid concentrations in the blood after protease supplementation to whey (Townsend et al., 2020, Oben et al., 2008) with seemingly contradicting results. Oben et. al. (Oben et al., 2008) showed an increase in postprandial aminoacidemia after adding a protease blend to 50g whey protein. Townsend et. al. (Townsend et al., 2020), however, demonstrated no benefit of protease supplementation to 26g of whey protein on exercise-mediated postprandial amino acid concentrations. Thus, more studies are needed, and to date, no studies have investigated the potential of microbial protease supplementation to enhance the digestibility/quality of plant-based proteins. Therefore, the purpose of the current study is to investigate the effects of co-ingesting a microbial protease blend with yellow-pea derived protein on the postprandial amino acids concentrations in healthy young adults. We hypothesized that ingesting isolated pea protein with a microbial protease blend will lead to greater postprandial

plasma concentrations of leucine, BCAA, EAA and total amino acids when compared to ingesting isolated pea protein alone.

4.3 METHODS

Participants and ethical approval

Twenty-four recreationally active, healthy adults (BMI 24.8 ± 1.9 $kg \cdot m^{-2}$; Age 27 ± 4 years; 12 male and 12 female) volunteered to participate in this study. Female participants performed the trials during the follicular phase of the menstrual cycle to eliminate any potential differences in baseline plasma amino acid concentrations (Draper et al., 2018). All participants were deemed healthy and physically active based on their responses to a routine medical screening questionnaire and International Physical Activity Questionnaire (IPAQ), respectively. All participants were informed about the experimental procedures, the purpose of the study, and potential risks before providing informed consent. All trials conformed to standards for the use of human participants in research as outlined in the Helsinki Declaration (Clinicaltrials.gov ID NCT04821557) and were approved by the Institutional Review Board at the University of Illinois at Urbana-Champaign (IRB # 21545).

Study design

A double-blind, randomized crossover design was used for this study. Two weeks before the experimental trial, participants reported to the laboratory in the morning after an overnight fast for measurement of body weight and height, and body composition by dual-energy x-ray absorptiometry (DEXA; Horizon W, Hologic Inc., Marlborough, MA, USA). After the preliminary testing session, participants were randomized to ingest 25g of pea protein isolate (NUTRALYS

S85F; 101 kcal, 20g protein, 2.2g fat) with either an added blend of three microbial proteases (P3) or a maltodextrin placebo (PLA) in a counterbalanced fashion. For allocation of the participants, we used a computer-generated list of random numbers. The study product was coded with a random number and remained blinded until after analysis was completed. Participants were instructed to refrain from any strenuous physical exercise 72 h and alcohol 48 h prior to each experimental trial. Participants were provided an identical standardized meal for consumption the evening before both trials (25-30% of energy requirement; 50% of energy from carbohydrate 25% energy from protein and 25% energy from fat). In addition, participants were instructed to maintain the same dietary intake for three days prior to each experimental trial, which was confirmed by the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool (version 2020; National Cancer Institute, Bethesda, MD, USA). Participants' subsequent trial days were separated by at least 7 d.

Treatment conditions

Both groups ingested 25g of powdered yellow pea protein (Nutralys S85F; Roquette, Lestrem, France). The manufacturer's report of the nutrient content of the pea protein was confirmed by proximate and amino acid analysis according to methods set forth by the Association of Official Analytical Collaboration by an independent lab (**Table 4.1**) (Latimer, 2016). The portion size of pea protein was selected to provide an amount of protein (20 g) known to robustly stimulate muscle protein synthesis (Moore et al., 2009). In the P3 condition 250 mg of a proprietary blend of three microbial proteases (OPTI-ZIOME™ P^3 HYDROLYZER; BIO-CAT, Inc., Troy, VA, USA) was added to the pea protein. In the PLA condition 250 mg of maltodextrin (BIO-CAT, Inc., Troy, VA, USA) was added to the pea protein. In both conditions the study products were thoroughly mixed

with 300 mL of water and ingested 5 min after adding either the protease blend or placebo. Participants were allowed to drink water ad libitum throughout the trial. Total water consumption was recorded, and an equal amount of water was provided during the second trial.

Table 4.1. Nutrient content of the investigational product (Yellow-Pea derived protein isolate; Nutralys S85F; Roquette, Lestrem, France).

Nutrition Information		**100 g**	**25 g**
Energy (kcal)		405	101
Amino Acids (g)			
	Taurine	0.03	0.01
	Hydroxyproline	0.35	0.09
	Aspartic Acid	9.10	2.28
	Threonine	2.79	0.70
	Serine	3.80	0.95
	Glutamic Acid	14.31	3.58
	Proline	3.39	0.85
	Lanthionine	0.05	0.01
	Glycine	3.18	0.80
	Alanine	3.33	0.83
	Cysteine	0.80	0.20
	Valine	4.28	1.07
	Methionine	0.73	0.18
	Isoleucine	4.07	1.02
	Leucine	6.63	1.66
	Tyrosine	2.89	0.72
	Phenylalanine	4.41	1.10
	Hydroxylysine	0.02	0.01
	Ornithine	0.04	0.01
	Lysine	6.07	1.52
	Histidine	1.98	0.50
	Arginine	6.89	1.72
	Tryptophan	0.75	0.19
	Total Amino Acids (calculated)	79.89	19.97
Protein (g)		80.2	20.0
Total Fat (g)		8.9	2.2
Carbohydrates (g)		0.9	0.2

Experimental trial

For both trials, participants reported to the laboratory between 0600 h – 0800 h after an overnight fast by car or public transport. Following assessment of blood pressure, a Teflon catheter was inserted into an antecubital vein, and an arterialized baseline blood sample was collected. Immediately after catheter placement and blood sample collection, participants consumed 25 g of pea protein powder dissolved in 300 mL of water with either microbial proteases or a maltodextrin placebo. Arterialized blood samples were collected in EDTA-containing tubes before (t = -5 min) and after treatment ingestion (t = 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240 and 300 min). Blood samples were centrifuged at 3000 g for 10 min at 4°C and the plasma were subsequently aliquoted and stored at -80°C for future analysis. Visual analogue scales (VAS) for measurement of appetite sensations were administered at baseline, after drink ingestion and subsequently every hour (t = -5, 0, 60, 120, 180, 240, 300 min) (Flint et al., 2000). A daily GI symptoms questionnaire was collected at the end of each trial day (Spears et al., 2021). The experimental protocol is shown in **Figure 4.1**.

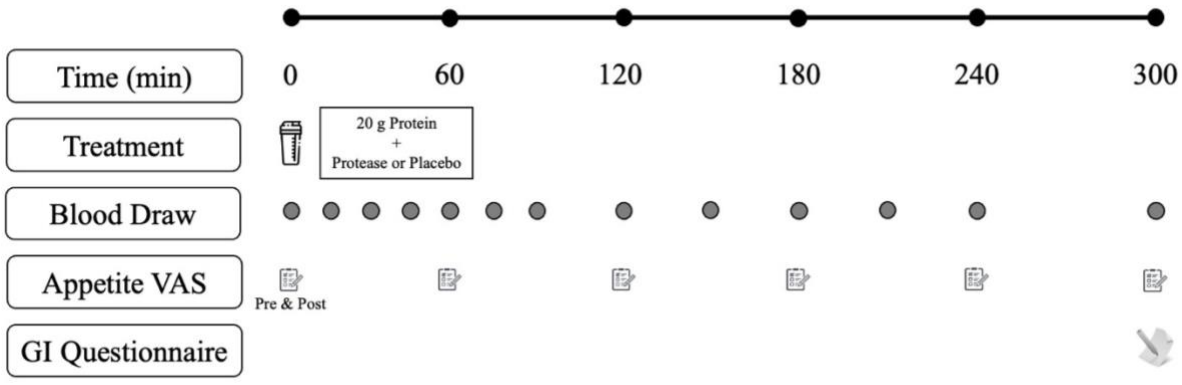

Figure 4.1. Schematic of the trial protocol. Participants (n=24) performed the trial in a double-blind randomized crossover fashion. VAS: Visual Analog Scale.

Plasma analyses

Plasma glucose concentrations were analyzed by an automated biochemical analyzer (2900 Stat Plus, YSI Life Sciences, Yellow Springs, OH). Plasma insulin concentrations were determined using commercially available enzyme-linked immunosorbent assays (Alpco Diagnostics; Salem, NH, USA).

Plasma amino acid concentrations were determined as follows: the Amino Acid standard solution (AAS18, Sigma, USA), containing 2.5 μmol·mL^{-1} each of L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine·HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L- threonine, L-tyrosine and L-valine, and 1.25 μmoL·mL-1 L-cystine and a custom mixture containing 2.5 μmol·mL^{-1} each of L-tryptophan, L-glutamine, L-asparagine, L-cysteine were used for the calibration curve. Plasma samples (50 μL) were deproteinized with methanol (940 μL) and 10 μL of internal standard (DL-p-Chlorophenylalanine, 1 mg·mL^{-1} 0.1 M HCL) was added to each sample. Subsequently, samples were vortexed and centrifuged at 20817 *g* for 10 min at 4°C and 700 μL of supernatant was transferred to a new tube. Supernatant was then evaporated under vacuum and re-suspended in 1 mL of 0.1% formic acid in water before instrument injection. Samples were analyzed by Thermo Altis Triple Quadrupole LC/MS/MS system. Software TraceFinder 4.1 was used for data acquisition and analysis. The LC separation was performed on a Thermo Accucore Vanquish C18+ column (2.1 x 100mm, 1.5μm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetontrile) and the flow rate was 0.2 mL/min. The linear gradient was as follows: 0-0.5 min, 0% B; 0.5-3.5 min, 60% B; 3.5-5.5 min, 100% B; 5.5-7.5 min, 0% B. The autosampler and HPLC column chamber were set at 10°C, 50°C, respectively. The injection volume was 1 μL. Mass

spectra was acquired under positive electrospray ionization (ESI) with the ion spray voltage of 3500 V. Selected reaction monitoring (SRM) used for the amino acid quantitation.

Statistical analyses

All data are presented as mean ± standard deviation (SD) and/or mean difference (95% confidence interval [CI]). A priori power analysis was conducted using G*Power version 3.1.9.2 (Faul et al., 2007). Based on previous research (Jäger et al., 2020) our power analysis showed that a sample size of 20 participants would be sufficient to detect differences in postprandial area under the curve (AUC) branched-chain amino acid concentrations between conditions when using a one-tailed t-test ($P < 0.05$, 85% power, $d = 0.62$). Accounting for a dropout rate of 15%, we recruited a total of 24 participants.

All data were assessed for normality before analysis via visual inspection of normal Q-Q plots and skewness kurtosis values. Differences in blood glucose, insulin and amino acid concentrations as well as appetite questionnaire results were analyzed using linear mixed effects models with time and group as fixed factors and participant intercept as a random effect. Bonferroni's post hoc test was used when significant main effects were identified. Differences in baseline dietary intake, peak plasma amino acid concentrations and plasma AUC glucose, insulin and amino acid concentrations were analyzed using a paired samples two-tailed t-test. The McNemar test was used to examine differences in GI symptoms questionnaire responses between treatments. The level of statistical significance was set at $P < 0.05$ for all analyses. All analyses were performed using IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA).

4.4 RESULTS

Participant characteristics and dietary intake

Participants' characteristics and dietary intake are presented in **Table 4.2**. Dietary intake during the three days leading up to the trial were similar for both conditions ($P < 0.05$).

Table 4.2. Baseline participant characteristics. Values are presented as mean ± SD.

	Male (n=12)	**Female (n=12)**	**Total (n=24)**
Age (y)	27 ± 5	26 ± 3	27 ± 4
Weight (kg)	83.0 ± 8.1	66.3 ± 6.7	74.6 ± 11.2
BMI ($kg \cdot m^{-2}$)	25.4 ± 2.0	24.2 ± 1.7	24.8 ± 1.9
Systolic Blood Pressure (mmHg)	116 ± 13	121 ± 11	119 ± 12
Diastolic Blood Pressure (mmHg)	67 ± 9	67 ± 6	67 ± 8
Body Fat (%)	21.6 ± 5.8	36.8 ± 5.3	29.2 ± 9.4
Lean Body Mass (kg)	61.9 ± 8.6	38.9 ± 4.5	50.4 ± 13.5
Fasting Glucose ($mmol \cdot L^{-1}$)	4.96 ± 0.52	4.84 ± 0.51	4.90 ± 0.52
Energy intake ($kcal \cdot d^{-1}$)	2,369 ± 562	2,009 ± 585	2,189 ± 595
Relative protein intake ($g \cdot kg^{-1} \cdot d^{-1}$)	1.5 ± 0.4	1.4 ± 0.7	1.5 ± 0.6
Carbohydrate ($g \cdot d^{-1}$)	252 ± 64	221 ± 73	236 ± 69
Fat ($g \cdot d^{-1}$)	102 ± 27	84 ± 24	93 ± 27

GI symptoms and appetite questionnaires

There were no significant differences in self-reported GI symptoms between conditions ($P \geq 0.480$). The only symptoms that were reported were nausea (n=3), vomiting (n=1), bloating (n=3) and flatulence (n=4). Collectively these symptoms ranged in severity from 1 to 5 on a 10-point scale (2.8 ± 1.5).

Self-reported feelings of hunger decreased after drink ingestion and increased over time (time effect: $P < 0.001$) with greater feelings of hunger in P3 when compared to PLA (CI: [0.012, 11.55] VAS score; condition effect: $P = 0.049$; **Figure 4.2A**). Self-reported feelings of fullness

increased after drink ingestion and decreased over time (time effect: $P < 0.001$) with no differences between conditions (CI: [-4.13, 5.86] VAS score; condition effect: $P = 0.733$; **Figure 4.2B**). Self-reported desire to eat decreased after drink ingestion and increased over time (time effect: $P < 0.001$) with no difference between conditions (CI: [-0.79, 10.86] VAS score; condition effect: $P = 0.090$; **Figure 4.2C**).

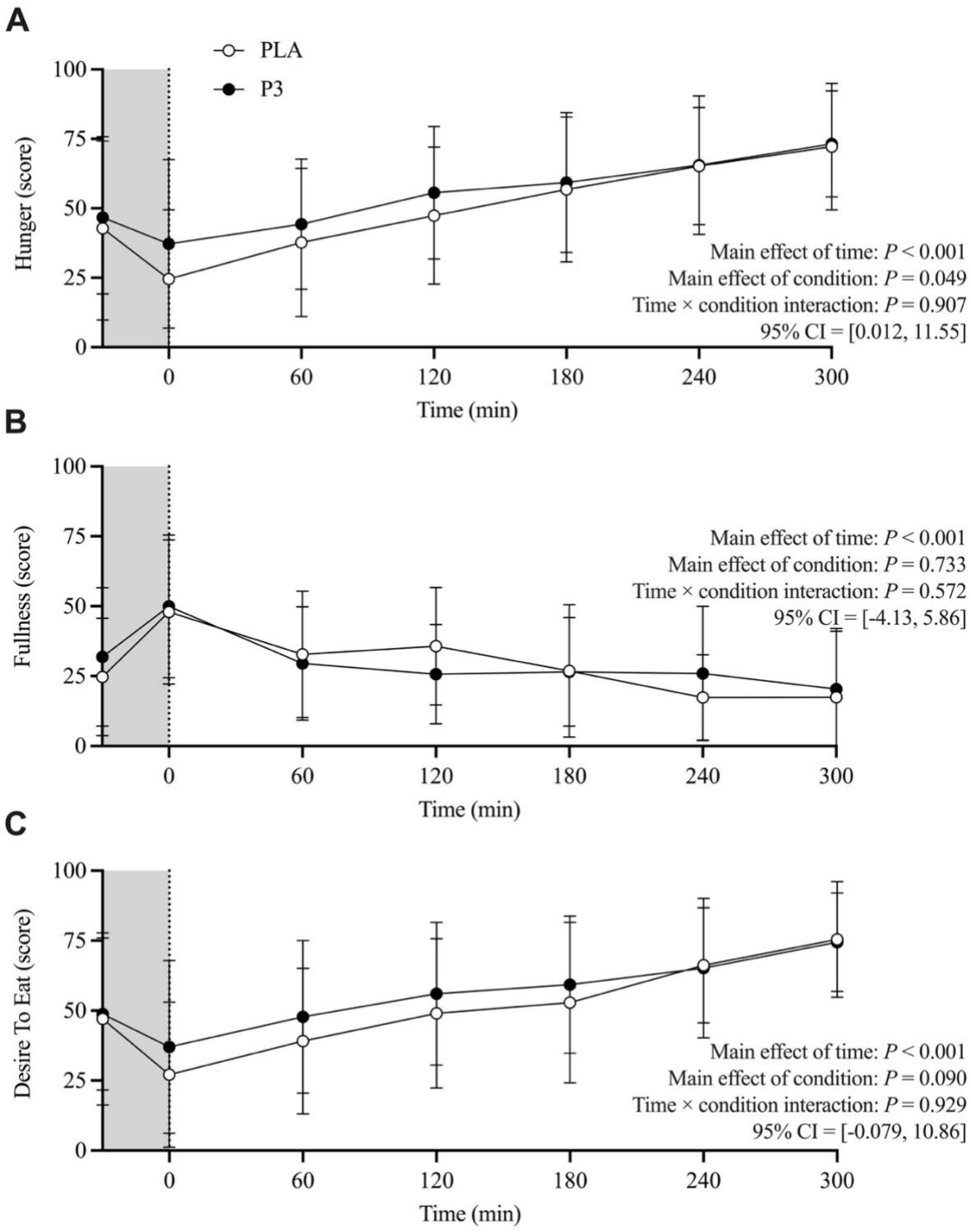

Figure 4.2. Self-reported feelings of hunger (A), feelings of fullness (B), and desire to eat (C) during the post-absorptive and postprandial period in healthy adults (n = 24). The post-absorptive period is shown in grey. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors and participant intercept as a random effect. Hunger: time effect, $P < 0.001$; condition effect $P = 0.049$. Feelings of fullness: time effect, $P < 0.001$. Desire to eat: time effect, $P < 0.001$.

Plasma glucose and insulin analyses

Plasma glucose concentrations decreased after drink ingestion (time effect: $P < 0.001$) with higher concentrations in P3 when compared to PLA (CI: [0.10, 0.24] mmol·L^{-1}; condition effect: $P < 0.001$; **Figure 4.3A**). Plasma glucose concentrations net AUC was 5% higher in P3 during the early (CI: [0.65, 48.68] mmol·120 min·L^{-1}; 0-2h; $P = 0.045$) postprandial phase when compared to PLA, with no differences between conditions during the total (CI: [-17.26, 117.44] mmol·300 min·L^{-1}; $P = 0.136$) postprandial phase.

Plasma insulin concentrations increased after protein ingestion (time effect: $P < 0.001$) with no differences between conditions (CI: [-1.63, 0.60] µIU·mL^{-1}; condition effect: $P = 0.362$; **Figure 4.3B**). Plasma insulin concentrations AUC did not differ between conditions during both the early (CI: [-243.95, 271.42] µIU·120 min·mL^{-1}; 0-2h; $P = 0.907$) and total (CI: [-382.39, 668.97] µIU·300 min·mL^{-1}; 0-5h; $P = 0.547$) postprandial period.

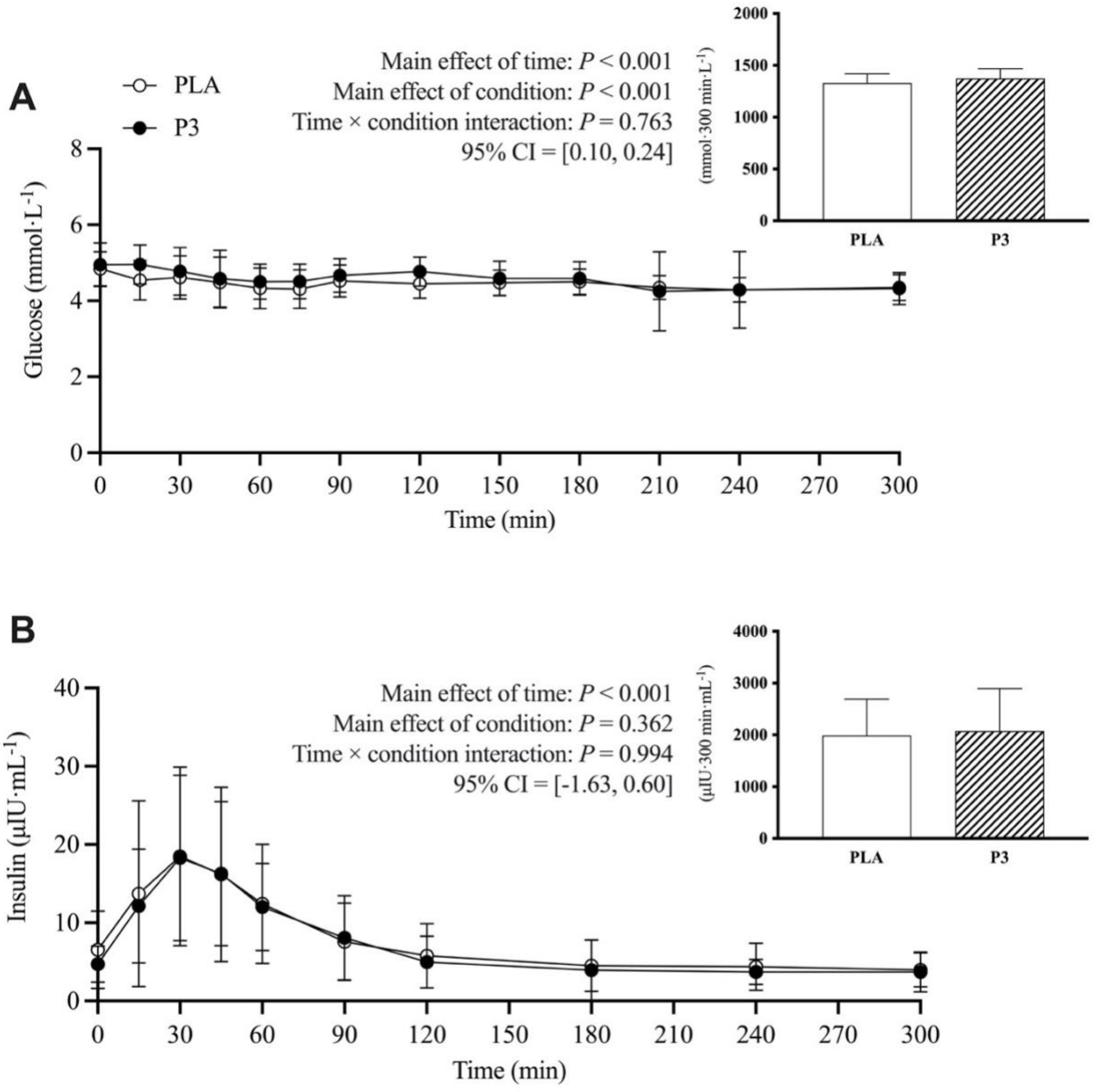

Figure 4.3. Plasma glucose (A) and insulin (B) concentrations during the post-absorptive and postprandial period in healthy adults (n = 24). Insets show area under the curve over the entire postprandial period. The post-absorptive period is shown in grey. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors and participant intercept as a random effect. Glucose: time effect, $P < 0.001$; condition effect, $P < 0.001$. Insulin: time effect, $P < 0.001$.

Plasma amino acid concentrations

Plasma leucine concentrations increased after P3 and PLA ingestion (time effect: $P < 0.001$) and returned to baseline at t = 210 min, with no differences between conditions (CI: [-2.99, 5.15] μmol·L^{-1}; condition effect: $P = 0.602$; **Figure 4.4**). Plasma leucine concentrations AUC was not statistically different between conditions during the first 120 min (CI: [-83.02, 1153.54] μmol·120 min·L^{-1}; 0-2h; $P = 0.086$) and total postprandial period (CI: [-1383.52, 1877.66] μmol·300 min·L^{-1}; $P = 0.753$).

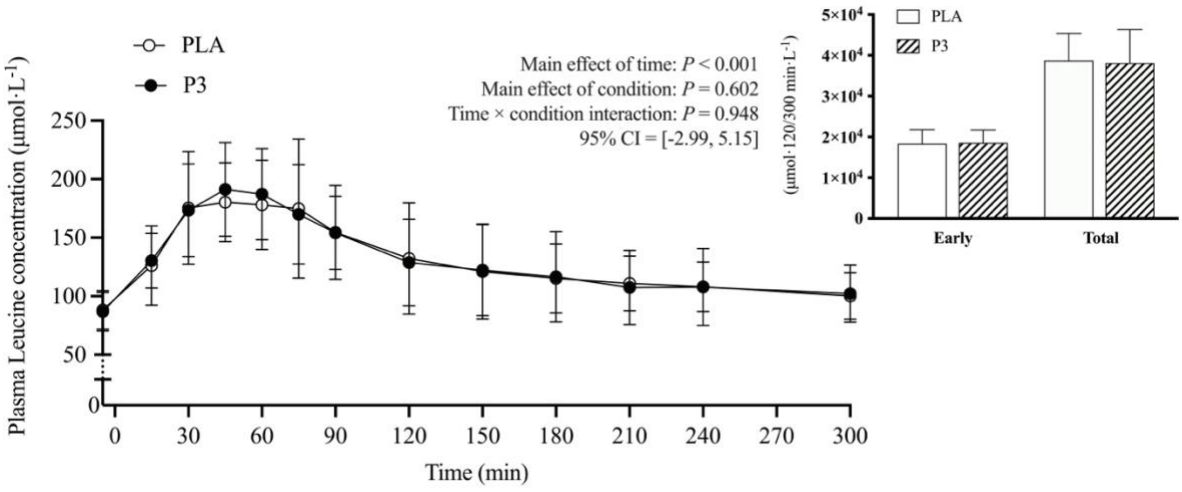

Figure 4.4. Plasma leucine concentrations during the post-absorptive and postprandial period in healthy adults (n = 24). Insets show the area under the curve over the early (120 min) and total (300 min) postprandial period. The post-absorptive period is shown in grey. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors and participant intercept as a random effect. A Bonferroni post-hoc test was used when significant differences were identified. Early: 0 – 120 min. Time effect, P < 0.001.

Plasma BCAA concentrations increased after P3 and PLA ingestion (time effect: $P < 0.001$) and returned to baseline at t = 300 min, with no differences between conditions (CI: [-7.94, 11.42] μmol·L^{-1}; condition effect: $P = 0.724$; **Figure 4.5**). Plasma BCAA concentrations AUC did not differ between conditions during the first 120 min (CI: [-793.33, 2128.95] μmol·120 min·L^{-1};

0-2h; P = 0.350) and total postprandial period (CI: [-5099.66, 3053.40] µmol·300 min·L^{-1}; P = 0.622).

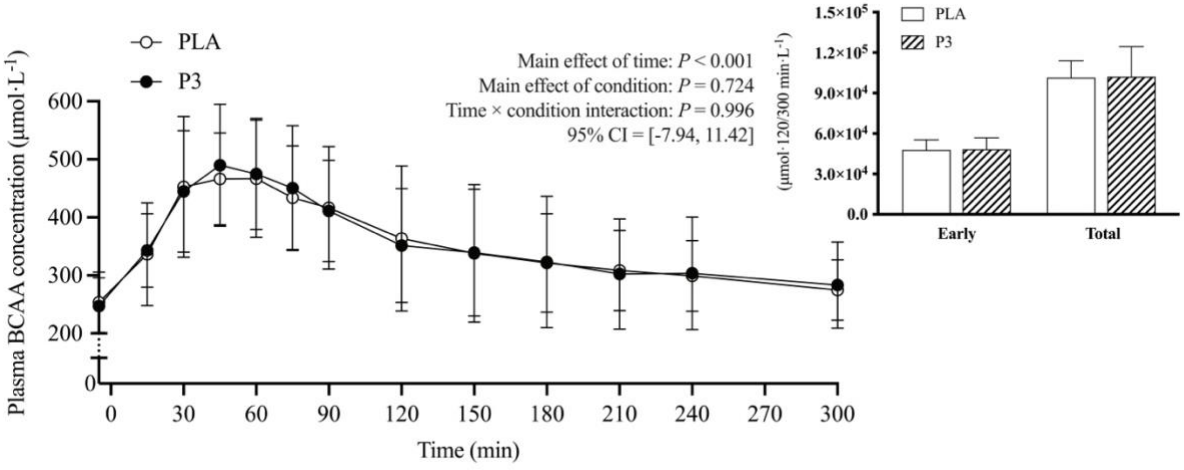

Figure 4.5. Plasma branched-chain amino acid (BCAA) concentrations during the post-absorptive and postprandial period in healthy adults (n = 24). Insets show the area under the curve over the early (120 min) and total (300 min) postprandial period. The post-absorptive period is shown in grey. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors and participant intercept as a random effect. A Bonferroni post-hoc test was used when significant differences were identified. Early: 0 – 120 min. Time effect, P < 0.001.

Plasma EAA concentrations increased after P3 and PLA ingestion (time effect: P < 0.001) and returned to baseline at t = 180 min, with no differences between conditions (CI: [-3.83, 31.19] µmol·L^{-1}; condition effect: P = 0.125; **Figure 4.6**). Plasma EAA concentrations AUC was 2% greater in P3 when compared to PLA during the first 120 min (CI: [158.16, 4989.30] µmol·120 min·L^{-1}; 0-2h; P = 0.038), with no differences between conditions during the total (CI: [-6383.60, 9348.43] µmol·300 min·L^{-1}; P = 0.883) postprandial period.

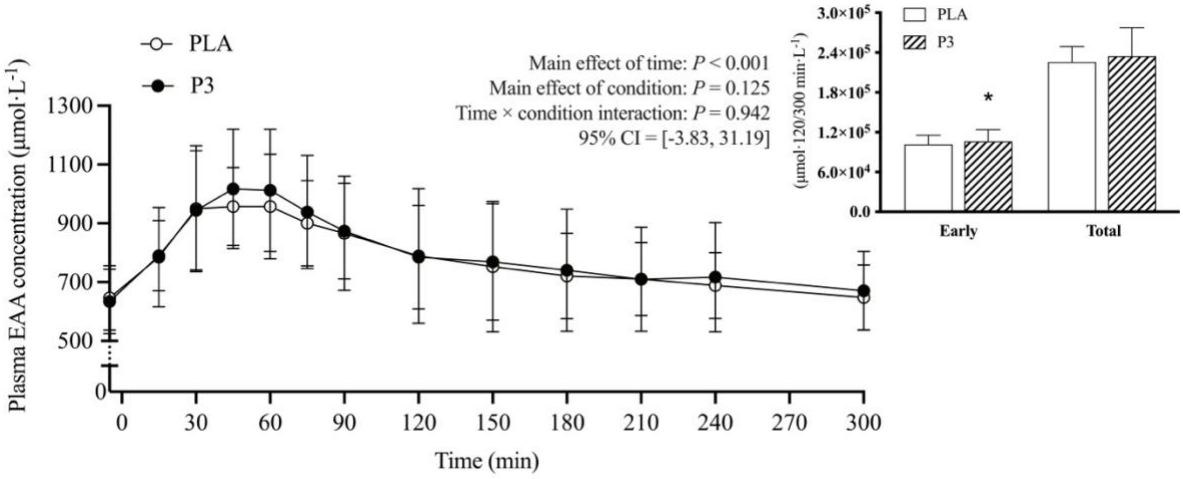

Figure 4.6. Plasma essential amino acid (EAA) concentrations during the post-absorptive and postprandial period in healthy adults (n = 24). Insets show the area under the curve over the early (120 min) and total (300 min) postprandial period. The post-absorptive period is shown in grey. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors and participant intercept as a random effect. A Bonferroni post-hoc test was used when significant differences were identified. * Indicates significant (P < 0.05) difference between conditions. Early: 0 – 120 min. Time effect, P < 0.001. Early AUC: P = 0.038; CI: [158.16, 4989.30].

Plasma total amino acid concentrations increased after drink ingestion (P < 0.001) and returned to baseline values at t = 150 min, with higher concentrations in P3 when compared to PLA (CI: [17.47, 86.58] μmol·L^{-1}; condition effect: P = 0.003; **Figure 4.7**). Peak total amino acid concentrations were 4% higher in P3 when compared to PLA (P = 0.028). Plasma total amino acid concentrations AUC was 4% greater in P3 when compared to PLA during the first 120 min (CI: [742.00, 16062.44] μmol·120 min·L^{-1}; P = 0.033), with no differences during the total (CI: [-8870.61, 32639.82] μmol·300 min·L^{-1}; P = 0.217) postprandial period.

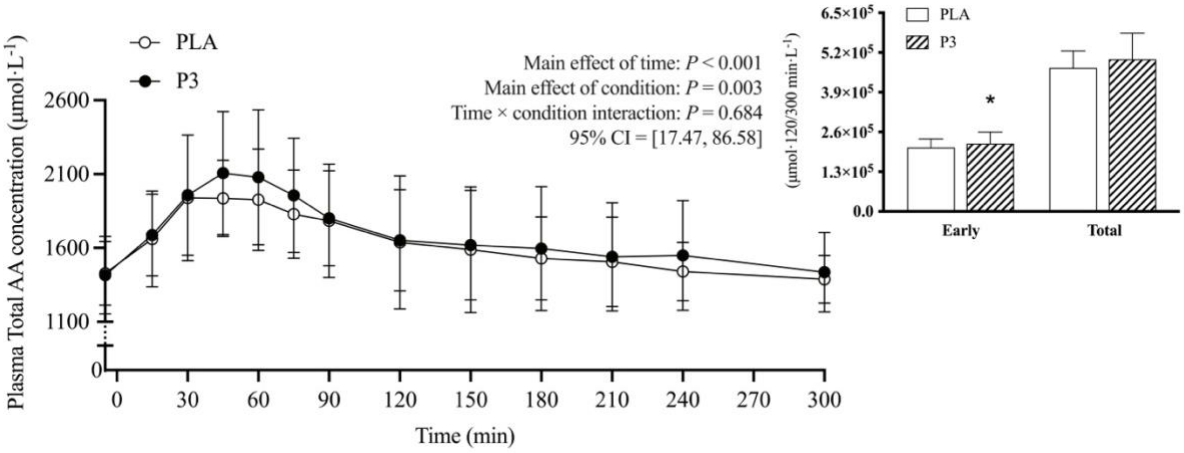

Figure 4.7. Plasma total amino acid (AA) concentrations during the post-absorptive and postprandial period in healthy adults (n = 24). Insets show the area under the curve over the early (120 min) and total (300 min) postprandial period. The post-absorptive period is shown in grey. Data are mean ± SD. Data were analyzed using a linear fixed effects model with time and group as fixed factors and participant intercept as a random effect. A Bonferroni post-hoc test was used when significant differences were identified. * Indicates significant (P < 0.05) difference between conditions. Early: 0 – 120 min. Time effect, P < 0.001; condition effect, P = 0.003. Early AUC: P = 0.033; CI: [742.00, 16062.44].

4.5 DISCUSSION

Proteolytic enzyme supplementation has recently emerged as a potential strategy for augmenting the bioavailability of amino acids after consuming dietary protein (Townsend et al., 2020, Oben et al., 2008). The current study sought to investigate the effects of co-ingesting a microbial protease blend with isolated pea protein on postprandial plasma amino acid concentrations. Ingesting a microbial protease blend with pea protein increased EAA and total amino acid concentrations during the early postprandial phase when compared to ingesting pea protein alone. No differences were observed in plasma leucine and BCAA concentrations between the P3 and PLA conditions.

Dietary protein is a main anabolic stimuli to skeletal muscle tissue (Rennie et al., 1982, Boirie et al., 1997, Holwerda et al., 2019b, Van Vliet et al., 2019) and thus has an important role in supporting physical performance (Morton et al., 2018), metabolic health (Camargo et al., 2020), and weight management (Paddon-Jones et al., 2008) due to its contribution to basal metabolic rate

and total daily energy expenditure. The anabolic potency of a protein source is largely based on its digestibility and the subsequent ability to deliver amino acids to skeletal muscle tissue. Indeed, various strategies have been studied to increase protein digestibility (Gorissen et al., 2016, Engelen et al., 2007) and/or to increase systemic release of amino acids in certain populations less sensitive to the anabolic potential of protein ingestion (Holwerda et al., 2019a, Engelen et al., 2007). Here, our results demonstrated a novel method of co-ingesting a microbial protease for increasing plasma amino acid concentrations (proxy for enhanced digestibility) (Groen et al., 2015). These digestive enzymes are believed to enhance cleavage of proteins into peptides and amino acids by increasing proteolytic activity as well as the endogenously produced proteases such as pepsin, trypsin, and chymotrypsin. We demonstrated that co-ingestion of triple protea blend with pea protein resulted in increased plasma EAA concentrations when compared to ingesting pea protein alone, as evidenced by a significantly greater net plasma EAA exposure (AUC) during the early postprandial phase (0-120 min) (**Figure 6**). Additionally, we observed an overall effect of time and condition for plasma total amino acids concentrations with higher plasma concentrations after the addition of a triple protease blend. In line with the plasma EAA concentrations, AUC analysis indicated the total amino acid differences occurred mainly during the first 2 hours of the postprandial period. Given that the plasma total amino acid concentrations returned to baseline at t = 150 min, these early AUC analyses are likely in line with the total postprandial period, after which participants returned to the postabsorptive state. These differences were not observed when examining BCAA or leucine concentrations in isolation.

Indeed, a limited number of studies in humans have examined the potential of protease co-ingestion with a protein source as a strategy to liberate more amino acids into the blood during the postprandial period. Our results are in line with previous findings from Oben et al. (Oben et al.,

2008) that demonstrated an increase in postprandial amino acid concentrations after co-ingesting either a low or high dose of a protease blend with 50g of whey protein when compared to ingesting 50g of whey alone. However, the authors reported a continuous increase in postprandial plasma total amino acid concentrations with peak concentrations at 4 h, the last recorded timepoint. Comparatively, in our study, total amino acid concentrations peaked at 56 ± 24 min, which is in line with typically observed time-to-peak amino acid concentrations after the ingestion of isolated protein sources (Burke et al., 2012, Gorissen et al., 2016, Boirie et al., 1997). In addition, Townsend et al. (Townsend et al., 2020), investigated the addition of a proprietary protease blend to 26 g of whey protein on the exercise mediated postprandial amino acid response in healthy young resistance-trained males. The authors reported no difference in postprandial plasma amino acid concentrations after co-ingesting a protease blend with whey when compared to ingesting whey alone. However, despite the pea protein isolate utilized in the current study showing excellent digestibility, with a digestible indispensable amino acid score (DIAAS) score of 100 (Guillin et al., 2021), whey is known to have higher digestibility scores when compared to plant-based protein sources like pea protein, with DIAAS digestibility scores well in excess of 100 (Mathai et al., 2017). The combination of a high-quality protein source like whey and increased exercise-induced gut-permeability (Mazzulla et al., 2017, Chantler et al., 2021) might explain the discrepancy between the results obtained by Townsend et al. with our study. Regardless, our study highlights the effectiveness of microbial proteases to enhance plasma amino acid concentrations after ingesting pea protein (**Figure 6 & 7**). These findings show promise for the use of microbial proteases as a tool to increase the bioavailability/digestibility of dietary proteins and underscore the need for more research into the long-term benefit microbial proteases might provide for lower-digestible proteins (e.g., plant-based proteins) or anabolic resistant populations.

Importantly, protease supplements were well-tolerated in the current study, with no differences in self-reported GI symptoms between conditions. Few reports of GI complaints were made, and those GI symptoms that did arise were of low severity (2.8 ± 1.5 on a 10-point scale). GI symptoms are often the result of undigested food components within the lumen of the intestine as a result of gas production and osmotic activity leading to distention (Tuck et al., 2019). The isolated protein source used within the current study lacks many other nutrients normally contained in whole foods and is known to be well tolerated (Babault et al., 2015). It remains to be determined if protease supplementation is able to reduce GI symptoms induced by other food sources.

Despite no differences between conditions in the postprandial rise of plasma insulin concentrations, we detected significantly higher glucose concentrations in P3 when compared to PLA. Specifically, when examining the total postprandial area under the curve of glucose concentrations, the differences seemed to occur during the early postprandial phase (0 – 120 min), which is in accordance with the higher plasma amino acid concentrations in P3 during this same time-period. We are not the first to show a discrepancy between plasma insulin concentrations and subsequent glucose clearance from the blood after meal ingestion despite unaltered peripheral insulin sensitivity (Lozinska et al., 2016). Thus, higher increases in plasma glucose concentrations in P3 after protein ingestion seems to be caused by insulin-independent mechanisms. One possible mechanism could be related to glucagon release. Calbet et al. have shown that glucagon concentrations are linearly dependent on plasma amino acid concentrations and are not influenced by gastric emptying, the source of protein or other constituents within an isolated protein source (Calbet and MacLean, 2002). Thus, the higher plasma amino acid concentrations in P3 could be at the root of increased plasma glucose release. Alternatively, there is some evidence to suggest that the proteolytic activity might be responsible for a decrease in GLP-1 secretion and thus lowered

glucose disposal at the liver, increasing plasma glucose concentrations (Santos-Hernández et al., 2018, Gillespie and Green, 2016, Geraedts et al., 2011). The release of glucose regulating hormone GLP-1, among other digestive hormones like cholecystokinin (CCK), is highly sensitive to the length of certain peptides within the intestine. Gillespie and Green showed that casein protein, a potent GLP-1 secretagogue, loses or significantly reduces its GLP-1 secretory activity upon hydrolysis with the proteases pepsin, trypsin and chymotrypsin (Gillespie and Green, 2016). In line, Geraedts et al. showed that multiple other protein sources, including pea protein, are potent GLP-1 secretagogues, but after the addition of trypsin, a protease occurring in the human duodenum, GLP-1 concentrations are reduced (Geraedts et al., 2011). Thus, as Geraedts et al. postulated, proteases might 'cleave' the dietary protein in its inducing site, thereby losing its effects to induce an increase in hormone release. This proteolysis induced reduction of GLP-1 and subsequent altered glucose concentrations would be in line with the reported increase in feelings of hunger and heightened glucose concentrations in P3 in the current study. Future studies are needed to clarify the implications of protease supplementation on glucose homeostasis.

In conclusion, these results provide the first insights into the effects of microbial protease supplementation on postprandial amino acid concentrations after the ingestion of plant-based proteins. Specifically, a blend of three microbial proteases added to 25 g of isolated pea protein increased postprandial plasma essential and total amino acid concentrations when compared to ingesting pea protein alone. The co-ingestion of microbial proteases did not lead to an increased availability of plasma leucine or BCAA concentrations. Additionally, microbial protease co-ingestion resulted in a lower reduction of postprandial plasma glucose concentrations as well as increased self-reported feelings of hunger. This work provides the basis for exploring the potential benefits of microbial proteases on increasing protein digestibility, improving muscle protein

turnover, and reducing food-related GI symptoms. Microbial protease co-ingestion may prove a potential option for food fortification in lower-quality proteins or in anabolic resistant populations given the improved amino acid availability after co-ingesting microbial proteases with 25 g of pea protein when compared to ingesting 25 g of pea protein alone.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5: GENERAL DISCUSSION

This dissertation outlined studies that investigated food-matrix effects and their regulation of changes in muscle protein turnover in humans. This dissertation contributed new information related to nutrient-nutrient interactions on the regulation of muscle protein turnover, the role of food components (i.e., di-peptides vs. single amino acids), and exogenous protease assisted digestion and absorption. This chapter will highlight major findings from the previously presented chapters and discuss any potential limitations of the studies. Finally, future directions for research in the area of whole food effects on human protein metabolism will also be discussed.

5.1 WHOLE PROTEIN FOODS VERSUS ISOLATED PROTEINS

In the first study of this dissertation, we aimed to evaluate the effect of the food matrix on the stimulation of post-exercise muscle protein synthesis and whole-body leucine oxidation by comparing the ingestion of salmon filet or its matched constituents in the form of crystalline amino acids, coconut oil, and fish oil. We expected that eating protein within its original food matrix would lead to greater stimulation of myofibrillar protein synthesis and lower whole-body leucine oxidation rates when compared to eating those same nutrients in isolation. With a randomized crossover approach, we tested male and female young healthy adults during a primed continuous infusion of L-[*ring*-$^{2}H_{5}$]phenylalanine and L-[1-$^{13}C_{6}$]Leucine when ingesting ~20 g of protein from either salmon or its isolated nutrients. Contrary to our hypothesis, we found no differences in muscle protein synthesis or whole-body leucine oxidation.

Investigations utilizing isolated nutrients have laid the foundation for our understanding of muscle protein turnover. Naturally, most of the dietary guidelines are based on these studies of

isolated nutrients and extrapolated to whole diet recommendations. Diets consist of whole foods and not isolated proteins, and food might be more than the sum of its parts. Research on whole food-based responses of muscle protein turnover is limited. Some studies have provided glimpses of a whole-food effect on protein metabolism (Elliot et al., 2006, Katsanos et al., 2008, Burd et al., 2015, Van Vliet et al., 2017), but none have directly investigated the food-matrix itself. For example, van Vliet et al. found that consuming an isonitrogenous amount of whole eggs or egg whites led to greater post-exercise protein synthetic rates and lower whole-body leucine oxidation rates when consuming whole eggs, despite having a similar protein content and amino acid profile (Van Vliet et al., 2017). These differences might be related to the food matrix. However, whole eggs contain several nutrients not available in egg whites making it hard to pinpoint what the driving factor is between the differential protein synthetic response. As the researchers pointed out, when teasing apart the nutrients within the egg it is evident that the non-protein components of the egg are mainly located within the yolk. On a macronutrient level the main difference is the lipid-rich profile of egg yolks when compared to egg whites. This lipid-rich matrix is of interest as previous work has shown that fatty acids (Yasuda et al., 2014) and cholesterol (Castellano et al., 2017) can regulate mTORC1 activation and lysosomal localization. Some evidence has suggested omega-3 fatty acids might play a role in the regulation of muscle protein turnover (Ferguson et al., 2021). The role of the lipid matrix in regulating muscle protein turnover is further supported by work from the same researchers showing greater plasma net exposure of cholesterol (AUC) and subsequent mTOR colocalization with the lysosome after post-exercise whole egg, but not egg white, ingestion (Abou Sawan et al., 2018).

In chapter 2 we compared the food matrix directly by using Salmon, a lipid-rich, nutrient-dense food, and compared it to the ingestion of its isolated nutrients which were matched for exact

amino acid profile, DHA and EPA, and total fat content. Salmon ingestion led to similar rates of muscle protein synthesis and whole-body leucine oxidation when compared to ingesting its isolated nutrients. More studies are needed to identify the mechanistical underpinnings of differential protein synthetic responses using whole foods in past literature. For example, we chose to use salmon filet with the skin removed, as this is how salmon is commonly consumed within the US. Keeping the skin on would have doubled the fat content of the food. In comparison, Van Vliet et al. had 18 g of fat in one serving of whole eggs, whereas in our study both interventional products contained 7 g of fat (Van Vliet et al., 2017). Future studies will need to elucidate the effects of other types of nutrient-dense whole foods to further pinpoint the role of the lipid matrix as a co-factor in the stimulation of muscle protein synthesis.

Despite equal amino acid profiles in both interventional products in our study in chapter 2, we saw differential plasma leucinemia profiles after ingesting salmon or its isolated nutrients. This is particularly interesting, as there was no divergent stimulation of muscle protein synthesis between groups. The current study thus adds to the body of evidence arising that the leucine trigger hypothesis might only apply to isolated protein sources and not whole foods (Burd et al., 2019, Zaromskyte et al., 2021). The current study design does not allow for further mechanistic insight into what other anabolic signals besides leucine might be underpinning the initiation of muscle protein synthesis. As described above, Van Vliet et al. (Van Vliet et al., 2017) were able to pinpoint differences in the colocalization of mTORC1 to the lysosome (Abou Sawan et al., 2018) when comparing whole egg to egg white ingestion, suggesting nonprotein dietary factors influence the postexercise regulation of mRNA translation on skeletal muscle. Unfortunately, we did not have the muscle tissue necessary to complete this type of analysis in the current study. It remains to be determined what role the mTOR colocalization to the lysosome plays and what specific dietary

components and nutrient interactions drive the muscle protein synthetic response after ingesting whole foods. It is likely that the specific effect of whole foods on the regulation of muscle protein synthesis rates cannot be attributed to an individual nutrient and is dependent on the sum and interaction of all its nutrients.

In chapter 2 of this dissertation, we compared the postprandial response in an exercised state. We administered the exercise stimulus with matched external work to standardize the physical activity between the two infusion trials before consuming one of the two treatment conditions. It is important to note that exercise, including resistance exercise (van Wijck et al., 2013), is known to increase gastrointestinal (GI) permeability (Chantler et al., 2021) and therefore it is likely to influence nutrient utilization. For example, under normal, rested circumstances there is little evidence that dietary bioactive peptides, other than possibly di-and tripeptides, can cross the gut wall and enter the circulation in physiologically relevant concentrations (Miner-Williams et al., 2014). However, in the exercised state it has been shown that large food-derived peptides can cross the epithelial barrier (JanssenDuijghuijsen et al., 2016). In addition, postprandial release of other non-energy providing components such as vitamins (Salles et al., 2013), minerals (Zhao et al., 2016), and microRNA (Wade et al., 2016) are known to be regulated by digestion as well and, as such, may be elevated during the postprandial period in the exercised state due to greater GI permeability. This is highly relevant to the effects of food ingestion on muscle protein metabolism as these compounds have been shown to play a role in protein turnover (Salles et al., 2013, Zhao et al., 2016, Wade et al., 2016). Whole foods, such as salmon, are rich in dietary bioactive peptides and other non-energy providing components (Neves et al., 2017). Therefore, to gain a full understanding of the potential of whole foods to impact muscle protein synthesis it is pertinent to maximize uptake of these nutrients. Despite more research being needed to fully

elucidate the effects of exercise on postprandial absorption of whole food components, consuming whole foods within the post-exercise state potentially elicits a more physiologically relevant result. Lastly, a holistic approach to health not only includes defining how the food-matrix helps regulate muscle protein turnover, but also important it is investigated in a state that reflects other components of a healthy lifestyle, such as exercise.

Our main result comparing the cumulative (0-5 h) muscle protein synthetic response after ingesting either salmon or its isolated nutrients illustrated a 24% difference in favor of salmon (Cohen's d = 0.44, 95% CI: [-0.033, 0.12], P = 0.308). Our previous study comparing the ingestion of whole eggs to an isonitrogenous amount of egg whites demonstrated a 42% (Cohen's d = 1.14) difference in favor of whole eggs, nearly double the response of the current investigation (Van Vliet et al., 2017). One possible explanation for these findings is that the intricacies of the whole-food matrix, when normalized for macronutrient content, lead to smaller effect sizes than previously seen when comparing isolated nutrients to whole foods. To account for this moving forward, the current investigation's effect sizes should be used when calculating the sample size needed to discern between results obtained when comparing whole food sources which are equal in macronutrient composition. Additional investigations are needed to determine whether the current results are unique or representative of whole food effects on muscle protein synthesis.

Ultimately, food plays a central role in our diets, not isolated nutrients. Different dietary patterns are composed of different foods and combinations thereof, which all have different components and interactions. Understanding how isolated nutrients work will be essential for our understanding of food-muscle interactions. However, it is important to analyze dietary patterns, and understand how the different parts of foods and diets have synergistic interactions on activating anabolic signaling pathways and stimulating the postprandial muscle protein synthetic

response. These possibly divergent findings between isolated proteins and whole foods are likely not attributable to one particular compound but may be complex and multi-factorial (**Figure 5.1**). This study lays the groundwork for future studies looking at interactions between nutrients, foods, and exercise.

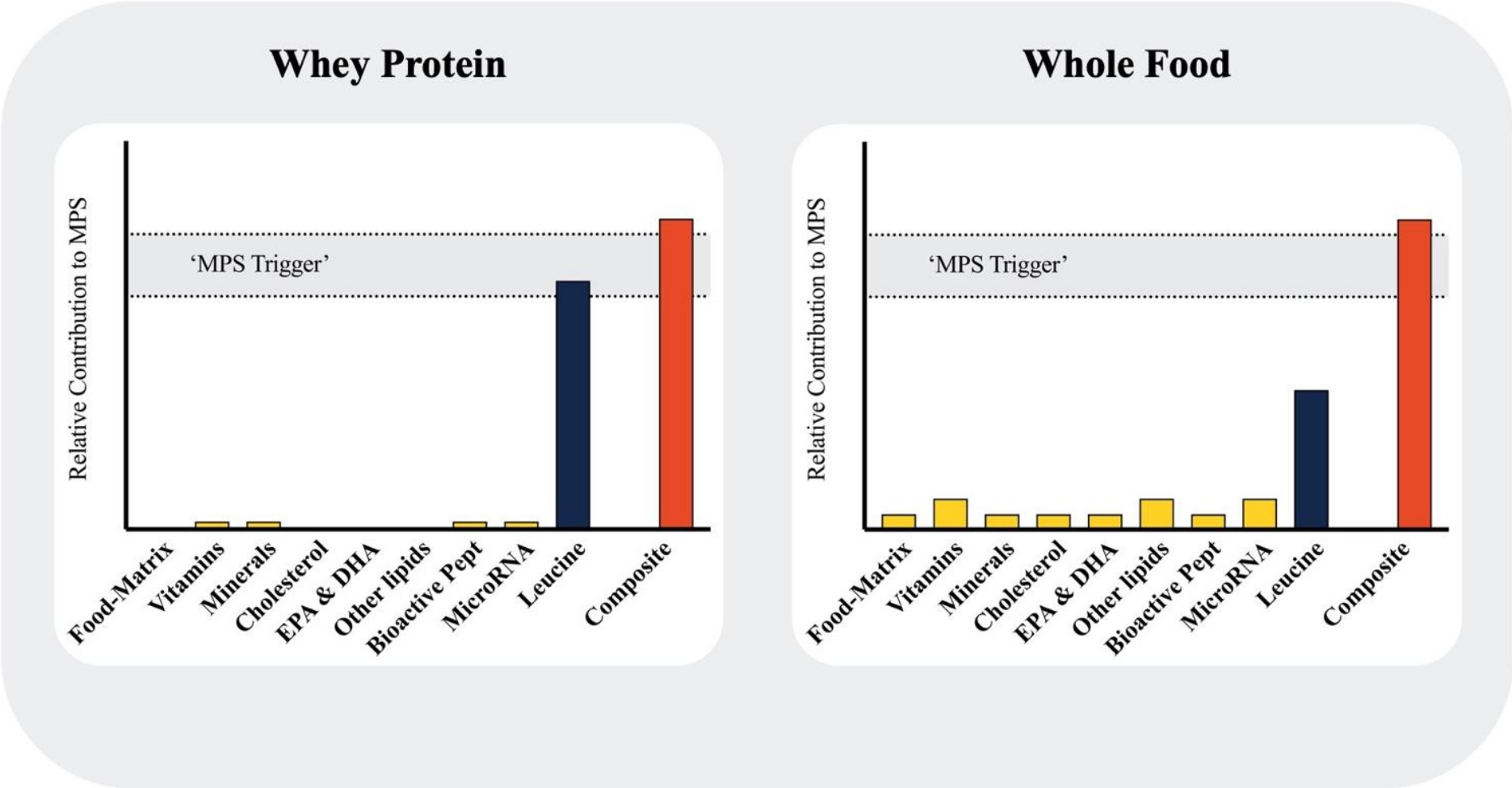

Figure 5.1. An illustration representing food components known to contribute to the stimulation of muscle protein synthesis. Historically defined as a 'leucine trigger', it was thought that the leucine content of a food would dictate the postprandial muscle protein synthetic response. Current literature (Zaromskyte et al., 2021) suggests that the leucine trigger hypothesis is mostly pertinent to isolated food sources and not whole food sources. Nutrient-nutrient interactions (Food-Matrix) within a whole food and non-protein components have been shown to impact the postprandial muscle protein synthetic response. Therefore, it is likely not the case that the stimulation of muscle protein synthesis is attributable to one single food component, but instead is multi-factorial and must be looked at from a holistic perspective.

5.2 DIPEPTIDES AND THE REGULATION OF MUSCLE PROTEIN TURNOVER

The second study of this dissertation provides the first insights into the effects of dipeptide ingestion on the subsequent regulation of muscle protein turnover. For this particular study, we chose dileucine, as leucine is known to be a potent signaling molecule for muscle protein synthesis when contained within protein or an amino acid mixture. We showed that certain foods known to be more anabolic (e.g., whey isolate) have high concentrations of this particular dipeptide. As a proof-of-concept we sought to identify if ingested dileucine could transfer into circulation and ultimately if it would have a role into regulating protein turnover.

We investigated the differences between ingesting 2 g of leucine or an isomolar amount of dileucine on muscle protein turnover in healthy men. Previous research on the anabolic potential of isolated leucine is contradictory. Wilkinson et al. have shown that the ingestion of 3.42 g of leucine leads to a robust stimulation of muscle protein synthesis in healthy males (Wilkinson et al., 2013), whereas Van Vliet and others showed that ingesting 0.0513 g·leucine·kg^{-1} fat free mass (~2.4 g) in older women was insufficient to stimulate muscle protein synthesis (van Vliet et al., 2018). We chose a dose of 2 g of leucine as this is the amount of leucine in an amount of whey protein (20 g) known to robustly stimulate muscle protein synthesis in healthy adults. Interestingly, we saw that ingesting this same amount of leucine in isolation is insufficient to stimulate myofibrillar protein synthesis in healthy young men. In our opinion, the most plausible theory for the lack of protein synthetic response when compared to previous studies feeding similar amounts of leucine as part of an amino acid or protein mixture (Churchward-Venne et al., 2012, Holwerda et al., 2019, Moore et al., 2009) is that the other EAAs required to build a functional protein are absent and need to be derived from protein breakdown (Wolfe, 2017). Conversely, when

consuming leucine in isolation it seems that higher doses are needed than the 2 g of leucine used in the current study (Wilkinson et al., 2013, Smith et al., 1992).

Given the rapid hydrolysis of dipeptides, one interesting finding is that absorption of intact dileucine is possible. This highlights the possible importance of food-borne dipeptides to be taken into consideration when investigating mechanisms underpinning the skeletal muscle protein synthetic response. Additionally, plasma dileucine concentrations after dileucine ingestion peaked faster than plasma leucine concentrations after leucine ingestion. Intracellular dileucine and leucine concentrations were not elevated. However, previous work has shown that in terms of leucine sensing it is the plasma concentrations, not muscle concentrations, that are of prime importance when it comes to stimulating the mTORC1 pathway (Kimball et al., 2016) and muscle protein synthesis (Bohé et al., 2003). In support, we show that 2 g of dileucine, not leucine was able to stimulate muscle protein synthesis. These findings are interesting, as we are the first to show the anabolic effect of dipeptides when compared to single amino acids.

We recognize that the lack of a mechanistic underpinning is a limitation of the current study. However, the study was designed with muscle protein synthesis and breakdown as primary outcomes in mind, and thus the current study design does not allow us to further investigate any regulatory roles of dipeptides on anabolic mechanisms underpinning muscle protein turnover. However, our work does provide the mechanistic basis for future work assessing how dipeptides regulate muscle protein turnover. In addition to any mechanistic underpinnings, future studies are needed to assess how food-borne dipeptides might play a potential role in the differential regulation of whole foods versus isolated nutrients on muscle protein metabolism.

In the second study of this dissertation, we decided to compare the effects of leucine and dileucine ingestion on muscle protein synthesis in the rested state. No studies in the past have

examined the postprandial response to dipeptide ingestion in humans. As such, this design was necessary as a proof-of-concept to delineate postprandial plasma leucine and dileucine concentrations and muscle protein turnover after ingestion of isolated dileucine. It has been well established that exercise significantly enhances muscle protein turnover when compared to hyperaminoacidemia alone (Biolo et al., 1997). This increase in muscle protein turnover is due to an increase in muscle protein synthesis as well as a reduction in muscle protein breakdown. It remains to be determined if exercise can sensitize the muscle to an extent where 2 g of isolated leucine ingestion is sufficient to stimulate muscle protein turnover. Conversely, some evidence suggests that exercise training leads to an increase in PepT-1 mRNA expression, potentially impacting direct dileucine uptake from the lumen of the intestine (Freitas et al., 2020). In addition, as described above, acute exercise impacts direct nutrient uptake within the intestine (van Wijck et al., 2013). Therefore, it remains to be determined whether a differential stimulation of muscle protein turnover when comparing leucine and dileucine ingestion still upholds, or whether the stimulation of muscle protein synthesis by dileucine is further increased by exercise.

5.3 PROTEIN DIGESTION AND EXOGENOUS PROTEOLYTIC ENZYMES

In the last study of this dissertation, we evaluated if microbial protease co-ingestion was able to liberate more amino acids from a yellow-pea derived protein source when compared to ingesting the pea protein alone. Specifically, twenty-four healthy adults consumed 20 g of protein from a pea protein isolate with either a blend of three microbial proteases or a maltodextrin placebo in double-blind randomized crossover fashion. Subsequently we assessed plasma amino acid concentrations during a 5 h postprandial period.

Literature on protease co-ingestion with dietary protein in humans is scarce. Only two studies to date have assessed plasma amino acid concentrations after co-ingesting a protease supplement with whey protein (Oben et al., 2008, Townsend et al., 2020). In vitro studies using gastrointestinal simulation models show promising results. However, the two studies in humans show contradicting findings. In addition, both studies found postprandial plasma amino acid profiles that did not match previous studies utilizing similar protein sources (Boirie et al., 1997, Gorissen et al., 2016, Burke et al., 2012). Townsend et al. increased plasma total amino acid concentrations 60 min after consuming 26 g of whey protein, with no increase in plasma amino acid concentrations at any other timepoint (Townsend et al., 2020). Oben et al., found continuously increasing plasma amino acid concentrations after ingesting 50 g of whey protein, with peak concentrations at 4 h postprandial, the last recorded timepoint (Oben et al., 2008). These discrepancies with established amino acid delivery profiles after whey protein ingestion are concerning and make it hard to compare them with the study outlined in chapter 4. Nevertheless, the goal of supplementing microbial proteases was to aid in digestion of dietary protein, with a specific interest in the BCAAs and EAAs. Whey is currently known as one of the most anabolic protein sources with whey protein isolate known to be almost 100 percent digested and reaching DIAAS scores of up to 1.30 (Rutherfurd et al., 2014). In addition, as discussed earlier, DIAAS scores are assessed in non-exercised conditions and so it could be postulated that exercise may even further increase these digestibility rates. Thus, exogenous proteases might provide little added benefit to a protein source with such high digestibility rates.

More interestingly, we used a plant-based protein source, which are known to have lower digestibility rates when compared to most animal-based sources. Despite the protein source in the current study already displaying high DIAAS scores of up to 1.00 (Guillin et al., 2021), we were

able to potentiate the postprandial plasma essential and total amino acid concentrations. This is especially promising since we investigated a young, healthy, well-active population. These results are exciting, as they show promise for future studies investigating less digestible protein sources or more compromised populations. Especially populations susceptible to anabolic resistance, such as older adults with sarcopenia, or protein digestive issues might benefit from increased digestibility and subsequent amino acid availability. Furthermore, from a food waste perspective the ecological potential for microbial protease co-ingestion is further encouraging as this would provide a more efficient use of dietary protein without any necessary increase in food production or consumption.

The lack of any measure of muscle-tissue specific metabolism might be a limitation to the translatability of our study. However, it should be recognized that studies assessing muscle protein turnover are costly, invasive and provide a substantial burden to the participant. With this study we provide the insight necessary to proceed with future studies investigating the implications of microbial protease co-ingestion further downstream at the muscle level. In addition, given the high tolerability of the protein source, and the healthy population recruited in our study, we were unable to determine if there is any benefit to protease co-ingestion on perception of GI tolerability. This is important to recognize, as enzyme supplementation is a common strategy to combat GI complaints after ingesting a variety of nutrients (Suarez et al., 1999, Rosado et al., 1984).

Ultimately, we showed that microbial proteases lead to increased plasma amino acid availability when co-ingested with isolated pea protein. These results highlight the anabolic properties exogenous proteases might have in potentiating dietary protein sources. Furthermore, these results emphasize the complexity and importance of food structure on digestion and

absorption and subsequently amino acid availability for protein metabolism at the tissue-specific level.

5.4 CONCLUSION

This dissertation investigated food matrix modulations and their effect on human protein metabolism. Each chapter provided a unique perspective on different parts of the food matrix and highlights the need for holistic approaches to nutrition and human protein metabolism. Although we did not see any differences in muscle protein synthesis or whole-body leucine oxidation in Chapter 2 of this dissertation, this work was just a step, and we hope this work proves to be a catalyst for future investigations. Furthermore, the work outlined in these studies, does highlight the complexity of food and its relation to protein metabolism. Food is more than just the sum of its parts.

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APPENDIX A: SUPPLEMENTARY FIGURES

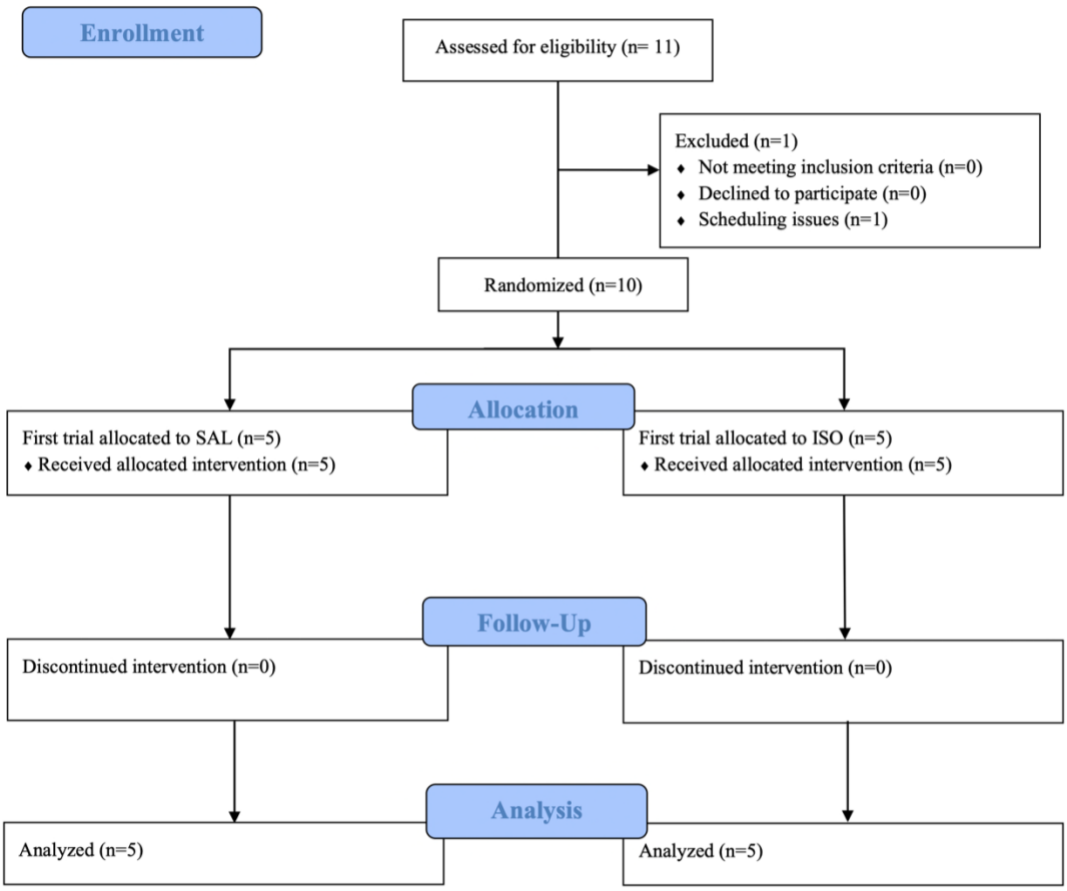

Figure A.1. CONSORT flow diagram. A double blind randomized controlled trial to assess the impact of the ingestion of 99 g of salmon filet or its isolated nutrients on the stimulation of muscle protein synthesis and whole-body leucine oxidation in healthy young adults. *n* = Participants per parameter.

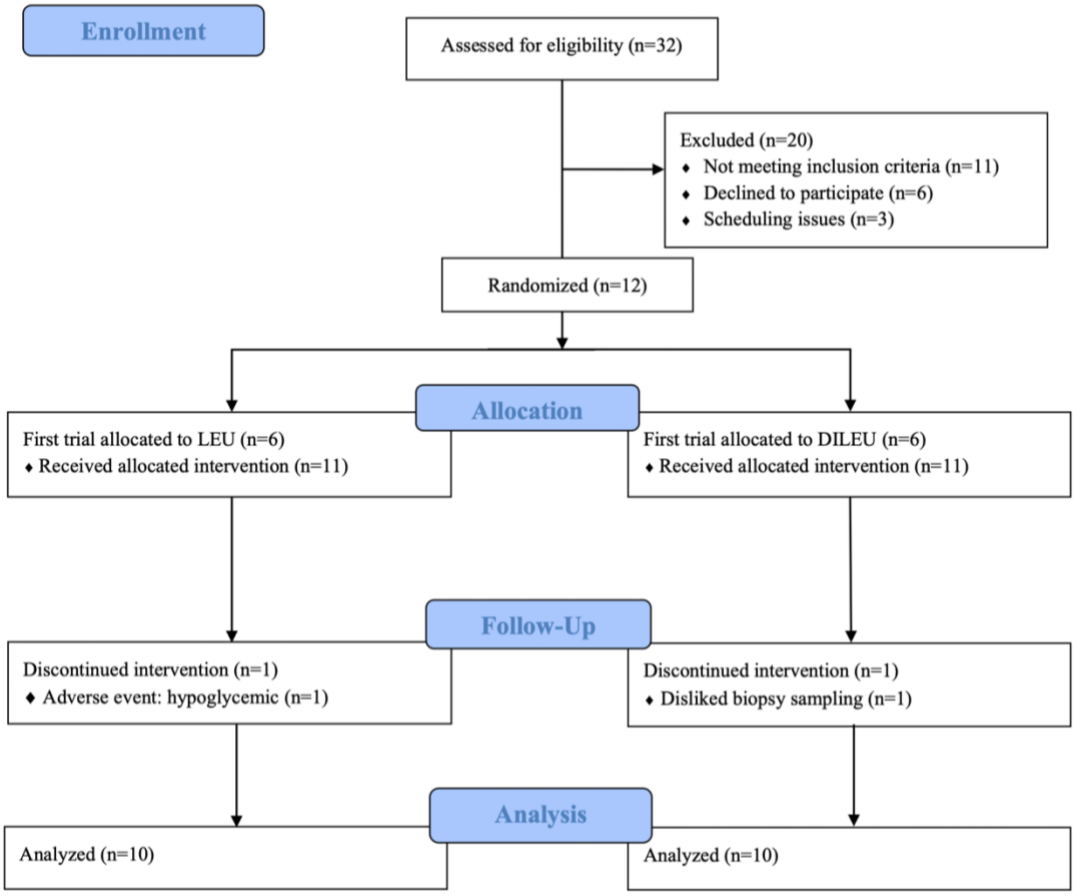

Figure A.2. CONSORT flow diagram. A double blind randomized controlled trial to assess the impact of the ingestion of 2 g of leucine or dileucine on the stimulation of muscle protein turnover in healthy young males. *n* = Participants per parameter.

APPENDIX B: SUPPLEMENTARY TABLES

Table B.1. Interventional products, study 1: A double blind randomized controlled trial to assess the impact of the ingestion of 99 g of salmon filet (SAL) or its isolated nutrients on the stimulation of muscle protein synthesis and whole-body leucine oxidation in healthy young adults.

		SAL
Energy (kcal)		**150.700395**
Amino acids (g)		
	Taurine	0.039689332
	Hydroxyproline	0.029766999
	Aspartic Acid	2.08368995
	Threonine	0.982310976
	Serine	0.744174982
	Glutamic Acid	2.788175599
	Proline	0.714407983
	Lanthionine	0
	Glycine	1.012077976
	Alanine	1.289903302
	Cysteine	0.248058327
	Valine	1.230369304
	Methionine	0.644951651
	Isoleucine	1.071611974
	Leucine	1.676874293
	Tyrosine	1.022000309
	Phenylalanine	0.912854645
	Hydroxylysine	0.019844666
	Ornithine	0.029766999
	Lysine	1.974544286
	Histidine	0.595339986
	Arginine	1.25021397
	Tryptophan	0.25798066
	Total Amino Acids	**20.61860817**

Table B.1. (Cont.)

		SAL
Fatty acids (g)		
	Myristic (C14:0)	0.406815657
	Myristoleic (C14:1 cis 9)	0.009922333
	C15:0	0.029766999
	C15:1 cis 10	0.009922333
	Palmitic (C16:0)	1.141068306
	Palmitoleic (C16:1 cis 9)	0.476271989
	Margaric (C17:0)	0.019844666
	C17:1 cis 10	0.019844666
	Stearic (C18:0)	0.248058327
	Elaidic (C18:1 trans 9)	0.009922333
	Oleic (C18:1 cis 9)	1.518116963
	Vaccenic (C18:1 cis 11)	0.25798066
	Linoelaidic (C18:2 trans 9, 12)	0
	Linoleic (C18:2 cis 9, 12)	0.41673799
	Linolenic (C18:3 cis 9, 12, 15)	0.15875733
	g-Linolenic (C18:3 cis 9, 12, 15)	0.009922333
	Stearidonic (C18:4 cis 6, 9, 12, 15)	0.138912663
	Arachidic (C20:0)	0.019844666
	Gonodic (C20:1 cis 11)	0.635029318
	C20:2 cis 11, 14	0
	Homo-a-linolenic (C20:3 cis 11, 14, 17)	0.019844666
	Arachidonic (C20:4 cis 5, 8, 11, 14)	0.029766999
	3n-Arachidonic (C20:4 cis 8, 11, 14, 17)	0
	EPA (C20:5 cis 5, 8, 11, 14, 17)	0.436582656
	Behenoic (C22:0)	0.009922333
	Erucic (C22:1 cis 13)	0.069456332
	C22:1 cis 11	0.575495319
	C22:2 cis 13, 16	0.099223331
	Adrenic (C22:4 cis 7, 10, 13, 16)	0
	Clupanodonic (C22:5 cis 7, 10, 13, 16, 19)	0.168679663
	DHA (C22:6 cis 4, 7, 10, 13, 16, 19)	0.585417653
	Lignoceric (C24:0)	0.009922333
	Nervonic (C24:1 cis 15)	0.049611665
	Total Fatty Acids	**7.580662484**

APPENDIX C: EXTRACTION PROTOCOLS

C.1 SKELETAL MUSCLE PROTEIN SUBFRACTIONATION

Reagent Notes

- 1M Sucrose -- maintain the osmolarity of the tissue/ potent oxidant
- 1M KCl – maintain ion balance
- 1M EDTA – ion sequestered Ca+, Mg+, non-specific grab everything
- 0.1 M EGTA/Tris – ion sequestered, specific to Mg+
- cOmplete-mini tablet – protease inhibitor for WB
- PhosStop – phosphatase inhibitor for WB

Table C.1. Stock Solutions

Soln	Storage	Amt	Reagent
1M Sucrose	4°C	17.115 g	sucrose
		50 mL	ddH2O
1M Tris/HCl	RT	6.07 g	Trizma
		7.4 pH	NaOH
		50 mL soln	ddH_2O
1M KCl	4°C	3.73 g	KCl
		to 50 mL	ddH2O
1M EDTA	4°C	18.61 g	EDTA
		30 mL	ddH2O
		7.4 pH	Tris base
		to 50 mL	ddH2O
0.1 M EGTA/Tris		2.5364 g	EGTA
		40 mL	ddH2O
		7.4 pH (~1.76g)	Tris base
		to 60 mL	ddH2O
10% NP-40		1.43 g	Tergitol 70%
		8.57 mL	ddH2O
0.3 M NaOH	RT	3 g	NaOH
		250 mL	ddH2O
1M Perchloric Acid (PCA)	RT	86.2 mL	70% PCA
		1000 mL	ddH2O
70% EtOH	RT	294.8 mL	95% EtOH
		to 400 mL	ddH2O
6M HCl	RT	37.14 mL	HCl (32.3M)
		to 200 mL	ddH2O

Table C.2. Buffer Solutions

Soln	Storage	Amt	Reagent
RIPA	-20°C	50 mL	ddH_2O
		790 mg	Tris base
		900 mg	NaCl
		7.4 pH (~1.76g)	HCl
		10 mL	10% NP-40
		0.5 g	Na deoxycholate
		0.1 g	SDS
		to 100 mL	ddH_2O
IB1	-20°C	670 uL	1M Sucrose
		500 uL	1M Tris/HCl
		500 uL	1M KCl
		170 uL	1M EDTA
		7.4 pH	NaOH or HCl
		to 10 mL	ddH_2O
IB2	-20°C	2.5 mL	1M Sucrose
		300 uL	0.1 EGTA/Tris
		100 uL	Tris/HCl
		7.4 pH	NaOH or HCl
		to 10 mL	ddH_2O

Supplies

- Homogenizer (glass if MITO, Teflon if no MITO)
- 4 mL screw top glass vials
- Labeled Eppendorf tubes

Homogenization

- Amt muscle aliquoted dependent on analysis being preformed
    - MITO: 80-100 mg
    - MYO: ~20 mg
    - WB: 50 mg

1. Weigh/record mass of wet muscle into MYO (COLL if applicable) Eppendorf
    a. Hole in cap if back in -80°C before homogenizing
2. Cool homogenizer in ice bucket
    a. If MITO/IB1 extraction: glass
    b. If MYO/RIPA: teflon
3. Transfer MYO muscle into homogenizer
4. If WB analysis: add enzyme inhibitors to buffers right before homogenizing
    a. 1 cOmplete-mini tablet per 10 mL buffer

    b. 1 PhosStop tablet per 10 mL buffer
    c. Vortex to mix
5. 10 uL buffer/1 mg wet muscle in homogenizer
    a. If MITO extraction: IB1
    b. If MYO only: RIPA
6. Pipette homogenate into MYO/COLL Eppendorf
    a. To COLL Eppendorf if collagen analysis
7. Centrifuge homogenate: 700 x g for 15 min at 4°C
8. Pellet: MYO and COLL extraction
    a. Can be FREEZED (-80°C) for further processing on a FUTURE date
    b. Hole in cap required!
9. Supernatant: aliquoting depends on analysis
    a. If MITO: all supernatant into MITO Eppendorf
        i. Follow "MITO Isolation" for further processing SAME DAY
    b. If SARC: 100 uL supernatant minimum
        i. Follow "SARC Isolation" for further processing SAME DAY
    c. If WB only:
        i. BCA: 6 uL
        ii. WB: rest of supernatant
        iii. Can be FREEZED (-80°C) for further processing on a FUTURE date

MITO Isolation

1. Centrifuge MITO Eppendorf: 14000 x g, 20 min, 4°C (at centrifuge max)
2. Supernatant →
    a. If SARC: SARC Eppendorf: 100 uL minimum
        i. Follow "SARC Isolation" for further processing
        ii. Store in -80°C for future processing
    b. If WB →
        i. BCA: 10-25 uL
        ii. WB: remaining
        iii. Store in -80°C for future processing
    c. If no SARC/WB: discard supernatant
3. Suspend pellet gently dispending 500 uL IB2 → do NOT vortex
4. Centrifuge MITO: 14,000 x g, 15 min, 4°C
5. Discard supernatant
6. Suspend pellet gently dispending 500 uL IB2 → do NOT vortex
7. Centrifuge MITO: 14,000 x g, 15 min, 4°C
8. Discard supernatant
9. Add 500 uL 95% EtOH; do NOT vortex
10. Centrifuge MITO: 14,000 x g, 5 min, 4°C
11. Discard supernatant
12. Transfer pellet to 4 mL screw top glass vials (using 1000 uL pipette)
13. Add 1.5 mL 6 M HCl
14. Hydrolyze overnight, 110°C (24h max)

SARC Isolation

1. Add 1000 uL 1M PCA to SARC Eppendorf; vortex
2. Centrifuge SARC: 10,000 x g, 5 min, 4°C
3. Discard supernatant
4. Add 1000 uL 70% EtOH; vortex
5. Centrifuge SARC: 10,000 x g, 5 min, 4°C
6. Discard supernatant
7. Add 1000 uL 70% EtOH; vortex
8. Centrifuge SARC: 10,000 x g, 5 min, 4°C
9. Discard supernatant
10. Transfer pellet to 4 mL screw top glass vials
11. Add 1.5 mL 6 M HCl
12. Hydrolyze overnight, 110°C
13. Can be stored at RT

MYO Isolation

1. Wash MYO pellet: 400 uL ddH_2O; vortex
2. Centrifuge: 700 x g, 10 min, 4°C
3. Discard supernatant
    a. Can freeze -80°C at this point for future processing
4. Add 1 mL 0.3 M NaOH; vortex
5. Heat at 37-50°C, 30 min
    a. Setting: low, 5-6
    b. Vortex every 10 min
6. Centrifuge: 10,000 x g, 5 min, 4°C
7. Transfer supernatant to MYO 4 mL screw top glass vials
8. Add 1.0 mL 0.3 M NaOH to MYO Eppendorf; vortex
9. *If samples appear pink:*
    a. Heat at 37°C, 10 min
10. Centrifuge: 10,000 x g, 5 min, 4°C
11. Transfer supernatant to "old" in 4 mL screw top
    a. Follow "MYO Recovery" for further processing
12. Pellet = COLLAGEN
    a. Freeze at -80°C for future processing

MYO Recovery

1. To MYO 4 mL screw top supernatant: Add 1.0mL 1 M PCA
    a. Should see "snow globe" effect
2. Centrifuge **un**capped: 3000 x g, 10 min, 4°C
    a. Accel: max Deaccel: 6
    b. Spinning too fast will break glass
3. Discard supernatant
4. Add 500 uL 70% EtOH; do not vortex
5. Centrifuge: 2000 RPM, 10 min, 4°C
6. Discard EtOH

7. Add 1.5 mL 6 M HCl
8. Hydrolyze overnight, 110°C

COLL Isolation

1. Add 1 mL 1 M PCA → vortex
2. Spin: 700 x g, 10 min, 4°C
3. Remove/discard supernatant
4. Add 1 mL 0.5 M Acetic Acid, 1 mL 0.1% Pepsin
5. Vortex mixer: overnight, 4°C
6. Spin: 700 x g, 10 min, TEMP?
7. Remove/discard supernatant
8. Add 1 mL 0.5 M Acetic Acid, 1 mL 0.1% Pepsin → vortex
9. 700 x g, 10 min, 4°C
10. Remove/discard supernatant
11. Add 0.1 M HCl, 1 mL Dowex slurry
    a. Purpose: softens hydrolysis, charges beads
12. Hydrolyze: overnight, 110°C

C.2 MUSCLE INTRACELLULAR RECOVERY AND EXTRACTION

Muscle intracellular free pool extraction protocol

Tissue needed: 15-20 mg

Supplies:

1. Teflon Pestle
2. 1.5 mL Eppendorf tube
3. 2.0 mL crimp top vial and cap
4. 4 ml screw top vial

Reagents:

1. 0.6 M Perchloric Acid (PCA)
2. 70% Ethanol (EtOH)
3. 6 M HCl
4. 1 M PCA
5. 0.3 M NaOH

Intracellular Pool Extraction:

1. Place chipped muscle in 1.5 mL Eppendorf tube on ice and add 500 µL 0.6 M PCA
2. Homogenize thoroughly with Teflon pestle
3. Spin at 4500 RPM for 5 min at 4 ˚C
4. Remove supernatant (IC–1st extraction) and place in 2 mL Eppendorf tube labelled IC
5. Add 500 uL 0.6 M PCA to pellet and vortex
6. Spin at 4500 RPM for 5 min at 4 ˚C
7. Remove supernatant (IC–2nd extraction) and place in same 2 mL Eppendorf tube labelled IC
8. Spin at 4500 RPM for 5 min at 4 ˚C
9. Remove supernatant (IC–3rd extraction) and place in same 2 mL Eppendorf tube labelled IC
10. At this point can leave the IC stored at room temperature if going to column the subsequent day or freeze at -80 ˚C if going to column at a future date

C.3 MIXED PLASMA PROTEIN EXTRACTION

Plasma needed: 200 µl

Supplies:

1. 1.5 ml Eppendorf tube
2. 18 G needle
3. 4 ml screw top vial

Reagents:

1. 100% Acetonitrile (ACN) or 2% PCA
2. 70% Ethanol (EtOH)
3. 6 M HCL

Plasma Protein Extraction:

1. Thaw baseline plasma samples on ice.
2. Add 500 µl ACN or PCA to Eppendorf.
3. Add 200 µl of plasma directly to ACN in Eppendorf.
4. Vortex
5. Spin at 10,000 RPM for 5 min (4˚C).
6. Discard supernatant
7. If sample is hazy:
    a. . (i.e., add 500 µl of ACN or PCA, spin, discard supernatant)
8. Wash pellet with 500 µl of ddH_2O.
9. Vortex
10. Spin at 10,000 RPM for 5 min (4˚C).
11. Discard supernatant.
12. Wash with 500 µl 70% EtOH and vortex
13. Spin at 10,000 RPM for 5 min (4˚C).
14. Remove and discard supernatant.
15. Poke hole in Eppendorf with needle.
16. Freeze dry pellet.
    I. Turn on pump
    II. Turn on Freeze-dryer (back button) (3001 mT); wait for vacuum
    III. Put Eppendorf in container and attach to freeze dryer; turn valve
    IV. Clean freeze-dryer after use
17. Place in 4 ml screw top vial.
18. Add 1.5 ml 6 M HCL and Hydrolyze overnight at 110˚C.
19. Purify amino acids over Dowex cation exchange column and convert to NAP derivative for analysis by GC-C-IRMS.

C.4 CATION EXCHANGE PURIFICATION

Plasma and Muscle AA Dowex purification prior to LC-MS/MS

Supplies:

1. 1000 mL beaker
2. Stir bar
3. pH paper
4. 5 mL syringes x n+1

Reagents:

1. Dowex™ 50WX8-200 ion-exchange resin
2. 4 M NH_4OH
3. 2 M NH_4OH
4. 1 M HCL
5. 0.1 M HCL

Dowex Slurry Preparation:

1. Weigh out 200 g of Dowex Resin in a 1000 mL beaker
2. Add 500 mL of ddH_2O
3. Stir for 5 min on stir plate
4. Wash with ddH_2O
    a. add ddH_2O → let resin sink to the bottom → discard water
5. Keep repeating until solution is neutral (check regularly with pH strips)
6. Add 500 mL of 4 M NH_4OH
7. Stir for 15 min on stir plate
8. Let Resin settle to bottom and discard NH_4OH
9. Check pH with strips—should be very basic
10. Wash with ddH_2O
    a. add ddH_2O → let resin sink to the bottom → discard water
11. Keep repeating until solution is neutral (check regularly with pH strips)
12. Store in fridge with a little layer of 0.1 m HCL on top of it
    ***ensures Resin does not dry out*

Column Preparation:

1. Pull out plunger of 5 mL syringe
2. Add small amount of glass wool to syringe
    a. Compact wool in syringe to 1 mL mark like picture below
    b. Push the wool down firmly with a pen/tweezers/plunger. Even amount between syringes, not creeping up on sides.

Column Clean Up:

1. Add 1 mL ddH_2O to syringe (press plunger in to "prime" the column)
2. Add 1 mL Dowex Resin with 5mL pipette (keep it consistent across all columns)
3. Add 1.5 mL 2 M NH_4OH to elute contaminants
    a. Tally volume added until pH paper blue (basic pH)
4. Add 2 mL ddH_2O until neutral

    a. Tally volume added until pH paper green (neutral pH); usually 6-8 mL
5. Add 1.5 mL 1 M HCL to charge column. Acidic columns retain samples
    a. Tally volume added until pH paper red (acidic pH); usually ~3 mL
6. Make test sample: 2 mL 6 M HCl + 1.5 mL ddH_2O
7. Add 1.5 mL ddH_2O to sample hydrolysates
8. Gently add the samples/test to respective columns
9. Add 1 mL ddH_2O to columns until neutral
    a. Tally volume added until all columns neutral (green pH paper)
    b. Usually requires ~6 mL
    c. Note if some samples require more volume to become neutral

Test Column ONLY: Elution

1. Add 2 M NH_4OH to your test column in 500 µL increments until basic
    a. Tally volume added until pH paper blue (basic pH)
    b. If only 500 µL needed, skip to "Sample Columns: Elution"
2. Subtract 500 µL from volume added to test
3. Add calculated volume of 2 M NH_4OH to sample columns

Sample Columns: Elution

1. Place newly labeled sample glass vials under sample-containing columns
2. Add 4 mL 2 M NH_4OH to elute all amino acids the column.
3. Wiggle the samples to fully expel all solution into 4 mL glass vials.
4. Dry down in Speedvac
    a. Heat time: 2h
    b. Run time: 6h at 45 °C
    c. Vacuum: 1.1

LC-MS/MS Preparation:

1. Reconstitute in 0.1 M HCL (50 mg/mL sample) and transfer to newly labeled Eppendorfs
2. Store in Freezer (-80°C)
3. When submitting for analysis by LC-MS/MS provide only 20 µL of total sample
    a. Eq. to 1000 µL for 50 mg muscle

C.5 PLASMA AMINO ACID EXTRACTION

Preparation for LC-MS-MS Analysis (Isotopic Enrichment and AA Quantification)

Items needed:

1. Pure methanol (930uL per sample)
2. 50uL of sample
3. 0.1% formic acid in H2O (Keep tightly closed) (1mL per sample)
4. LCMS-IS 20µL * # samples * 2 (e.g. 20 samples = 800 µL)
5. 2 pair of Epp (1,1; 2,2; 3,3…etc.) with number and record file in Drive.
6. LCMS ready vials labelled with sample codes (handwritten, no labels).

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 50µL of sample to labelled epp.
4. Add 930µL of pure methanol to labelled epp.
5. Add 20µL of LCMS-IS (vortex the IS before) – (DL-p-Chlorophenylalanine - Prepared with 0.01mg to 1mL of 0.1 M HCl)
6. Vortex for 10 seconds.
7. Set Centrifuge:
    a. 4°C
    b. Max Relative Centrifugal Force (RCF)
    c. 10minutes
    d. *** Centrifuge again if supernatant is not clear.
8. Pipette 700 µL of Supernatant to new Epp. (the rest will be precipitated protein)
9. Discard Pellet.
10. Dry in the SpeedVac (Settings):
    a. 45°C.
    b. 2 hours (Heat Time)
    c. 8 hours (Run Time)
    d. Click Auto Run
        a. If the samples are wet, re-run SpeedVac: 1-2hours (Heat Time) – 4 Hours (Run Time), Temp: 45°C.
11. Re-suspend with 1mL of 0.1% formic acid in H2O.
12. Vortex for 10 seconds.
13. Set Centrifuge:
    a. 4°C
    b. Max Relative Centrifugal Force (RCF)
    c. 10minutes
    d. *** Centrifuge again if supernatant is not clear.
14. Remove from centrifuge and transfer 500 µL supernatant to LCMS ready vials.

C.6 PLASMA α-KIC EXTRACTION

Plasma Preparation for LC-MS-MS Analysis (α-[^{13}C]ketoisocaproate)

**For α-[13C]ketoisocaproate we can use the same protocol as for amino acids, but we need to avoid any acidic conditions that will destroy the target. Additionally, we should start with more sample (100 µL) and use methanol as the deproteinization solvent.*

Items needed:

1. Pure methanol (300uL per sample)
2. 100uL of sample
3. 0.1% formic acid in H2O (Keep tightly closed) (1mL per sample)
4. LCMS-IS 10µL * # samples * 2 (ex. 20 samples = 800 µL)
5. 2 pair of Epp (1,1; 2,2; 3,3…etc.) with number and record file in Drive.
6. LCMS ready vials labelled with sample codes (handwritten, no labels).

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 100µL of sample to labelled epp.
4. Add 300µL of pure methanol to labelled epp.
5. Add 10µL of LCMS-IS (vortex the IS before) – (DL-p-Chlorophenylalanine - Prepared with 1mg to 1mL of 0.1 M HCl)
6. Vortex for 10 seconds.
7. Leave at -20 °C for 2 hours
8. Set Centrifuge:
    a. 4°C
    b. Max Relative Centrifugal Force (RCF)
    c. 10 minutes
    d. *** Centrifuge again if supernatant is not clear.
9. Pipette 300 µL of Supernatant to new Epp. (the rest will be precipitated protein)
10. Discard Pellet.
11. Dry in the SpeedVac (Settings):
    a. 45°C.
    b. 2 hours (Heat Time)
    c. 8 hours (Run Time)
    d. Click Auto Run
        1. Check if the samples are wet or not.
            a. If the samples are wet, re-run SpeedVac: 1-2hours (Heat Time) – 4 Hours (Run Time), Temp: 45°C.
12. Re-suspend with 1mL of 0.1% formic acid in H2O.
13. Vortex for 10 seconds.

14. Set Centrifuge:
    a. 4°C
    b. Max Relative Centrifugal Force (RCF)
    c. 10minutes
    d. *** Centrifuge again if supernatant is not clear.
15. Remove from centrifuge and transfer 500 µL supernatant to LCMS ready vials.
16. Submit for LC-MS-MS analysis.

C.7 PLASMA FATTY ACID EXTRACTION

Plasma preparation for fatty acid concentration analysis by GC/MS

Items needed:

1. Pure methanol:chloroform (1:2 v/v; 500uL per sample)
2. 150uL of sample
3. 2 pair of Epp (1,1; 2,2; 3,3…etc.) with number and record file in Drive.

Procedures

1. Code a pair of Eppendorf tubes (1,1;2,2;3,3 etc.) for sample separation
2. Add 150 μL of plasma sample to respective tube
3. Add 0.5 mL of methanol:chloroform (1:2 v/v) to sample
4. Vortex for 5 seconds
5. Centrifuge sample (4 °C for 10 min)
    a. 4 °C
    b. Max RCF
    c. 10 min
6. Collect 0.4 mL of the supernatant (Bottom phase) into a new tube and discard pellet
7. Evaporate under vacuum or N2
    a. 45 °C
    b. 2 hours (Heat Time)
    c. 8 hours (Run Time)
    d. Auto Run
8. Store at -20C

APPENDIX D: IRB APPROVAL LETTERS

ILLINOIS

OFFICE OF THE VICE CHANCELLOR FOR RESEARCH

Office for the Protection of Research Subjects
805 W. Pennsylvania Ave., MC-095
Urbana, IL 61801-4822

Notice of Approval: New Submission

November 26, 2018

Principal Investigator	Nicholas Burd
CC	Kevin Paulussen; Amelia Woods
Protocol Title	*Food first approach to stimulate muscle protein synthesis in healthy adults*
Protocol Number	19293
Funding Source	Division of Nutritional Science
Review Type	Full Board
Status	Active
Risk Determination	more than minimal risk
Approval Date	11/26/2018
Expiration Date	11/25/2019

This letter authorizes the use of human subjects in the above protocol. The University of Illinois at Urbana-Champaign Institutional Review Board (IRB) has reviewed and approved the research study as described.

The Principal Investigator of this study is reponsible for:

- Conducting research in a manner consistent with the requirements of the University and federal regulations found at 45 CFR 46.
- Requesting approval from the IRB prior to implementing modifications.
- Notifying OPRS of any problems involving human subjects, including unanticipated events, participant complaints, or protocol deviations.
- Notifying OPRS of the completion of the study.

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN
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ILLINOIS

OFFICE OF THE VICE CHANCELLOR FOR RESEARCH

Office for the Protection of Research Subjects
805 W. Pennsylvania Ave., MC-095
Urbana, IL 61801-4822

Notice of Approval: New Submission

July 26, 2018

Principal Investigator	Nicholas Burd
CC	Colleen McKenna; Amadeo Salvador; Amelia Woods;
Protocol Title	*Skeletal muscle protein synthetic response to amino acid and dipeptides*
Protocol Number	18897
Funding Source	Unfunded
Review Type	Full Board
Status	Active/Data Analysis Only
Risk Determination	more than minimal risk
Approval Date	07/26/2018
Expiration Date	07/25/2019

This letter authorizes the use of human subjects in the above protocol. The University of Illinois at Urbana-Champaign Institutional Review Board (IRB) has reviewed and approved the research study as described.

The Principal Investigator of this study is reponsible for:

- Conducting research in a manner consistent with the requirements of the University and federal regulations found at 45 CFR 46.
- Requesting approval from the IRB prior to implementing modifications.
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OFFICE OF THE VICE CHANCELLOR
FOR RESEARCH & INNOVATION

Office for the Protection of Research Subjects
805 W. Pennsylvania Ave., MC-095
Urbana, IL 61801-4822

Notice of Approval: New Submission

March 14, 2021

Principal Investigator	Nicholas Burd
CC	Kevin Johannes Maria Paulussen
Protocol Title	*BIO-CAT: Effect of Microbial Protease Supplementation on Post-Prandial Amino Acid Levels in Healthy Adults*
Protocol Number	21545
Funding Source	BIO-CAT, Inc.
Review Type	Full Board
Status	Active
Risk Determination	More than minimal risk
Approval Date	March 14, 2021
Expiration Date	March 13, 2022

This letter authorizes the use of human subjects in the above protocol. The University of Illinois at Urbana-Champaign Institutional Review Board (IRB) has reviewed and approved the research study as described.

The Principal Investigator of this study is responsible for:

- Conducting research in a manner consistent with the requirements of the University and federal regulations found at 45 CFR 46.
- Using the approved consent documents, with the footer, from this approved package.
- Requesting approval from the IRB prior to implementing modifications.
- Notifying OPRS of any problems involving human subjects, including unanticipated events, participant complaints, or protocol deviations.
- Notifying OPRS of the completion of the study.

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